EXPOSURE TO MERCURY IN SLOVENIA AND NEURODEVELOPMENT OF CHILDREN

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Doctoral Dissertation
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IZPOSTAVLJENOST PREBIVALCEV SLOVENIJE ŽIVEMU SREBRU IN POVEZAVA Z NEVROPSIHOLOŠKIM RAZVOJEM OTROK

Doktorska disertacija

Supervisor: Prof. Dr. Milena Horvat

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The Journey Matters More Than the Destination.

Pot je pomembnejša od cilja.

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Abstract

The present work deals with Hg exposure and evaluates its association with neurodevelopment of children exposed prenatally. The work combines the outcomes of human biomonitoring (HBM) and complementary epidemiological studies carried out as national or international research projects. The framework for assessment arose from the methodological part of the thesis, which defined the analytical quality requirements for HBM and the national reference population to derive background levels of internal exposure to Hg and other potentially toxic (Pb, Cd, As) and essential (Se, Cu, Zn, Mn) elements. The latter was selected from men and women of childbearing age, living in 12 geographically distinct areas, divided into rural, urban and potentially contaminated (n=1084). This is the first reference population established in Slovenia. Analytical performance of the methods to determine elemental concentrations in most commonly used biological matrix, venous blood, was evaluated using measurement uncertainty, inter-laboratory variability and their contribution to the required sample size as compared to the variability within a given population.

The second part of the thesis evaluated an association between low-level Hg exposure in the most vulnerable period for developmental toxicity, the prenatal period, and neurodevelopment of children in a birth cohort study from the western Mediterranean area (n=1282). Neuropsychological development was monitored at 18 months of age using Bayley Scales of Infant and Toddler Development (Bayley III). The association assessment took into account non-genetic (demographic, socio-economic, dietary factors, and co-exposure to Pb and status of beneficial micronutrient Se), and in a subset of the study population, the Slovenian and Croatian population groups, also genetic (apolipoprotein E – *APOE* – polymorphism) factors (n=361). Prenatal Hg exposure in the overall population was predominated by fish consumption and showed no negative associations with cognitive and language development, with the majority of children being within the normal scales of development. However, stratification by the *APOE* genotype revealed negative association with cognitive score in children carrying ε4 allele. A weak but significant inverse relation between total Hg levels and developmental motor scores at 18 months was observed, which was independent of the genotype. The study groups residing in the former Hg mine area (pregnant women and school-age children) had higher internal exposure than the residents of the rural control areas, particularly to inorganic or elemental Hg, but most of them did not exceed the values that could be associated with biological and/or potential adverse health effects, and pregnant women showed normal oxidative response.

The overall work demonstrates the efficiency of HBM as a tool to monitor low-level internal exposure, its use in classical epidemiological and exposome-based studies and the key role of strict quality control. It further indicates an importance of accounting for simultaneous exposure to different neurotoxic chemicals, non-genetic (e.g. diet) and genetic factors that could influence exposure and/or health outcomes, particularly at low-levels of chronic exposures. Complementary to the standardized neuropsychiatric assessments, on-going and future research are designed to use non-targeted approach to reveal also early alterations in biological pathways that could lead to clinical manifestations later in life.

Povzetek

Pričujoče delo obravnava izpostavljenost Hg in njegov morebitni vpliv na nevrološki razvoj otrok v prenatalni dobi. Delo združuje rezultate humanega biomonitoringa (HBM) in epidemiološke študije, ki smo jih izvajali v sklopu nacionalnih ali mednarodnih raziskovalnih projektov. Prvi del disertacije predstavlja metodološki okvir za oceno izpostavljenosti, ki opredeljuje analitične zahteve za HBM in nacionalno referenčno populacijo za določitev ravni ozadja izpostavljenosti Hg in drugim potencialno strupenim (Pb, Cd, As) ter esencialnim (Se, Cu, Zn, Mn) elementom. Analitična zmogljivost metod za določanje elementarnih koncentracij v najpogosteje uporabljeni biološki matrici, venski krvi, je bila ovrednotena z merilno negotovostjo, medlaboratorijsko variabilnostjo in njihovim prispevkom k zahtevani velikosti vzorca v primerjavi z variabilnostjo znotraj določene populacije. Kot referenčno populacijo pa smo izbrali moške in ženske v rodni dobi iz 12-ih geografsko ločenih območij, razdeljenih na podeželska, mestna in potencialno kontaminirana območja (n = 1084). Omenjena populacija je omogočila postavitev prvih referenčnih vrednosti za Slovenijo.

V drugem delu naloge smo ocenili povezavo med izpostavljenostjo nizkim odmerkom Hg v najranljivejšem obdobju življenja, prenatalnem obdobju, in nevropsihološkim razvojem otrok, in sicer v študijski populaciji otrok z območja zahodnega Sredozemlja (n = 1282). Nevropsihološki razvoj smo spremljali pri starosti 18 mesecev z uporabo lestvic zgodnjega razvoja (Bayley III). Ocena je upoštevala ne-genetske (demografske, socialno-ekonomske, prehranske dejavnike, hkratno izpostavljenost Pb in status mikronutrienta Se) ter na podskupini študijske populacije iz Slovenije in Hrvaške tudi genetske dejavnike (polimorfizem apolipoproteina E - *APOE*) (n = 361). Prisotnost živega srebra v popkovni krvi je bila večinoma na račun uživanja rib v nosečnosti in ni pokazala negativnih povezav s kognitivnim ali govornim razvojem otrok. Stratifikacija glede na genotip *APOE* pa je pokazala negativno povezavo s kognitivnim razvojem pri otrocih, ki so nosili alel ε4, čeprav so bili otroci v okvirih normalnega razvoja. Poleg tega smo opazili gensko neodvisno in šibko, vendar statistično pomembno negativno povezavo med Hg v popkovni krvi in gibalnim razvojem pri 18 mesecih. Nosečnice in otroci z območja nekdanjega rudnika Hg so imeli višje vrednosti Hg kot prebivalci podeželskih območij, zlasti anorganskega ali elementarnega Hg, vendar pa večina ni presegla vrednosti, ki bi lahko bile povezane z biološkimi in/ali možnimi škodljivimi učinki na zdravje, skupina nosečnic pa je pokazala normalen oksidativni odziv.

Doktorska naloga demonstrira učinkovitost HBM kot orodja za spremljanje notranje izpostavljenosti na nizki ravni, njegovo uporabo v klasičnih epidemioloških in 'exposome' študijah, poleg tega pa prikazuje pomembno vlogo nadzora kakovosti laboratorijskih analiz. Nadalje navaja pomen upoštevanja sočasne izpostavljenosti drugim potencialno strupenim kemikalijam, ne-genetskim (npr. prehrana) in genetskim dejavnikom, ki bi lahko vplivali na izpostavljenost in/ali zdravstvene izide, zlasti na nizki ravni kronične izpostavljenosti okoljskim onesnažilom. Kot dopolnitev standardiziranim nevropsihiatričnim ocenam pa so bodoče raziskave zasnovane v smeri ne-ciljnega pristopa z namenom odkriti tudi zgodnje spremembe, ki bi lahko vodile do kliničnih manifestacij pozneje v življenju.

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Abbreviations

8-OHdG	urinary 8-hydroxy-deoxyguanosine
ADME	absorption, distribution, metabolism and elimination
ALAD	γ-aminolevulinic acid dehydratase
APOE	apolipoprotein E
AS3MT	arsenic (III) methyl transferase
BDNF	brain-derived neurotrophic factor
BPA	bisphenol A
BSID-II	Bayley Score of Infant Development, 2nd Edition
Bayley-III	Bayley Score of Infant and Toddler Development, 3rd Edition
CAT	catalase
CDC	Centers for Disease Control and Prevention
CI	confidence interval
CYP3A	cytochrome p450 3A
CNV	copy number variant
CPOX	coproporphyrinogen oxidase
CROME	Cross-Mediterranean Environment and Health Network
EPA	Environmental Protection Agency
GCL	glutamyl-cysteine ligase
GM	geometric mean
GSH	glutathione
GST	glutathione S-transferase
GPx	glutathione peroxidase
GWAS	genome-wide association studies
HBM	human biomonitoring
MT	metallothionein
PAH	poly-aromatic hydrocarbons
PBTK	physiologically-based toxicokinetics
PFAS	perfluorinated alkyl substances
PHIME	Public Health Impact of Long-term Mixed Element Exposure in Susceptible Population Strata
PON1	paraoxonase 1
POPs	persistent organic pollutants
PGR	progesterone receptor
SG	specific gravity
SNP	single nucleotide polymorphism
SOD	superoxide dismutase
TF	transferrin
VDR	vitamin D receptor

Chapter 1

Introduction

The present work deals with exposure to mercury (Hg) in the general, non-occupationally exposed population residing in Slovenia, however it does not exclude other potentially toxic and essential elements. It evaluates the link between low-level Hg exposure during the most vulnerable period of life – prenatal period – and health outcome – neurodevelopment of child – taking into account non-genetic and genetic confounders that could influence exposure and/or the outcome. The methodology for this work is based on the human biomonitoring (HBM) – a tool to assess human exposure to environmental pollutants and potential health effects of such pollutants.

The introductory part (Chapter 1) therefore starts from the methodological framework covering basic principles of HBM, different stages of HBM (including analytical quality requirements, identification of biomarkers, laboratory analysis, data analysis and interpretation) and continuous with accompanying use of data from epidemiological studies, providing health outcome data to study associations with exposures (Sections 1.1 and 1.2). Scientific background of Hg is described in a separate section (1.3) to provide background toxicological information, sources of exposure and possible adverse outcomes associated with the exposure. Basic information for other potentially (neuro)toxic non-essential elements is added as the investigated associations may be confounded by co-exposure to other neurotoxic elements and also the status of beneficial elements (nutrients). We refer to the relevant published data to describe the world-wide trends and information available for the Slovenian population. Biomarkers to monitor internal exposure are described in Section 1.4. The epidemiological part is dealt with in an additional section (1.5), which focuses on neurotoxicity of Hg and the role of non-genetic and genetic confounders as modulators of exposure levels and/or possible impact on health outcome. The introductory part of the thesis is followed by aims and hypothesis (Chapter 2).

The main part of the thesis (Chapter 3) combines outcomes of complementary HBM and epidemiological studies carried out as national or international research projects at the Department of Environmental Sciences, Jožef Stefan Institute. The outcomes are presented by separate scientific papers and a book chapter and are organized into two parts to cover the methodological aspect as well as the assessment of exposure and its link to the health outcome. The first part provides the scientific background of Hg exposure assessment, sets the analytical quality requirements for HBM allowing to compare exposure between different population groups and/or on a time scale and to perform association studies; and defines a study population representative for the Slovenian population of childbearing age to establish background (i.e. reference) values for internal exposure to Hg and other non-essential elements and status of essential elements. The second part investigates a link between low-level Hg exposure in the prenatal period and neurodevelopment of a child.

Although low, exposure to a potentially neurotoxic element in this critical period of development could trigger sub-clinical or even clinically relevant adverse response(s), depending on an increased/decreased susceptibility of the subject. The latter includes, but is not limited to, concomitant exposure to other neurotoxic elements, essential element's intake, and genetic predisposition. These factors were accounted for as confounders in assessing exposure to Hg and/or subsequent health outcomes in the birth cohort study presented within Chapter 3. Chapter 4 discusses and concludes the overall work following the specific aims and hypothesis of the thesis and on the basis of data gaps identified provides recommendation for future work.

1.1 Human Biomonitoring – Basic Principles

Human biomonitoring is an efficient and cost-effective tool to assess human exposure to environmental pollutants and potential health effects of such pollutants. HBM can measure these chemicals, their metabolites or reaction products in human specimens, mostly in blood or urine (CDC, 2005; Knudsen, Hundebøll, & Merlo, 2012). It can identify their spatial and time trends, life-style contributing factors and specific at-risk groups (WHO, 2015). Exposure to environmental pollutants occurs through different routes (inhalation, ingestion, dermal absorption), and the body burden of a specific pollutant depends on factors, such as route of exposure, pollutant's concentration in an environmental compartment, its physical and chemical properties, timing and duration of exposure, and also individual factors influencing absorption, distribution, metabolism and elimination (ADME) of a pollutant. Intrinsic by its nature, HBM data reflects the total body burden or biological effect resulting from all exposure routes, and inter-individual variability in exposure levels, metabolism and excretion rates (Knudsen et al., 2012; WHO, 2015). Therefore, in comparison to environmental monitoring (measures of pollutants in air, water, soil, or food), HBM results are intrinsically associated with a person and are more relevant to the health outcome studied and thereby allow direct and more precise assessment of the distribution of risk in the population (Dennis et al., 2017; NRC, 2006; WHO, 2015). HBM has therefore become a key tool of exposure assessment in many epidemiological studies that attempt to link exposures to health outcome(s) (Dennis et al., 2017). A new concept emerged in 2005 named the "exposome", which involves the characterization of the totality of exposures to environmental chemicals through a lifespan (Wild, 2005). In view of the fragmented epidemiological studies, each focusing on a specific environmental compartment (e.g. air, water, food), it was recognized that a more comprehensive and quantitative approach is needed in order to discover the major causes of chronic diseases attributable to environmental pollutants (Rappaport & Smith, 2010). The exposome approach aims to capture thousands of biologically active chemicals people are exposed to daily through direct chemical exposures or consequences of exposures (e.g. hormones, reactive chemicals generated inside the body) arising from all sources (Dennis et al., 2017; Rappaport, 2011). However, xenobiotics are present at much lower levels than chemicals from food, drugs or endogenous sources. Their detection in an untargeted manner and linking these measures to an exogenous sources is therefore particularly challenging. Based on the discussions within a group of scientists gathered in the Exposome Workshop in 2015 (Dennis et al., 2017), coupling the traditional HBM, with well-established targeted analysis, and broader exposomic approaches, with untargeted discovery of unknown chemicals of biological importance, seems the right approach towards improvement of exposure assessment (Dennis et al., 2017).

In the beginning, HBM has been mainly used to monitor exposure and assess health risk in occupational settings, for example blood lead levels in industries with high lead

exposures (Knudsen et al., 2012). For the last couple of decades HBM studies have been applied worldwide on national and regional levels to monitor exposures of the general population to environmental pollutants, and just recently also internationally. A number of datasets including newborns, children and adults is increasing. Within Europe, repeated national HBM activities have been carried out in several countries: Germany (the German Environmental Survey – GerES), Belgium (Flemish Environment and Health Study – FLEHS), France (Population- and/or pollutant-specific HBM studies since 1980, multipollutant approach – ENNS), Spain (BIOAMBIENT.ES), Czech Republic (national HBM), Italy (PROBE) and Romania (focus on Pb). In Slovenia, several HBM activities have been conducted to assess the extent of exposure of Slovenian population, with the pilot phase of the first national HBM program starting in 2007 (Snoj Tratnik, Mazej, & Horvat, 2012). The latter was completed in 2015 and the assessment of exposure to selected elements in the selected study population is given in the present dissertation (*"Results of the first national human biomonitoring in Slovenia: trace elements in men and lactating women, predictors of exposure and reference values"*). Outside Europe, the well-established national programs include the U.S. National Health and Nutrition Examination Survey (NHANES), the Canadian Health Measures Survey (CHMS), and the Korean National Environmental Health Survey (KoNEHS). The main HBM studies are listed in Table 1.1.

Table 1.1: Summary of main HBM studies in and outside Europe.

Country	Study	Population (N)	Biomarkers	Reference
Belgium	FLEHS I (2002-2005)	Mother-newborn pairs (1196)	POPs in serum or plasma (cord blood), Pb, Cd, in blood and urine; hydroxypyrene in urine	Schoeters et al., 2017
		Adolescents (14-15 y) (1679)		
		Adults (50-65 y) (1583)		
	FLEHS II (2007-2011)	Mother-newborn pairs (225)	POPs in serum or plasma (cord blood), Pb, Cd, As, Tl in blood and urine; hydroxypyrene, phthalates in urine	
		Adolescents (14-15 y) (210)		
	FLEHS III (2013-2014)	Mother-newborn pairs (281)		
		Adolescents (14-15 y) (208)		
		Adults (50-65 y) (209)		
Germany	GerES I (1985-86)	Adults 25-69y (2731)	Metals and essential elements in blood and urine, chlorophenoles in urine,	Kolossa-Gehring et al., 2012
	GerES II (1990-92)	Adults 18-79y (4287)		
		Children 6-17y (812)		
	GerES III (1998)	Adults 18-69y (4822)	Metals and essential elements in blood and urine, POPs, in blood; chlorophenoles, BPA, phthalates, PAH, organo-P and pyrethroid metabolites in urine[1]	
	GerES IV (2003-2006)	Children 3-14y (1790)		

Country	Study	Population (N)	Biomarkers	Reference
	ESB (1985-annually)	Students (120 each year)	Metals and essential elements in blood and urine[2], POPs, chlorophenoles, perfluoroalkyl-caboxylates and sulfonates in blood, chlorophenoles, BPA and phthalates in urine[2]	
France	HBM studies on specific pollutants	Since 1980	Metals (e.g. Pb in blood of children), PCBs and dioxins in serum of adults	Fréry et al., 2012
	ENNS (2006-2011)	Adults 18-74y (1949)	Metals, PCBs, pesticides in blood, urine, hair of adults, and hair of children; nutrition biomarkers	
		children 3-17y (1364)		
	National HBM (2008-)	Children and adults (6-74 y) (anticipated 5000 participants); neonates from ELFE study	Blood, urine, hair: environmental chemicals (>100) and nutrition biomarkers	
Italy	PROBE (2008-2010)	Adolescents 13-15y (252), urban areas of Latium region	Metals in blood	Pino et al., 2012
Spain	Pilot study (2007)	Adults 23-66y (170)	Pb, Cd, Hg in urine	Castaño et al., 2012
	BIOAMBIENT (2009-2010)	Working population 18–65 y (1880)	Pb in blood (n=1880), Cd in urine (n=1770)	Cañas et al., 2014; López-Herranz et al., 2015
Czech Republic	CZ-HBM (1994-2015)	Children 8-10y (5262)	Blood, urine and hair[3]	Cerná et al., 2017
		Adults 18-58y (6443)	Blood and urine[3]	
		Breastfeeding mothers (6054)	Breast milk[3]	
Romania	Various HBM activities in contaminated sites; Initiation of national HBM in 2008		Mostly Pb and As	Gurzau et al., 2012
Slovenia	National HBM (2007-2015)	Adults 18-49y (1084)	Trace elements in blood, urine, breast milk, Hg in hair	*Present thesis*
U.S.	NHANES (1999-2016)	All age ranges, from 1 year onward	A range of persistent and non-persistent chemicals in urine and blood	CDC, 2018
Canada	CHMS (2007-2019)	All age ranges, 3-79 years	279 chemicals in blood, urine or hair	Haines et al., 2017

Country	Study	Population (N)	Biomarkers	Reference
South Korea	KoNEHS (2009-2011)	19 years and older (6311)	Metals, BPA, PAHs, phthalates, pyrethroid insecticide, VOCs	Park et al., 2016
	KoNEHS (2012-2014)	19 years and older (6478)	21 chemicals (metals, PAHs, VOCs, phthalates, phenols, pesticides, tobacco smoke metabolites)	Choi et al., 2017

[1]morning urine; [2]24-h urine; [3] spectrum of biomarkers varied through time (metals were included in all campaigns).

The above-listed programs and other HBM activities use different study protocols and recruitment strategies, different population groups are recruited. Objectives of different HBM surveys span from assessing distribution of exposure, identifying and monitoring contaminated sites, monitoring spatial patterns and time trends, detecting emerging threats, monitoring the effects of policy interventions, to supporting epidemiological research (WHO, 2015). As a consequence, results cannot be compared nor can comparable health impact assessments be carried out. It has been recognized only recently that harmonized approaches are needed, including support activities to guarantee the acquisition of comparable results. The recent DEMOCOPHES study was the first attempt to perform harmonized HBM on a European scale, from study design, protocols to laboratory measurements and data analysis (Becker et al., 2014; Den Hond et al., 2015; Schindler et al., 2013). Furthermore, the on-going European HBM Initiative with its Horizon 2020 project HBM4EU is developing harmonized approaches for HBM in Europe (https://www.hbm4eu.eu/).

As described above, HBM is a key tool of exposure assessment in many epidemiological studies. The National Research Council (NRC, 2006) stated that the ultimate objective of biomonitoring is to link information on exposures, susceptibility, and effects to understand the public health implications of exposure to environmental chemicals. This can occur when HBM data is used with available epidemiologic, toxicological, and pharmacokinetic modeling data to estimate how much of a chemical has been absorbed into the body and to provide an indicator of potential health risk. However, epidemiological, toxicological, and exposure assessment studies have not adequately incorporated biomonitoring data for interpretation of health risks at the individual, community and population levels (NRC, 2006).

Regarding the epidemiological data, some studies, mostly birth cohorts, have been completed in Europe, some of them still on-going. Among the largest are the national French birth cohort study (ELFE) launched in 2011, the Spanish Environment and Childhood Research Network INMA finished in 2008, and the Public Health Impact of Long-term Mixed Element Exposure in Susceptible Population Strata (PHIME). The latter included birth cohorts from Greece, Italy, Croatia and Slovenia and continued with the Cross-Mediterranean Environment and Health Network (CROME). Among the main health outcomes, neurodevelopment was investigated in all these studies, the nervous system being the primary target for many environmental chemicals, particularly in the prenatal or early postnatal period of life.

The most recent ongoing epidemiological studies in Europe that aim at characterizing complete exposures to environmental chemicals following the "exposome" approach are the "Health and Environment-Wide Associations based on Large population Surveys" (HEALS), "The Human Early-Life Exposome" (HELIX) projects and EXPOsOMICS, all financed by the European Commission. In the U.S., Health and Exposome Research Center:

Understanding Lifetime Exposures (HERCULES) has been established by the National Institute of Environmental Health Sciences. Among them, particularly the HEALS project is focused on integrating 'omics' data and traditional HBM measurements with alterations in health outcomes to asses environmental exposures and human health associations. The projects bring together epidemiologists, chemists, molecular and systems biologists and bioinformatists, and include different 'omics' technologies, such as high-resolution metabolomics, (epi)genomics, adductomics, etc., to link exogenous exposures to internal chemical environment.

1.2 Overview of the Stages of Human Biomonitoring

The four stages of the HBM are study design, study conduct, data analysis, and communication and implementation of results. Each stage incorporates several steps, which are presented in Figure 1.1. The steps follow in chronological order and require interaction of several disciplines and processes. All the main steps, from study population and biomarker selection, laboratory and data analysis, to interpretation of the results, depend on the objectives of the study. Communication and ethical considerations are intrinsic in the design of HBM studies and present some of the most significant challenges with respect to interpretation and use of HBM data (NRC, 2006).

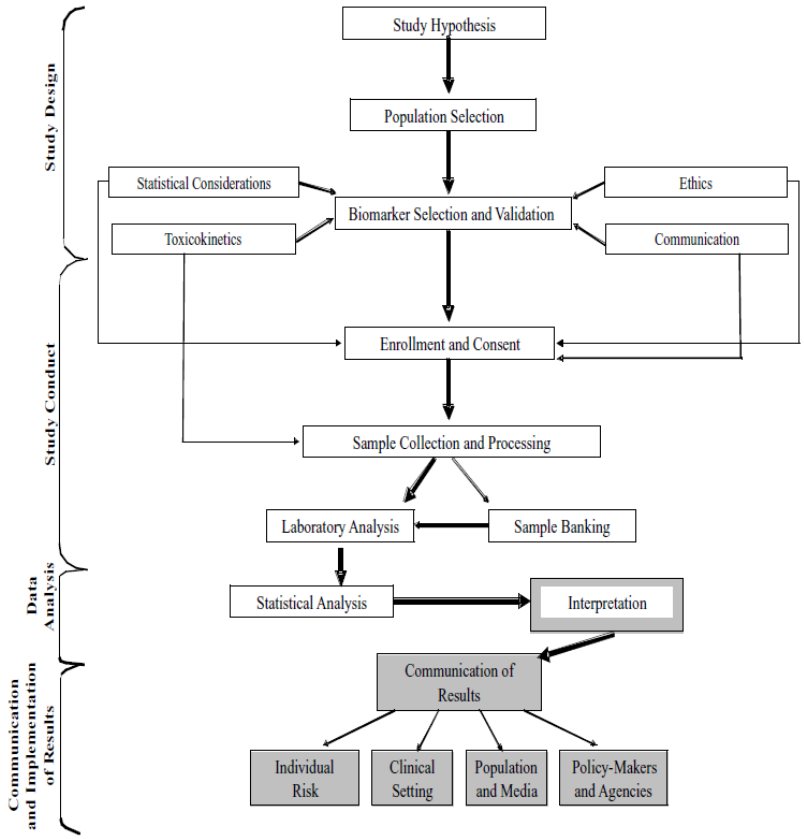


Figure 1.1: Stages of a biomonitoring study. Human biomonitoring study consists of study design, study conduct, data analysis and communication and interpretation of results (NRC, 2006).

1.2.1 Study design

Within the study design, *biomarker selection and validation* is a critical step and usually follows from study hypothesis and population selection. It is informed by statistical considerations, toxicokinetics, ethics, and communication. Biomarker selection should be driven by scientific criteria, such as expected health outcomes, available guidelines, expected exposure ranges, and availability of valid analytical techniques with appropriate detection limits (WHO, 2015).

The term "biomarker" was used by the WHO in 1993 in relation to risk assessment, as almost any measurement reflecting an interaction between a biological system and an environmental agent, which may be chemical, physical or biological. Interaction of chemicals with biological systems is documented by analysis of tissues and body fluids for chemicals, metabolites of chemicals, enzymes and other biochemical substances, referred to as "biomarkers" (WHO, 1993). Three classes of biomarkers were identified with respect to environmental chemicals:

- Biomarkers of exposure: an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism;
- Biomarkers of effect: a measurable biochemical, physiologic, behavioral, or other alteration within an organism that, depending on the magnitude, can be recognized as associated with an established or possible health impairment or disease;
- Biomarkers of susceptibility: an indicator of an inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic substance.

Relationship between exposure to a toxic chemical and its relevant health effects is illustrated in Figure 1.2 as a series of steps along a continuum. The further to the right in the continuum the biomarker, the greater the clinical or health relevance of its measurement (NRC, 2006). This also illustrates the potential use of biomarkers within the continuum, starting from measurements confirming and assessing the exposure to a particular substance, providing a link between *external exposures* and *internal dosimetry* (WHO, 1993). Secondly, biomarkers of effect can be used to document either preclinical alterations or adverse health effects elicited by external exposure and absorption of a chemical (WHO, 1993), whereat biomarkers of early effect may predict the occurrence of delayed adverse effects (Knudsen et al., 2012). Inter-linked to all steps in the continuum, biomarkers of susceptibility help elucidate the degree of the response to exposure elicited in individuals (WHO, 1993). The linkage between biomarkers of exposure and effect contributes to the definition of cause-effect and dose-response relationships used in the health risk of chemicals, which is the ultimate objective of the HBM, monitoring purposes and clinical diagnosis (Bernard, 1995; WHO, 1993). The relationships may be applied whether exposure has been from dietary, environmental or occupational sources.

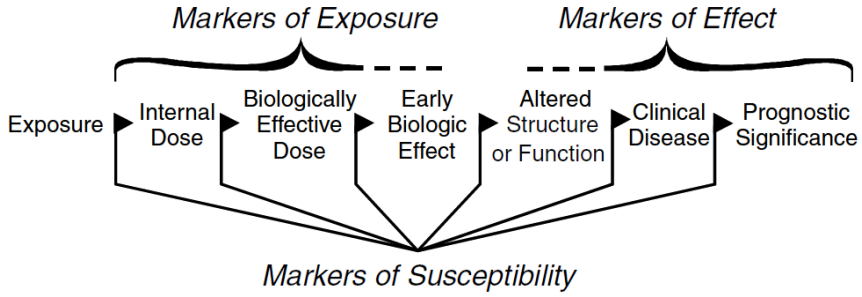


Figure 1.2: Simplified flow chart of classes of biomarkers (NRC, 2006).

Biomarkers of exposure are presented in Section 1.4 (Internal exposure assessment), with toxicokinetic background and selection of appropriate biomarkers for assessing exposure to Hg. Detailed information is provided in the book chapter "Mercury: Biomarkers of exposure and Human Biomonitoring" as part of this thesis in Section 3.1. Biomarkers of effects and susceptibility are summarized in Section 1.5 (Neurodevelopment), with a focus on Hg and neurodevelopmental outcomes, and the role of genetic variability in exposure and/or effect(s) of exposure.

1.2.2 Study conduct

Study conduct starts with the enrollment of study participants from the selected study population (recruitment). Based on the ethical requirements, each participant is informed about the study and signs the consent (WHO, 2015). Following the consent of the study participants, collection of the samples defined by the study design begins. This step is usually referred to as a fieldwork. Along with the study design, study conduct and implementation of the fieldwork has been well described for the pilot study of the HBM on the European scale, the DEMOCOPHES, by Becker et al. (2014). It includes a systematic approach, which can be applied also to many other studies. In relation to neurodevelopment and Hg exposure, the study design of the Mediterranean study PHIME with the fieldwork is described in detail by Valent et al. (2013). The fieldwork will not be explained in detail in this thesis.

Along with the fieldwork, laboratory analysis of the collected biological material is an important part of the study conduct. With the progress of the analytical technologies, low concentrations of chemicals in the body can be detected in relatively small quantities of biological material (e.g. urine, blood), therefore uncertainty of analytical measurements plays an important role, not only in the implementation of the study, but already in the study design. Measurement uncertainty refers to the variability of measurements of a specific analyte (JCGM, 2012) and is normally estimated for a defined concentration range within one laboratory. It is important because it assesses reliability of the measured result, its confidence, and comparability of the results. Variability of measurements, along with the variability within a population, defines how many subjects are required to observe significant differences between population groups or within one population on a time scale. The sample size is therefore defined by the study objectives, and requires statistical considerations.

We estimated measurement uncertainty of determination of total Hg in urine and blood by the commonly used method (cold vapor atomic absorption spectrometry, CVAAS) for the purpose of HBM studies in the general population, which has been published by WHO (WHO, 2018). As part of this thesis, analytical requirements for HBM were evaluated. We compared single-laboratory variability (measurement uncertainty), inter-laboratory

variability, and variability within a given population and demonstrated their relative contributions to the sample size required. The work is presented in the paper in Section 3.2. *"Analytical Quality Requirements in Human Biomonitoring Programs: trace elements in human blood".*

1.2.3 Data analysis and interpretation

Data analysis step includes collection and management of the data obtained throughout the study conduct, from measurements of selected chemicals in the collected specimens and accompanying data obtained from questionnaires filled in by the study participants. The latter includes socio-economic data, life-style and nutrition data, residential characteristics, etc. Analysis of the data has the potential to reveal major sources of exposure, geographic and temporal trends, and also identify risk factors. When connected to environmental monitoring data, multiple exposure sources can be quantified more accurately. Demonstration of identifying predictors of exposure has been done for the selected study population, representative for the adults in childbearing age residing in Slovenia and is presented in the publication *"Results of the first national human biomonitoring in Slovenia: trace elements in men and lactating women, predictors of exposure and reference values"* (Section 3.3). The paper adds to the methodological framework, as it builds statistical models from the available questionnaire data, as well as existing environmental monitoring data (elemental concentrations in soil, drinking water and moss samples).

Interpretation of the HBM data includes comparing the measured biomarker concentrations to health-relevant biomonitoring reference values, which is then the basis for risk assessment and measure(s) or intervention(s) taken up by the policy makers. However, health-based values, which are based on human exposure-response relationships, exist only for few chemicals (Angerer et al., 2011; NRC, 2006). For example, the German Human Biomonitoring Commission established HBM values (HBM-I and HBM-II values) which are either based on exposure-effect relationships (epidemiological data) or derived from tolerable daily intake values. The latter are based on the external dose (IPCS, 1994), therefore the derivation requires availability of basic toxicokinetic human data (Angerer et al., 2011). In cases health-based values are not available, population-based reference values or ranges are used to determine whether some study subjects have higher exposures than others. The population-based reference values are statistical descriptions of the ranges of concentrations typically observed in a specified reference population and are not relevant to health. They are intended to characterize the upper margin of the current background exposure; usually the 90^{th} or 95^{th} percentiles of the concentration values are defined as reference values (Ewers et al., 1999). The German HBM Commission uses the 95^{th} percentile of the measured pollutant concentration in the relevant matrix of the reference population, which is rounded off within the 95% confidence interval (CI) (Angerer et al., 2011). The theory of the reference values is described by the International Federation of Clinical Chemistry (IFCC) (Solberg, 1987a, 1987b). There are different types of reference values/intervals proposed, however, the most commonly used are those defined by fractiles. Central 0.95 fraction of the reference distribution is a frequently used convention for reference intervals, with reference limits as the 0.025 and 0.975 fractiles, accompanied by CIs (Solberg, 1987a). In case of potentially toxic elements, the 0.95 fractile (=95^{th} percentile) rounded off within the 95% CI is used as described above.

The representative population to establish the background exposure level for the Slovenian population by deriving population-based reference values (toxic elements) and intervals (essential elements) was selected from the adults in childbearing age living in 12 geographically distinct areas of Slovenia. Among women, *primiparous* mothers in the lactating phase were selected to enable estimation of background levels for infant's intake

through breast milk. This is the first time a reference population has been defined in Slovenia. The work is described in the paper "*Results of the first national human biomonitoring in Slovenia: trace elements in men and lactating women, predictors of exposure and reference values"* (Section 3.3.). This work is important also because the literature lacks consensus on values for the *prenatal* and *postpartum* periods. Most reference values are available for the general population of adults and children, e.g. for the German and Canadian populations (Saravanabhavan et al., 2017; Schulz et al., 2011), but not for the most vulnerable exposure periods. For the chemicals that pass placenta at least to a certain extent, it is believed that there are no safe limits for maternal blood levels (Taylor et al., 2014). The only recommendations that exist are for the Pb in blood of children and pregnant women, issued by the US Centers for Disease Control and Prevention (CDC), and for the Hg in cord or maternal blood issued by the US Environmental Protection Agency (EPA).

We have to keep in mind that reference values are informative by their nature: a measured value that is above the given reference value does not necessarily present a health risk, and a value below the reference value does not necessarily mean there is no risk. The HBM data is more challenging to interpret than external exposure data (e.g. personal air sampling data), because HBM data provides information on internal doses that are integrated across different routes of exposure and reflect the amount of chemicals that undergo ADME processes in the human body (NRC, 2006). For this reason it is important that exposure assessment accounts for as much confounding factors as feasible. Potential confounding factors are discussed in Sections 1.5.2-1.5.4.

1.2.4 Communication and implementation of the results

Communication plays an important role throughout the course of the HBM study. The strategy of communication depends on the objectives of the study and the target audience (WHO, 2015). This thesis does not deal with the communication aspect specifically, anyway, the communication was an integral part of all the studies conducted to deliver the presented results. It included informing study participants about their individual results, (unless they refused to receive their results), with a clear explanation of the individual results and in case of elevated exposure. The explanation was accompanied by relevant recommendation(s) to reduce the exposure and was given by a medical doctor, competent for trace elements exposures. Aggregated results were communicated to the general public and policy–makers through various workshops. As stated in the WHO publication on HBM, these interactions stimulate efforts to reduce exposure(s), they serve the specific policy needs in the country, and are important to discuss the public health significance of the data and provide basis to develop interventions (WHO, 2015).

1.3 Selection of Chemicals for HBM

Chemicals for HBM studies are usually selected based on the general criteria which include toxicity of a chemical (hazardous properties), public concern, regulatory demand, predicted exposure level, technical feasibility and a possibility to intervene, and usually follow recommendations of the agencies or organizations, such as World Health Organization, European Environmental Agency, etc. Among the top 10 chemicals of highest concern on The ATSDR Substance Priority List, which in addition to above-mentioned criteria prioritizes substances also based on their occurrence on the National Priority Lists, arsenic (As), lead (Pb), and Hg are ranked the first three, while cadmium (Cd) is ranked seventh (ATSDR, 2017). They are of high concern because they are toxic also at low doses, they

bioaccumulate in different organs and tissues, and can act as neurotoxicants. Methyl Hg, Pb, As and Mn have been recognized as developmental neurotoxicants, together with polychlorinated biphenyls, toluene, fluoride, chlorpyrifos, dichlorodiphenyltrichloroethane, tetrachloroethylene, and polybrominated diphenyl ethers (Grandjean & Landrigan, 2006; 2014). Although Mn is an essential element, it may act as a neurotoxicant in cases of excessive exposure, reported mainly in adults' occupational exposures (Dobson et al., 2004; Horning et al., 2015). Additionally, some epidemiological studies have indicated certain association between Cd and child's development with limited evidence. This element does not readily cross the blood-brain barrier, but indirect mechanisms are proposed (reviewed by Nordberg et al., 2018).

Mercury, Cd, Pb, and As have been recognized as priority substances also in the most recent on-going Europe-wide HBM project (HBM4EU, www.hbm4eu.eu). Other chemical groups from the HBM4EU priority list include phthalates and DINCH, bisphenols, flame retardants, poly-aromatic hydrocarbons (PAHs), perfluorinated alkyl substances (PFAS), hexavalent chromium (Cr(VI)), aprotic solvents, mycotoxins, pesticides, benzophenones and acrylamide.

1.3.1 Mercury

Mercury is among the many environmental chemicals which are known to have adverse impacts on human health via various toxicological mechanisms. The doses at which adverse health effects take place are not well established for many of these chemicals. Infants and children are especially susceptible due to their increased exposures to some chemicals, increased absorption rates and diminished ability to detoxify many exogenous compounds (Grandjean & Landrigan, 2006). However, based on the assessment of Hg exposure from seafood consumption in European countries, it was estimated that between 0.2 to 1.8 million children are born every year within the EU, with exposures exceeding the established threshold limits for developmental neurotoxicity (2.5 µg/g, 1.0 µg/g, and 0.58 µg/g, respectively), which is 4%–35% of the total EU annual births. In Slovenia, this number is assessed to be up to 4796 children each year (Bellanger et al., 2013).

Fish and other seafood is the most important source of non-occupational human exposure to Hg (Horvat et al., 2012; NRC, 2000). Most of the Hg consumed in fish or other seafood is in the organic form of highly absorbable monomethyl Hg (NRC, 2000; UNEP/WHO, 2008). Because of higher consumption of fish and other seafood, inhabitants of the Mediterranean countries are known to have higher Hg levels in their blood and/or hair than residents of other European countries (Den Hond et al., 2015; Miklavčič et al., 2013). As reported by the EFSA in 2012, tuna, swordfish, cod, whiting and pike were the major contributors to methyl Hg dietary exposure in the adult age groups, while the same species and hake were the most important contributors in the child age groups (EFSA CONTAM Panel, 2012). In the Slovenian market, the highest total and methyl Hg concentrations were also found in organisms at the top of the food chain, such as tuna, cod, conger, frog-fish, goatfish, swordfish, John Dory and European hake (Miklavčič et al., 2011). However, the mean content of Hg in both canned fish (0.130 mg/kg) and fresh fish (0.174 mg/kg) was low compared to the maximum allowed levels of 1 mg/kg (Regulation (EC) No. 87/2005). Based on the frequency of fish consumption and the levels of methyl and total Hg determined, the authors concluded that the fish available on the Slovenian market are not expected to represent a health risk (Miklavčič et al., 2011).

Dental amalgam fillings are the main source of exposure to elemental Hg in the general population (Clarkson, 2002; UNEP/WHO, 2008). Exposures to elemental and inorganic Hg forms can occur also due to the use of some skin-lightening creams and soaps, the presence of Hg in some traditional medicines, use of Hg in cultural practice, and due to various

accidental Hg spills in homes, school or other locations; in occupational settings (e.g. chlor-alkali plants, Hg mines, Hg-based small-scale gold and silver mining, refineries, thermometer factories, dental clinics with poor Hg handling practices, production of Hg-based chemicals); and through environmental contamination (Basu et al., 2018; UNEP/WHO, 2008). In Slovenia, highly elevated soil Hg concentrations in the Idrija town as a consequence of over 500 years of mining and smelting activities and of its natural geological occurrence, resulted in elevated Hg levels in foodstuff from that area (Kocman et al., 2011; Miklavčič et al., 2013), which present the potential pathway of inorganic Hg exposure in the residents of the town. Besides, certain locations within the town still have elevated Hg vapor levels in the air, particularly in the summer time, when higher temperatures occur. The town of Idrija has been thoroughly investigated in terms of environmental and biological monitoring (Hg concentrations in soil, water, air, and biota) and monitoring of occupational exposure in the mine workers. However, after the closure of the mine, none of the studies investigated the actual exposure in the residents of the town, non-occupationally exposed to Hg.

Detailed scientific background on Hg exposure is given in the book chapter presented among the published work (Chapter 3) in Section 3.1. Based on the background summarized in the present section, Hg exposure and its association with health outcome in this thesis refers to the general population mainly exposed to Hg from fish and other seafood consumption and partially also from dental amalgams. It is covered by papers presented in Sections 3.4 and 3.5. Exposure due to contamination from the past mining activities was assessed in the residents of the Hg-contaminated area of Idrija town and is presented in a separate paper in Section 3.6. Analysis of Hg in biological specimens presented in this thesis refers to the measurements of total Hg, and excludes any speciation of Hg. This is justified by the selection of appropriate exposure biomarkers that reflect exposure source and/or Hg species. Selection of biomarkers is given in Section 1.4 that follows.

1.3.2 Co-exposure to other potentially neurotoxic elements

In addition to Hg, other elements, potentially toxic and essential were included in the thesis, namely in the evaluation of quality requirements of HBM studies (Cd, Pb) and setting the background exposure levels for the representative Slovenian population of childbearing age (Cd, Pb, As, Se, Zn, Cu, Mn) (Sections 3.2 and 3.3). Co-exposure to these trace elements can occur through ambient air, foods, drinking water, soil or/and dust in the areas with various or even mixed industrial activities (past and/or current). In Slovenia, the two well-known heavily polluted areas are the town of Idrija with the former Hg mine, and the Upper Mežica Valley with residual pollution from a closed Pb and Zn mine with a smelting plant (Finžgar & Leštan, 2008; Miler & Gosar, 2012). Mixed-element exposure sites from past and current industrial activities are more or less spread across the country and include Lower Mežica Valley and Jesenice with an ironworks industry; Šaleška Valley with its thermo-power plant Šoštanj and the industrial area of Celje with its metallurgy, chemical-processing company and ironworks; and Sava Valley with its coal-mining activities, former antimony mine, thermo-power plant, cement plant, chemical company and glassware.

Lead is generally the most extensively studied toxic agent, with occupational and widespread exposure in the general environment (Skerfving & Bergdahl, 2015). Although worldwide blood Pb levels have decreased significantly over the past two decades, mainly due to the elimination of Pb in petrol in the beginning of the 90s (Jakubowski, 2012), substantial numbers of European residents still have levels of Pb in excess of national guidance or reference levels (WHO, 2015). In Slovenia, the 'hotspot' area of the Upper Mežica Valley has been monitored since 1976. In 2001-02, monitoring of three-year-old

children still showed higher blood levels as compared to children from the lower part of the valley, with more than one third of the children studied having blood Pb levels above 100 µg/L (Eržen & Janet, 2005). In contrast, a recent assessment of exposure to toxic inorganic species of As in 3-12-year-old children in the vicinity of the former Znojile antimony mine did not show elevated As levels in their urine samples (Perharič et al., 2017).

Apart from the site-specific environmental contamination, sources of exposure to trace elements in the general population can arise from diet and other life-style habits such as smoking, with several elements sharing the source. For example, exposure to As in the general population follows a similar exposure pathway as organic Hg – through fish and other seafood (Hughes, 2006) – but in the latter, As is mostly present in non-toxic organic forms, which are much less harmful to humans than inorganic As (Horvat et al., 2012). Food is a common exposure source in the general populations also for Pb (fruits, vegetables, and grains grown in Pb-containing soils) and Cd (shellfish and organ meats) (ATSDR, 2007, 2012). Moreover, drinking water in houses containing Pb pipes may contain Pb, especially if the water is acidic (ATSDR, 2007). Smoking (active or passive) is the major source of Cd exposure in the general population, and contributes to a smaller extent also to Pb exposure (ATSDR, 2007, 2012). Based on the available HBM data, Cd exposure of the majority of the European population is below the given health-based values, however a certain proportion of the population exceeds them. In the pilot European HBM study (DEMOCOPHES), the levels in the Slovenian mothers were rated 3rd among the 16 participating countries, while in children they were below the overall average (Berglund et al., 2015).

Other trace elements were considered in the thesis not only because of the mixed exposure due to geological background and/or industrial activities, but also because of interaction between the elements: either acting synergistically or antagonistically by diminishing the toxic effects. The investigation of association between Hg exposure and neurodevelopment therefore took into account co-exposure to Pb as a known developmental neurotoxicant, and Se, as an essential element with a known detoxifying role in Hg exposure (Sections 3.4–3.5). Mercury-Se interaction is further explained in Section 1.5.2.

1.4 Internal Exposure Assessment

Biomarker of exposure usually presents an analyte measured in human specimen (matrix), such as blood, urine, hair, nails, etc. Selection of an appropriate analyte, biological specimen and sampling time depends on the kinetics of a chemical of interest, which at the same time allows to determine whether the biomarker of exposure is likely to reflect the recent exposure or to integrate the exposure over a certain period of time. Toxicokinetic data must provide information on the absorption of the substance by the lung, the skin or the gastrointestinal tract, its distribution and possibly accumulation in the main body compartments, its biotransformation and, finally, its main routes of excretion (Bernard, 1995). The absorption, metabolism, distribution and elimination (ADME) processes in the human body can be quantitatively described through a variety of mathematical models (Bernard, 1995), among which the most advanced pharmacokinetic models are called the Physiology-Based Pharmacokinetic (PBPK) models, with a focus on the effective dose at the expected target site (described in WHO, 2015). They can significantly improve modern risk assessment (Sarigiannis & Karakitsios, 2019).

As a general rule, biomarkers of exposure to compounds that remain stable in the human body (e.g. metals, dioxins, PCBs) are measurements of the original compound concentrations in blood, plasma, serum or urine. For chemicals that are metabolized rapidly (e.g. phthalates, bisphenols, parabens, organophosphate pesticides, PAHs), one or more

metabolites of the original compound are often used as biomarkers of exposure, and are usually measured in urine (WHO, 2015). In contrast to biomarkers with short half-lives, for which the time of sampling is very critical, cumulative chemicals, such as metals, reflect exposure over a relatively long time (a few weeks up to a few months or years). For the latter, the sampling time is not critical (Bernard, 1995).

Most assessments of internal exposure rely on urine or blood samples, because of their accessibility. Within the exposome approach, assessment of multiple samples/biomarkers provides the best insight into overall exposures (Dennis et al., 2017). For this purpose, available biomarkers of exposure for a broad range of environmental stressors were summarized within the exposome project HEALS (Steckling et al., 2018). In the following paragraphs, matrices commonly used in HBM are critically reviewed.

Urine is a readily available matrix which is easily accessible in relatively large volumes. Urine sample can be collected directly by the donor which simplifies the fieldwork (WHO, 2015). Beside the rapidly metabolized and excreted chemicals, urine is also a useful matrix for some metals, particularly As and inorganic Hg (WHO, 2015). Problems related to urine as a biological matrix are related to the inter-individual variability in excretion rates, as well as daily variability within an individual (Aylward et al., 2014). For example, the assumption that urinary Cd concentration reflects the lifetime accumulation of the metal in the body has been questioned by recent studies revealing that low-level urinary Cd varies widely within and between individuals depending on urinary flow, urine collection protocol, and recent exposure, and can increase with increased excretion of plasma proteins (proteinuria) and essential elements deficiencies (Bernard, 2016). Although it is known that creatinine normalized levels predict the daily excretion of trace elements better than non-normalized levels for most of the elements due to systematic variation in urinary flow rates (Hays, Aylward, & Blount, 2015), specific gravity (SG) appears to be a more reliable alternative without the risk of over-adjustment and with fewer uncertainties associated with its use (Hoet et al., 2016; Stajnko et al., 2017).

Blood has the advantage that it is in contact with all tissues and in equilibrium with organs and tissues, therefore, it has been used extensively for various research and survey purposes (Smolders et al., 2009), mainly indicating recent exposure. For the metals that are mostly bound to red blood cells, the whole blood concentrations are normally used due to practicality reasons, however the packed cell volume (hematocrit) may vary among individuals. It is therefore advisable to consider the hematocrit or hemoglobin level (Skerfving & Bergdahl, 2015), especially when a high level of precision is required or in conditions that affect the hematocrit (e.g. anemia). Cord blood as a matrix for HBM incorporates the exposure history of both the mother and the early exposure of the newborn infant. Besides, it has the advantage that standard procedures for peripheral blood can be applied and at the same time provides a non-invasive sample (Smolders et al., 2009). However, depending on the sampling technique, the collected sample consists of (a) mixed - arterial and venous cord blood, (b) venous or (c) arterial cord blood. Concentration of the chemicals may differ significantly or insignificantly between the three types of samples, therefore, a standardized sampling procedure to collect venous blood through venipuncture has been described by the WHO standard operation procedure for prenatal Hg exposure assessment (WHO, 2018).

Hair is a simple widely-used non-invasive matrix to monitor both internal and external exposure to a variety of organic and inorganic pollutants (Smolders et al., 2009), and is particularly preferred in the case of Hg exposure through fish consumption (UNEP/WHO, 2008). As hair grows about one centimeter per month, analysis of hair provides an integrative and non-invasive sample for estimating mid- to long-term average exposure, depending on the hair segment analyzed. Hair is also useful in assessing long-term Pb exposure (Sanders et al., 2009). Although exogenous contamination and unreliable reference

values have limited the utility of scalp hair as a biomarker of chemical elements exposure, its use in various fields is growing and becoming more extensive (Pozebon et al. 2017). Fingernails and toenails can also be used as useful biomarkers, which has been demonstrated for prenatal organic Hg exposure, especially during the third trimester of gestation (Sakamoto et al., 2015) and in various studies examining long-term Pb exposure (Sanders et al., 2009). Likewise in the case of hair, it may be difficult to distinguish between contaminants taken up and those related to external contamination. Teeth are also a promising biomarker for prenatal exposures to metals and some organic chemicals, but like in the case of nails, standardized protocols and reference materials are lacking (Dennis et al., 2017).

Human milk is considered one of the most acceptable matrices for monitoring persistent bio-accumulating toxicants. In the lactation period, human milk is a major uptake route for environmental contaminants due to its fatty character, especially for *primiparous* mothers. Human milk is easy to collect, enriched in lipophilic compounds and represents the main exposure source for breast feeding infants (Smolders et al., 2009). Also other matrices, such as meconium, placenta, cord tissue saliva offer great potential for routine application in human biomonitoring, but their use and applicability is much less documented in the literature and standardized procedures for collection and exposure assessment are lacking.

1.4.1 Biomarkers of mercury exposure

Biomarkers to assess exposure to Hg are selected depending on the Hg species, route of exposure and the dose. The toxicokinetics of Hg is described in Section 3.1 of this thesis "*Mercury: Biomarkers of Exposure and Human Biomonitoring*".

Based on the distribution of Hg within the human body, exposure to elemental Hg is well represented by the presence of Hg in urine. As inorganic Hg is slowly excreted from kidneys through urine, urinary Hg reflects not only recent exposure to inorganic Hg, but also exposure that occurred in the past (Barregard, 1993; Mason et al., 2001). In an occupationally non-exposed population, the number of amalgam surfaces was found to be the best predictor for urinary Hg (Langworth et al., 1992). The presence of Hg in the blood indicates recent or current exposure to both, organic and inorganic Hg. In the general population, methyl Hg from fish and seafood, and Hg vapor from dental fillings contribute to Hg concentration in blood, while in occupationally exposed people, Hg vapor is the major contributor to blood Hg concentrations. Hg in hair is a good indicator for methyl Hg exposure through fish consumption, providing a simple, integrative, and non-invasive sample for estimating mid- to long-term average exposure (depending on the length of a hair sample) (UNEP/WHO, 2008). Assessment of methyl Hg exposure through measuring total Hg in hair is based on the assumption that methyl Hg in hair is proportional to the blood methyl Hg concentration and that almost 100 % of the total Hg in hair is methyl Hg (NRC, 2000). The latter is only true in populations with high fish consumption, whereas in populations with relatively low fish consumption, inorganic Hg might constitute a larger fraction of hair Hg. Besides, the exact relationship between Hg exposure and Hg excretion in hair follicles is not known, and it can vary greatly among individuals. Hair-to-blood ratios ranging from 140 to 690 have been reported for low to mediate exposure levels (Berglund et al., 2005; Horvat et al., 1991). Therefore, Hg concentration in blood is considered as a more accurate indicator of recent Hg exposure. As blood Hg represents both methyl and inorganic Hg exposure, speciation analysis is required to characterize the exposure, but not in cases below e.g. 1 µg/L as such exposure presents little risk and is analytically challenging (Horvat et al., 2012a).

As a marker of prenatal Hg exposure, Hg in umbilical cord blood is preferentially used. It represents methyl and metallic Hg exposure, as the two species move freely across placenta. Cord blood methyl Hg is almost twice as high as maternal blood methyl Hg, which is due to higher hematocrit and also hemoglobin levels in cord blood (Horvat et al., 2012a).

Infant exposure to Hg through breast milk is low compared to fetal exposure. However, based on the reported milk-to-plasma ratios, it seems that inorganic Hg is transported more easily to breast milk than methyl Hg (Björnberg Ask et al., 2005).

Although the present work excludes speciation analysis of Hg, the biomarkers of exposure have been selected to distinguish between different Hg species as justified above. Moreover, it has been demonstrated previously using exposure data in the Slovenian birth cohort that total Hg levels in cord blood and hair are suitable biomarkers of low-level Hg exposure through fish consumption (Miklavčič et al., 2011). However, speciation of Hg has been done in a separate work by Trdin et al. (2019), and will add to the refined characterization of exposure in the future studies, particularly for the purpose of association studies.

Reference doses or tolerable limits are focused mainly on methyl Hg exposure; the Reference Dose established by US EPA (0.1 µg/ kg day) corresponds to total Hg concentration of about 1 µg/g hair for children and women of child-bearing age or 5.8 µg/L cord blood, while updated calculations, supported by recent studies of developmental neurotoxicity at exposure levels close to the background, resulted in adjusted biological limit corresponding to 0.58 µg/g hair (Bellanger et al., 2013). On the other hand, a tolerable limit proposed by the WHO, which corresponds to a hair Hg concentration of approximately 2.5 µg/g takes into account the possible compensation of methyl Hg toxicity by beneficial nutrients in seafood.

The health-based values established for the Hg levels in urine are from the Human Biomonitoring Commission of the German Environmental Agency, and are HBM I 7 µg/L (5 µg/g creatinine) and HBM II 25 µg/L (20 µg/g creatinine).

1.5 Neurodevelopment

Although the exposure of people to various harmful substances in the environment is well recognized, effects on organism, its integrity and functioning are not entirely clear, particularly at low doses of exposures. The potentially neurotoxic elements, Hg, Pb, As, Mn, and with limited evidence also Cd, are known to have adverse impacts on human health via various toxicological mechanisms, however the doses at which adverse health effects take place are not well established. Their common mechanisms on a cellular level include mimicry, production of free radicals, and adduct formation with DNA or proteins (Keil et al., 2011). Free radicals production that is triggered by the presence of toxic metals, induces lipid peroxidation. This way, toxic metals can indirectly affect the nervous system. Brain is especially susceptible to oxidative attack because of the high lipid content, high rate of oxidative metabolism and low-level of free radical scavenging enzymes such as metallothionein, glutathione, heme oxygenase, and other stress proteins (Castoldi et al., 2001; Naarala et al., 1995). A number of studies have suggested the implication of such mechanisms in the neurotoxicity of methyl Hg. Methyl Hg-induced generation of reactive oxygen species seems to be a crucial event towards damage and cell death in different cell types, including neurons (Castoldi et al., 2001). Further, the capability of several toxicants to interfere with biochemical processes lies in the reactivity with sulfhydryl (SH-) groups of thiol-containing molecules (proteins, cysteine, and glutathione), changing the structure and therefore the function of proteins (Castoldi et al., 2001; NRC, 2000). The high affinity

of cation form of Hg (methyl Hg^{+}, Hg^{2+}) for SH- groups causes mitochondrial changes, lipid peroxidation, microtubule disruption, and disruption of protein synthesis. Another possible mechanism of interfering is the substitution for other polyvalent cations in their binding sites (Ca^{2+}, Zn^{2+}), whereat selectivity is limited to ions that are normally present in the cells. Mercury (and also Pb and Cd) cations are known to mimic cellular cations (Garza et al., 2006). All of the above-described mechanisms are responsible for damage that occurs in the nervous system and causes permanent dysfunction. The damage is mediated through direct promotion of cell death (disruption of microtubules; disruption of calcium homeostasis), primary damage of axons, secondary damage of neurons affected by neurotoxicants that target other cells in the nervous system, effects on myelin formation, and toxicant interactions with the signaling mechanisms of neurotransmission (Blake, 2004).

Various dysfunctions of nervous system have been reported from acute or chronic high-dose occupational exposures in adults and accidental poisoning episodes of certain industrial chemicals in the past. In contrast to this, we are now facing the so called global, silent pandemic of neurodevelopmental toxicity, important contributors of which are industrial chemicals widely distributed in the environment (Grandjean & Landrigan, 2006; Grandjean & Landrigan, 2014). Chronic low exposures to environmental neurotoxicants in *prenatal* or early *postnatal* periods can lead to various neuro-psychological decrements. In case of methyl Hg exposure, developmental dysfunctions in the domains of language, attention, and memory, and to a lesser extent in visual-spatial and motor functions were observed in a Faroe Islands birth cohort (Grandjean et al., 1997), which is along with the Seychelles and New Zealand studies, one of the major longitudinal studies on Hg exposure performed. It showed mean cord blood Hg of 23 µg/L, and mean maternal hair Hg 4.3 µg/g. A number of other cross-sectional and prospective cohort studies assessed the neurodevelopmental effects of chronic low-to-moderate prenatal methyl Hg exposure from fish consumption, but inconsistent findings have been reported (Valent et al., 2013). Despite this, there is evidence that low levels of prenatal Hg exposure may cause early childhood neurocognitive effects (Karagas et al., 2012). The overview of the recent epidemiological studies dealing with low-level chronic exposure to Hg, mainly from fish consumption, is given in Table 1.2. Although the central nervous system is an equally sensitive target of Hg° vapor exposure as it is for methyl Hg, and the transfer of inhaled Hg° to the developing fetus has been observed (Clarkson, 1989; Clarkson, 2002), most of the studies on potential neurotoxic effects of Hg° vapor exposure refer to occupational settings in adults. Therefore, the given overview of epidemiological studies on prenatal Hg exposure is limited to methyl Hg exposure from seafood consumption.

Based on the evidence it is clear that at such low levels of chronic exposure, diet and therefore nutrient status plays an important role as a non-genetic factor in exposure and consequentially its response. Although a major source of methyl Hg, fish are considered especially important to prenatal development of the human nervous system due to their contents of n-3 polyunsaturated fatty acids (PUFAs), particularly docosahexaenoic acid, as important components of neuronal membranes (Janssen & Kiliaan, 2014; Mozaffarian & Rimm, 2006). This has raised numerous debates on risks and benefits of fish consumption during pregnancy. Strain et al. (2015) hypothesized and confirmed that n-3 PUFA and other nutrients contained in fish might exceed possible neurotoxic effects. Furthermore, a physiological balance between n-6 and n-3 PUFAs is important, as both compete for the same enzymes in the biosynthesis and incorporation into cell membranes. The n-6 PUFAs act pro-inflammatory, and a high n-6/n-3 ratio may increase any possible inflammations in the brain resulting from the oxidative damage caused by methyl Hg exposure (Janssen & Kiliaan, 2014).

Table 1.2: Overview of recently published (2015-) associations between prenatal Hg exposure and neuropsychological performance of children, accounting for non-genetic confounders related to fish intake.

Cohort study	Biomarker, Mean level	Nutritional confounders	Psychological assessment (age)	Association with internal exposure to Hg	Reference
Seychelles Child Development Study N=1265	Maternal hair Hg, 3.9 µg/g	-	Different ages, up to 24 years	No direct associations between prenatal Hg and neurodevelopmental outcomes at different ages	Wijngaarden et al., 2017
		PUFAs	BSID-II (20 months)	Lower PDI in children with higher n-6/n-3 PUFA ratio	Strain et al., 2015
Korean prospective Cohort N=1076	Maternal blood Hg, 3.3 µg/L	Fish intake, n-6, n-3 PUFAs	BSID-II (6 months)	Lower early cognition (MDI, PDI) (n=847)	Kim et al., 2018
		Fish intake	WPPSI-R (60 months)	Lower verbal IQ (n=445)	Jeong et al., 2017
INMA, Spain N=1362	Cord blood Hg, 8.8 µg/L	-	MSCA (4-5 years)	Doubling in cord blood Hg associated with higher scores in most of the MSCA scales	Sabrina Llop et al., 2016
		Fish intake	MSCA (4-5 years)	Lower MSCA scores in children, whose mothers ate less than 3 fish servings/week during 1st semester of pregnancy	
		n-6, n-3 PUFAs	MSCA (4-5 years)	Lower motor scores in children with an n-6/n-3 FA ratio above the median	
Norwegian mother and child cohort N=46,750	calculated from reported fish intake	Fish intake, n-3 PUFAs	Dale and Bishop grammar rating, ASQ	unintelligible speech, weaker communication skills	Vejrup et al., 2018
Mediterranean birth cohort[1] N=1308	Cord blood Hg, 3.6 ng/g; Maternal hair Hg, 704 ng/g	Fish intake	Bayley-III (18 months)	inverse relation with motor scores; no evidence of detrimental effects of Hg for cognitive and language outcomes	Barbone et al., 2019 (*present thesis*)

[1]Includes Italy, Slovenia, Croatia and Greece.

PUFAs – poly-unsaturated fatty acids; FAs – fatty acids; BSID-II – Bayley Scales of Infant Development, 2nd Edition; Bayley-III - Bayley Scales of Infant and Toddler Development, 3rd Edition; WPPSI-R - Wechsler Preschool and Primary Scale of Intelligence; MSCA – McCarthy Scales of Children's Abilities; ASQ – Ages and Stages communication scale; PDI – Psychomotor Developmental Index; MDI – Mental Developmental Index

Overall, a prevalence of neurodevelopmental disabilities seem to be increasing and among the known causes for this rise are environmental pollutants, such as methyl Hg, Pb, As, and also Mn. Exposures to pollutants with neurotoxic potential are of particular concern in the fetal life and early infancy (*prenatal* and early *postnatal* periods), and may contribute importantly to causation of latent impairments later in life, causing for example Parkinson's and Alzheimer's disease (Chen et al., 2016; Landrigan & Todd, 1994). Mercury (organic forms) is also among the most prominent possible causes of autism spectrum disorders, of course in combination with other factors (e.g. diet, genetics) (Schofield, 2016).

1.5.1 Monitoring of alterations in neurodevelopment

In line with the above-given definition of a biomarker of effect (»a measurable biochemical, physiologic, behavioral, or other alteration within an organism that, depending on the magnitude, can be recognized as associated with an established or possible health impairment or disease«), a biomarker of neurotoxicity could therefore be any significant biochemical change that occurs in the nervous system and which precedes permanent dysfunction of the nervous system.

Biomarkers of effects generally used in HBM or epidemiological studies are summarized by Smolders et al. (2009) and include from DNA damage biomarkers and gene expression profiling of the fetus using placenta and cord blood; and newer 'omics' technologies, like metabolic profiling (metabolomics and metabonomics) in urine. The non-specific responses elicited by potentially toxic elements can be detected through oxidative stress biomarkers (e.g. activity of antioxidant enzymes, such as glutathione peroxidase – GPx, superoxide dismutase – SOD and catalase – CAT), and urinary 8-hydroxy-deoxyguanosine (8-OHdG) which has been widely used as a biomarker of the DNA repair response to oxidative stress (Flora, 2014). Activities of antioxidant enzymes have been monitored as part of this thesis in a small group of pregnant women residing in the former Hg mine area to assess biological response as a result of hypothetically elevated exposure to Hg vapor (Section 3.6).

Common tests for assessing neurotoxic effects are classified as observational, neurobehavioral, neurophysiological (nerve conduction velocity, evoked potentials, electro-encephalogram), physiological (muscle strength, temperature regulation, reflexes), and biochemical (alternations in levels of hormones, enzymes or other biological markers) (Fan et al., 1995). Behavioral and functional tests are quite sensitive, capable of detecting effects of chemicals that would be overlooked using standard pathology measures. However, in contrast to most structural changes observed in pathology examinations, not every change in a measured parameter is toxicologically relevant (Fan et al., 1995). In addition, neurobehavioral examinations are difficult to localize the site of toxic action (e.g. sensorial-motor tests can indicate either neuromuscular or psychomotor damage) (Blake, 2004). Electrophysiological techniques can help to focus an investigation to the site of a lesion, and characterize electrical dysfunction within the damaged nerves. Neuro-imaging methods can produce images of the brain that show structural changes in volume/density of a specific region, or determine functional biochemical changes in processes like receptor binding (using radioactive tracer molecules) (Blake, 2004). The use of these non-invasive techniques may be of certain usefulness in particular cases, but widespread application to neuro-toxicological investigation is doubtful (Costa & Manzo, 1995). For example, in the Croatian birth cohort established in the PHIME project, neurosonographic exam has been used and descriptive analyses showed maternal pregnancy hair Hg >1 µg/g was associated with a smaller cerebellar volume (Cace et al., 2011).

However, since neurotoxicants cause biochemical and molecular events which indicate early stage effects well before or well below the induction of a clinically detectable effects, monitoring of these early events has been developing to assess sub-clinical effects. However,

the use of biochemical markers in neurotoxicology is problematic due to the complexity of the central nervous function, the multistage nature of events, and inaccessibility of target tissue (Manzo et al., 2001).

Because of the feasibility issues and cost and at the same time sufficient sensitivity, neuro-epidemiological studies, including those listed above, most often rely on neurobehavioral tests and some also on neurophysiological examinations. In our birth cohort study, associations between prenatal Hg exposure and neurodevelopment of children at 18 months of age were assessed using a standardized individually administered developmental assessment – the Bayley Scales of Infant and Toddler Development, Third Edition (Bayley-III, sometimes abbreviated as BSID-III) (Bayley, 2006). The study is presented in the papers presented in Sections 3.4 and 3.5. The first paper assessed the overall association at low levels of Hg exposure accounting for general, non-genetic, factors that could confound the association; while the second paper additionally accounted for genetic background of apolipoprotein E. Potential non-genetic and genetic factors used in HBM and epidemiological studies are described in the following sections.

1.5.2 Non-genetic factors influencing the assessment of internal exposure and its effects

Dietary components, namely n-3 PUFAs in fish, have already been explained above as an important factor that could influence developmental effects of methyl Hg, particularly at low levels. However, there are many other components, like essential elements and vitamins that can interact with toxic metals (and vice versa), influencing their absorption, distribution, metabolism and elimination in the human body and consequentially the biological response on the developmental level.

An essential element Se, that is a well-known Hg antagonist, is also contained in relatively high amounts in fish. It protects cells against the toxic effects of Hg in a number of different organisms (Chen et al., 2006; Falnoga & Tušek-Žnidarič, 2007), acting through Se-proteins or by forming biologically inactive Se-Hg complexes (Goyer, 1995; Suzuki et al., 1998). Several epidemiological studies have already demonstrated potential involvement of Se status in the development of children: a prospective birth cohort study set in the rural Bangladesh showed low prenatal Se status as disadvantageous for children's psychomotor and language development assessed at 1.5 years using BSID-II (maternal erythrocyte Se 0.19-0.87 mg/g hemoglobin) (Skröder et al., 2014); moreover a low maternal blood Se (≤0.90 µmol/L or 71 µg/L) in pregnancy was found to be associated with a lower psychomotor score (assessed using ASQ) at 6 months in children of otherwise healthy mothers from Norway (Varsi et al., 2017).

Similarly as in the case of Hg toxicity, Se can protect also against the harmful effects of As by forming inactive complexes (Zeng et al., 2005). Besides Se, other essential elements (e.g. Cu, Zn) also act through their protective role against free radicals damage (through metallothioneins, superoxide dismutases, etc.), which is triggered by toxic elements in general. Deficiency of essential elements can therefore increase the manifestation of the toxic effect of non-essential metals (Goyer, 1995; Rahman et al., 2019). Furthermore, the status of essential elements (particularly Zn and Fe) influences intestinal absorption of Cd and Pb. For example, iron (Fe) deficiency is known to increase Pb absorption, because Pb competes with Fe for ferritin (intra-cellular Fe-storing protein) binding sites. It is therefore a critical factor in children who are at risk for Pb toxicity (Goyer, 1995). A tendency towards higher blood Pb levels was demonstrated in a group of children from an industrial area in Katowice, Poland, who had low serum ferritin levels (Osman et al., 1998). Body Fe influences also intestinal absorption of Cd (Rahman et al., 2019). Furthermore, adequate vitamin D is associated with higher absorption of essential elements, but also enhanced

absorption of potentially toxic elements, e.g. Cd and Pb (Moon, 1994; Schwalfenberg & Genuis, 2015). Of course, it should be noted that essential metals can also be toxic in cases of excessive exposure.

Along with the specific chemical factors, exposure and its response can be confounded by more general confounders, like sex, socio-economic status, mental stress, climate, etc., which are in the exposome era considered as the "general external exposome" (Wild, 2012). These factors are usually obtained through interviews or questionnaires filled in by study participants. Also the data on frequency of consumption of specific dietary items, which is a crucial component of assessing the contribution to exposure from a specific source and a putative confounding of nutritional components in association studies. However, questionnaire data have limited utility (uncertainty of data due to inaccurate recall or misunderstanding of questions), but are often the only source of information available (Coggon, 1995). The validity is critical particular for the dietary information. With the advancement of analytical methods, promising tools are available that could increase the accuracy of dietary information. For example, PUFAs can be determined in biological specimens using isotope-ratio techniques (13C/12C) and enable identification of the source (marine vs. terrestrial). However, the methods are in progress of validation (Jagodic et al., *submitted*).

Among the listed confounders, the present thesis includes monitoring of essential elements (Se, Cu, Zn and also Mn) as part of the methodological framework of the HBM, which enabled establishment of the background levels in the Slovenian general population. Furthermore, Se was used as an important component in assessing prenatal Hg exposure in relation to methyl Hg from fish and inorganic Hg from the contaminated environment and its association with neurodevelopment (Sections 3.5 and 3.6). Adequate levels of micronutrients that are required to maintain the physiological function, are particularly important in pregnancy or *postpartum* – the periods of higher physiological demand for micronutrients (Izquierdo Álvarez et al., 2007). If the requirements are not met, this could lead to enhanced absorption of potentially toxic elements and/or increased manifestation of their effects. The thesis accounted also for the general confounders, which were obtained from carefully prepared questionnaires, namely sex, age, birthweight, weight and height of the mother, residential information, socio-economic status, education, smoking status and dietary habits. Specific confounders are explained in the separate papers as part of the thesis.

1.5.3 Genetic factors influencing the assessment of internal exposure and its effects

Besides nutritional and other non-genetic confounders, an increasing amount of evidence demonstrates that genetic variability of proteins involved in absorption, transport, elimination or distribution of Hg and other potentially toxic metals in the human body significantly influences (either decrease or increase) the manifestation of toxic effects in an individual, especially at chronic low exposures. Furthermore, the modifying effect of genetic predisposition can be direct, influencing the exertion of the toxic effect itself (Basu et al., 2014; Julvez & Grandjean, 2013).

In general, many common, complex traits are believed to be a result of the combined effect of genes, environmental factors, and their interactions (Murcray et al., 2009). Large scale studies of genetic epidemiology have been enabled after completion of the Human Genome Project and HapMap Project and of course rapid advances of genotyping technology (Reid-Lombardo & Petersen, 2010). The following large-scale high-throughput sequencing studies, such as the 1000 Genomes Project, have already identified millions of

genetic variants among individuals from different populations (The 1000 Genomes Project Consortium, 2015), which is creating a deep and robust catalogue of human genetic variation (Reid-Lombardo & Petersen, 2010).

Genetic variation can determine the human's unique susceptibility to a disease or toxic effect of an environmental pollutant. It is a result of mutations in DNA throughout the whole genome in the coding and noncoding parts of DNA. If these variations do not cause any severe alternations, they can persist over generations. If a given variant increases to >1% frequency in a population, it is called a polymorphism (Reid-Lombardo & Petersen, 2010). Frequently studied variants/polymorphisms are insertion or deletions, copy number variations (CNVs), and single nucleotide polymorphisms (SNPs). A SNP is the most common type of polymorphism, and it has two alternative forms – alleles – where the adenine (A) base is replaced by guanine (G), and thymine (T) by cytosine (C) (Figure 1.3). The frequency of the least frequent allele of a SNP in a population is termed the minor allele frequency, which can vary among populations (Reid-Lombardo & Petersen, 2010). Every person has a large number of variants, but the minor allele frequencies of most individual variants are relatively rare. Understanding relations between genetic polymorphisms and environmental exposures can help identify high-risk subgroups and also gives better insight into pathway mechanisms (Murcray et al., 2009).

Associations between genome and environment and/or disease can be studies using two general approaches (Reid-Lombardo & Petersen, 2010). The first approach is the genome-wide association study (GWAS), where a large number of SNPs is screened to identify potential susceptibility genes, comparing subjects with high exposure / disease (cases) and subjects with low exposure / without the disease (controls). The ultimate goal of GWAS is to identify novel pathways or susceptibility genes. The second approach is the candidate gene association study and unlike GWAS it is hypothesis driven, with a priori selection of genes. The genes are usually selected from the knowledge on pathways involved in disease / toxic effect or ADME of environmental pollutants. So far, significant associations between environmental exposures and genetic polymorphisms have been demonstrated by various studies using the second – targeted – approach.

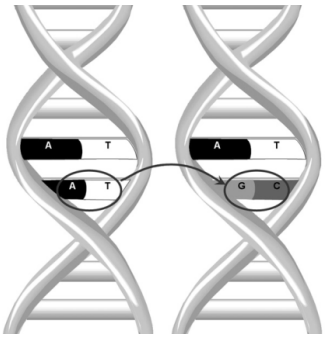


Figure 1.3: Illustration of single nucleotide polymorphism (SNP) (Reid-Lombardo & Petersen, 2010).

Firm associations have been established between several polymorphic genes and Hg exposure and/or effects caused by the exposure. Many studies reported associations for glutathione (GSH) metabolism related genes, metallothionein (MT) genes and others related to toxicokinetics of Hg or other elements (Table 1.3). Direct associations with neuropsychological performance have been extensively studied in the longitudinal UK cohort, ALSPAC, where an absence of any Hg neurotoxicity at low average exposure levels was observed, but the study discovered adverse associations among carriers of certain SNP

allelic variants (Julvez et al., 2013). Four SNPs functionally related to the brain-derived neurotrophic factor (*BDNF*), paraoxonase 1 (*PON1*), transferrin (*TF*) and progesterone receptor (*PGR*) genes appeared to modify the Hg-outcome associations with cognitive deficits in children carrying minor alleles, as indicated by decreased IQ scores. The 10-fold increase in cord blood Hg resulted in decrease in IQ for 25 points in children with at least 4 mutations (Julvez et al., 2013). Further, the importance of such genetic predisposition is illustrated by the fact that 21 % of the cohort subjects had at least four minor alleles in the four SNPs identified. Many other gene polymorphisms have been shown by different studies to modify neuropsychological outcome caused by potentially toxic metals, particularly Hg or Pb. The studies are reviewed in Table 1.3 below.

Table 1.3: Gene mutations affecting absorption-distribution-metabolism-elimination of metals and neuropsychological performance.

Polymorphic genes	Role of enzyme/protein	Associations between polymorphisms and exposure
GCL (glutamyl-cystein ligase) genes: *GCLC, GCLM*	The first and rate-limiting enzyme for GSH synthesis	*GCLM* – Hg in erythrocytes (Schläwicke Engström et al., 2008); *GCLC* – Hg in cord tissue (Julvez et al., 2013), or adult's blood (Gundacker et al., 2009); *GCLM* – Hg in hair and blood (Barcelos et al., 2013)
GST (glutathione-S-transferase) genes: *GSTP1, GSTM1, GSTT1, GSTO,…*	Catalyse conjugation of GSH to xenobiotic substrate	*GSTP1* – Hg in erythrocytes (Schläwicke Engström et al., 2008); *GSTM1* – Hg in blood and hair (Barcelos et al., 2013); GSTT1, GSTM1 – Hg in blood (Gundacker et al., 2007); GSTT1 – Hg in urine of dentists; GSTP1 – Hg in hair of dentists (Goodrich et al., 2011)
MT (metallothionein) genes: *MT2A, MT3, MT4, MT1A, MT1M,…*	Metal-binding protein involved in regulation of metal homeostasis (Zn, Cu); toxic metal binding; protection against oxidative stress	*MT4* – Hg in urine (Gundacker et al., 2009); *MT2A* - general cognitive functioning (Julvez et al., 2013); Cd, Pb and Zn in blood (Kayaaltı et al., 2011); MT1M, MT2A – modification of association between Hg in urine and neurobehavioral effects in children (Woods et al., 2013)
ABC transporter (multidrug resistance) genes: *ABCC1 (=MRP1), ABCC2 (=MRP2), ABCB1 (=MDR1)*	Responsible for active transport of xenobiotics (and other compounds) across biological membranes	*ABCC2* – Hg in urine of gold miners (Engström et al., 2013); *ABCB1*, *ABCC1*, *ABCC2* - Hg in cord blood (Sabrina Llop et al., 2014)
Selenoprotein genes: *SEPP1* (Selenoprotein P), *GPx1,3,4,..* (GSH peroxidase)	Se homeostasis, delivery in tissues (particularly brain and testes), antioxidant activity, decrease of lipid peroxides, metal binding	*SEPP1* - hair or urine Hg levels of dental professionals (Goodrich et al., 2011)
ALAD (γ - aminolevulinic acid dehydratase),	Pb uptake and distribution in the body:	*ALAD2* carriers higher binding capacities for Pb in red blood cells;

Polymorphic genes	Role of enzyme/protein	Associations between polymorphisms and exposure
VDR (vitamin D receptor)	ALAD (enzyme required in heme synthesis; presumably the major intracellular ligand for lead in red blood cells), VDR (intestine calcium absorption and storage in bone)	*VDR* influences Pb accumulation in bones (reviewed by Onalaja & Claudio, 2000); *VDR* and *VDR/ALAD* – Pb in urine (Gundacker et al., 2009)
AS3MT (As (III) methyl transferase)	Methylation of As	AS3MT haplotype – As methylation efficiency (MMA and DMA in urine) (Engström et al., 2011b; Schlebusch et al., 2015)
APOE (apolipoprotein) gene *Apoe* alleles: ε2, ε3, ε4	A lipid-transporting protein, with highest expression in liver and brain (astrocytes, microglia); ε4 allele associated with poor neural repair function	*APOE* –association between Hg and neurodevelopment and behaviour (Ng et al., 2013, 2015)
CPOX (coproporphyrinogen oxidase)	Impacts biosynthesis of heme (catalyses decarboxylation of coproporphyrinogens to precursor of heme) - a likely mitigating factor in neurological signalling.	*CPOX4* – affected association between Hg in urine and neuropsychological performance of dentist professionals (Echeverria et al., 2010, 2006); Hg in urine and neurobehavioral effects of children (Woods et al., 2012); Hg and general cognitive function of children (Julvez et al., 2013)
COMT (catechol O-methyltransferase)	Catecholamine catabolic enzyme important in the methylation of endogenous and exogenous catechol compounds, including catechol neurotransmitters (dopamine, norepinephrine, epinephrine) and oestrogens	*COMT* - Hg in umbilical cord (Julvez et al., 2013); affected association between Hg in urine of dentist professionals and self-reported symptoms and mood (Heyer et al., 2009)
BDNF (brain-derived neurotrophic factor)	Growth and differentiation of neurons and synapses; supports survival of existing ones	Among 40 SNPs analysed, *BDNF*, *PON1*, *TF* and *PGR* showed modification of associations between Hg in cord and cognitive deficits in children with the minor alleles (Julvez et al., 2013).
PON1 (Paraoxonase 1)	Inhibits oxidation of lipoproteins through hydrolysis of lipid peroxides	
TF (transferrin)	Iron uptake	
PGR (progesterone receptor)	Brain development and neurotransmitter metabolism	
CYP3A (cytochrome p450 3A)	Oxidative metabolism of xenobiotics	*CYP3A7* and *5* – Hg in cord blood and improved neurodevelopment (Llop et al., 2017)

Apolipoprotein E (APOE) is the major lipid-transporting protein with the highest expression in liver and brain (mainly in astrocytes). Being involved in delivery of cholesterol to neurons, it has a major role in neurite outgrowth and synaptogenesis (Xu et al., 2014). All those events are important for learning, memory formation and neuronal repair, as they depend on availability of cholesterol (Mauch et al., 2001). There are three major isoforms

of APOE, encoded by the alleles epsilon 2 (ε2), epsilon 3 (ε3) and epsilon 4 (ε4) (Zannis & Breslow, 1981). They give rise to three homozygous (ε2/ ε2, ε3/ ε3, and ε4/ ε4) and three heterozygous phenotypes (ε3/ ε2, ε4/ ε3, and ε4/ ε2) (Mahley, 1988). The isoforms differ in the two amino acids at residues 112 and 158 of the protein: APOE2 has two cysteine residues, APOE3 one cysteine and one arginine, and APOE4 two arginine residues. Epsilon 3 allele is presently the most common one (Fonseca Arrifano et al., 2018). *APOE* ε4 allele is recognized as one of the strongest genetic risk factors for the development of late onset Alzheimer's disease (Buttini et al., 1999; Xu et al., 2014). However, research with younger populations of ε4 carriers has suggested that the ε4 allele may in fact be beneficial in earlier ages (Tuminello & Han, 2011). *APOE* is capable of influencing either neurodevelopment or modify the response to maternal diet or maternal exposure to environmental pollutants (Wright et al., 2003). The authors suggested that ε4 allele has a beneficial effect on neurodevelopment because higher scores were observed among ε4 carriers in comparison to either ε2 or ε3 carriers at 24 month of age. In addition, the Bayley mental development score of ε4 carriers had a less steep dose-response to cord Pb levels than the response in subjects with either the ε2 or ε3 allele (Wright et al., 2003). On the other side, (Ng et al., 2013) reported that the ε4 carriers prenatally exposed to methyl Hg (median cord blood total Hg was 12.2 µg/L) had poorer neurodevelopment at 2 years of age as assessed by Comprehensive Developmental Inventory for Infants and Toddlers than ε2 and ε3 carriers. One of the latest studies has found also neurobehavioral problems associated with traffic-related pollution (PAH, NO_2) in ε4 carriers (Alemany et al., 2018).

The potential role of *APOE* genetic polymorphism as a modifying factor in Hg exposure and neurodevelopment in the Slovenian and Croatian birth cohorts was investigated in the paper »*Prenatal mercury exposure, neurodevelopment and apolipoprotein E genetic polymorphism*« (Snoj Tratnik et al., 2017) as part of this thesis.

1.5.4 Genetic tolerance

The above-described aspect of genetic susceptibility focuses mainly on increased susceptibility to exposure/potential effects in relation to genetic variability. However, several studies showed that in certain populations or individuals, decreased susceptibility (tolerance) can be observed as a result of continuous environmental contamination. For example, the most frequent *AS3MT* haplotype in an Argentinian population consuming drinking water containing about 200 µg As/L was associated with higher methylation efficiency which decreased the risk for As toxicity (Engström et al., 2011). Similarly, in an exposed Vietnamese population (groundwater As levels 0.7-502 µg/L) three *AS3MT* SNP variants were found to have a strong association with higher As methylation capacity (Agusa et al., 2009). Just recently, the protective *AS3MT* haplotype was confirmed also in the Slovenian population of women in childbearing age and their children (Stajnko et al., 2019).

Chapter 2

Aims and Hypothesis

The main aim of the thesis was (1) to assess Hg exposure of the Slovenian population of childbearing age and their children, and (2) its association with neurodevelopment, referring to the exposure in the prenatal period. We aimed to assess also co-exposure to other potentially toxic elements (Pb, Cd, As), essential elements (Mn, Se, Zn, Cu), and the *APOE* genotype (4 versus 2/ 3), as these factors can confound exposure and/or potential effects of Hg. To identify potential sources and other predictors of exposure, the data obtained from the questionnaires and available environmental databases was used.

2.1 Specific Aims

- Set the methodological framework for the assessment of low-level Hg exposure and its link to neurodevelopment. This includes evaluation of analytical quality requirements; definition of the reference population living in Slovenia to assess the background exposure; and evaluation of biomarkers of exposure including confounding factors.
- Assessment of exposure to Hg and other potentially toxic elements in the Slovenian population, focusing on the most vulnerable population groups – adults in childbearing age and their children.
- Construction of statistical model to assess association between low-level prenatal Hg exposure and neurodevelopment (cognitive, language and motor), including all potential factors that could influence exposure and/or neurodevelopmental outcome (co-exposure to other potentially neurotoxic elements, essential elements status, life-style habits).
- Evaluation of the role of *APOE* genetic polymorphism in association between the Hg exposure and neurodevelopment.
- Assessment of Hg exposure in the residents of the Idrija town – the former Hg mine area – and comparison to other study areas.

2.2 Hypothesis

The Slovenian childbearing population and their children have low to moderate exposure to Hg, as consumption of fish and other seafood is low, and contribution from dental amalgam fillings is moderate. The exposure of the residents of the Idrija town is expected to be higher than in the residents of other areas, particularly to elemental or inorganic Hg, however, the majority of the residents are not expected to exceed the values above which elevated health risk is possible.

Low-level exposure to organic form of Hg from seafood consumption in the prenatal period can influence neuropsychological development, however benefits of consuming seafood, which contains poly-unsaturated fatty acids and Se, and at the same time moderate amount of Hg, can prevail over potentially negative effects of Hg. The influence of Hg on neuropsychological outcome is confounded by (1) co-exposure to other potentially toxic pollutants and micronutrients, and by (2) *APOE* genetic polymorphism which can modulate exposure and/or neurodevelopment itself.

Chapter 3

Publications

The publications selected for the thesis follow the structure as given in the introductory part, representing (1) HBM methodological framework to assess exposure and (2) association between exposure and health outcome. They have resulted from different studies carried out as national and international projects coordinated by the Department of Environmental Sciences of JSI; University of Udine, Italy; University in Lund, Sweden; and Aristoteles University in Thessaloniki, Greece. I contributed to all the activities considerably, however, only publications most relevant for the scope of the thesis are given here and are divided into two parts as given above. The first part, dealing with HBM as a methodological tool, includes:

- a published book chapter which summarizes comprehensively the basics of Hg exposure assessment in HBM programs (Section 3.1);
- a manuscript defining analytical quality requirements for HBM programs, taking into account a variability in Hg exposure within a given population; uncertainty of measurements within a single laboratory; and variability in measurements of Hg in blood (as most commonly used biomarker of exposure) among different laboratories (Section 3.2);
- and a published paper which presents the first national HBM survey that established a representative Slovenian population for men in childbearing age and *postpartum* lactating women and set the first national reference values for trace elements in venous blood, urine, breast milk and Hg in hair, giving the background exposure for the Slovenian population. Moreover, it evaluated biomarkers of exposure for the use in association studies (Section 3.3).

The second part links exposure assessment to health outcome, building on the HBM framework. It brings together the papers resulting from the prospective cohort study PHIME on prenatal exposure to Hg, and the parallel cross-sectional study to evaluate the prenatal exposure and exposure of school-age children in the former Hg mining area – the town of Idrija. Specifically, the three published papers present:

- the overall assessment of association between low-level Hg exposure due to fish consumption and neurodevelopment in the Northern Adriatic (Slovenia, Croatia, Italy) and Aegean area (Greece) (Section 3.4);
- further evaluation of the association between prenatal exposure and neurodevelopment in the Slovenian and Croatian study populations, stratifying the association based on the genotype of APOE (Section 3.5);
- assessment of Hg exposure due to the past mining activities in the most vulnerable population groups living in the town of Idrija (Section 3.6).

3.1 Mercury: Biomarkers of Exposure and Human Biomonitoring

This section includes a book chapter which was published in the "Biomarkers and Human Biomonitoring. Volume 1: Ongoing Programs and Exposures" (Editors L. E. Knudsen, D. F. Merlo) by the Royal Society of Chemistry in 2012. The chapter was authored by M. Horvat, J. Snoj Tratnik and A. Miklavčič.

This work summarizes comprehensively the Hg properties, exposure sources, toxicokinetics, biomarkers of exposure and association to potential health effects. It gives scientific background for assessment of Hg exposure and its link to health outcome, and substantially complements the introductory part of this thesis.

In this book chapter, I wrote the part on Human Biomonitoring of Mercury Exposure (3H.5), Predictors and Identification of High Risk Populations (3H.6), and Associations of Biomonitoring Data and Adverse Effects (3H.7).

CHAPTER 3H

Mercury: Biomarkers of Exposure and Human Biomonitoring

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3H.1 Background

3H.1.1 Chemical Structures, Compound Classes

Mercury in the environment exists in several forms. These forms can be grouped under three headings:

- metallic mercury (also known as elemental mercury);
- inorganic mercury;
- organic mercury.

Several forms of mercury occur *naturally in the environment*. The most common natural forms of mercury found in the environment are metallic mercury, mercuric sulfide (cinnabar ore), mercuric chloride, and methyl mercury. Some microorganisms and natural processes can change mercury in the environment from one form to another. The most common organic

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mercury compound that microorganisms and natural processes generate from other forms is methyl mercury. Methyl mercury is of particular concern because it can build up in certain edible freshwater and saltwater fish and marine mammals to levels that are many times higher than the levels in the surrounding water, thus resulting in exposure among fish-eating populations, often at levels exceeding what is regarded as safe.

3H.1.1.1 Metallic Mercury (or Elemental Mercury)

Metallic mercury is a heavy, shiny, silver–white metal that is a liquid at room temperature. Metallic mercury is the elemental or pure form of mercury. Metallic mercury is the liquid metal used in thermometers and some electrical switches. At room temperature, some of the metallic mercury will evaporate and form mercury vapour. Mercury vapour is colourless and odourless.

3H.1.1.2 Inorganic Mercury

Many salts of divalent mercury (Hg^{2+}) are readily soluble in water, such as mercury sublimate ($HgCl_2$), and are thus highly toxic. In contrast, the water solubility of HgS (cinnabar) is extremely low, and, correspondingly, HgS is much less toxic than $HgCl_2$. The extremely high affinity of Hg^{2+} for sulfhydryl groups of amino acids such as cysteine and methionine in enzymes explains its high toxicity.

Monovalent mercury is found only in dimeric salts such as Hg_2Cl_2 (calomel), which is sparingly soluble in water and, again correspondingly, much less toxic than $HgCl_2$ (sublimate).

3H.1.1.3 Organic Mercury Compounds

When mercury combines with carbon, the compounds formed are called *"organic"* mercury compounds or organomercurials. There is a potentially large number of organic mercury compounds; however, by far the most common organic mercury compound in the environment is monomethyl mercury (known as methyl mercury). The term "methyl mercury" is used throughout this text to represent monomethyl mercury compounds. In many cases, the complete identity of these compounds is not known except for the monomethyl mercury cation, CH_3Hg^+, which is associated either with a simple anion, such as chloride, or a large charged molecule (*e.g.* a protein). In the past, the aryl organic mercury compound phenyl mercury was used in some commercial products. *Ethyl mercury* is used as a preservative in vaccines. Another organic mercury compound called *dimethyl mercury* is also used in small amounts as a reference standard for some chemical tests. Dimethyl mercury is very harmful to humans and other animals.

3H.1.2 Production and Use

Mercury is available as a commodity on the world market from several sources. Mine production of primary mercury (extracted from ore) is still carried out in Algeria, Kyrgyzstan, and until only recently in Spain and China. There are also reports of small-scale artisanal mining of mercury in China, Russia (Siberia), Outer Mongolia, Peru and Mexico, mainly serving local demand.

Mercury also occurs as a *by-product of mining or refining* of other metals (such as zinc, gold, and silver) or minerals, as well as refining of natural gas. Reprocessing or secondary mining of historic mine tailings containing mercury occurs. *Recycled mercury* is recovered from spent products and waste from industrial processes. Private *stocks* include the mercury used in the chlor-alkali and other industries.

Examples of the uses of mercury, in no particular order, are listed below.

As a metal (among others):

- for extraction of gold and silver (for centuries);
- as a cathode in the mercury-cell process for chlor-alkali production;
- in electrical and electronic switches;
- in fluorescent lamps;
- in discharge lamps (*e.g.* streetlights and some automobile headlights);
- in thermometers;
- in thermostats;
- in manometers for measuring and controlling blood pressure (sphygmomanometers);
- in barometers;
- in dental amalgam fillings.

Metallic mercury is still used in some herbal or religious remedies in Latin America and Asia, and in rituals or spiritual practices in some Latin American and Caribbean religions such as Voodoo, Santeria, and Espiritismo. These uses may pose a health risk from exposure to mercury both for the user and for others who may be exposed to elemental mercury vapour in contaminated air.

As a chemical compound (among others):

- in batteries;
- in vaccines (as a preservative in the form of ethyl mercury in thimerosal);
- biocides/fungicides in the paper industry, paints and on seed grain;
- in pharmaceutical antiseptics;
- laboratory analysis reagents;
- catalysts (*e.g.* to produce vinyl chloride monomer);
- pigments and dyes (may be historical);
- detergents (may be historical);
- soaps and creams (as a bactericide and/or whitening agent);
- explosives (mercury fulminate detonators; may be historical).

Most of these uses have been reduced significantly in many industrialized countries, particularly during the last two decades, because of their adverse impacts on humans and the environment. However, many of the uses discontinued in OECD countries are still alive in other parts of the world.

3H.1.3 Sources of Mercury in the Environment

Mercury is a naturally occurring element found throughout the environment. It enters the environment as the result of the normal breakdown of minerals in rocks and soil from exposure to wind and water, forest fires, and from volcanic activity. Mercury releases from natural sources have remained relatively constant in recent history, resulting in a steady rise in environmental mercury. However, it should be noted that a part of today's emissions from soil and water surfaces is composed of previous deposits of mercury from both anthropogenic and natural sources. The mercury found today in air, water, soil and biota may come from both natural sources and human activity.

Human activities since the start of the industrial age (*e.g.* mining, burning of fossil fuels) have resulted in additional release of mercury to the environment. Estimates of the total annual mercury release that result from human activities range from one-third to two-thirds of the total mercury releases. A major uncertainty in these estimates is the amount of mercury that is released from water and soils that were previously contaminated by human activities, as opposed to new natural releases.

The level of mercury in the atmosphere is very low and does not pose a health risk; however, the steady release of mercury has resulted in current levels that are *three to six times higher* than the estimated levels in the preindustrial era.

Approximately 80% of the mercury released from human activities is elemental mercury emitted to the air, primarily from fossil fuel combustion, mining and smelting, and from solid waste incineration. About 15% of the total is released to the soil from fertilizers, fungicides and municipal solid waste (for example, from waste that contains discarded batteries, electrical switches, or thermometers). An additional 5% is released from industrial wastewater to water bodies in the environment.

With the exception of mercury ore deposits, the amount of mercury that exists naturally in any one place is usually very low (Figure 3H.1). In contrast, the amount of mercury that may be found in soil at a particular hazardous waste site because of human activity can be very high (over 200 000 times natural levels).

3H.1.4 Environmental Fate

Most of the mercury found in the environment is in the form of metallic mercury and inorganic mercury compounds. Metallic and inorganic mercury enters *the air* from mining deposits of ores that contain mercury, from the emissions of coal-fired power plants, from burning municipal and medical

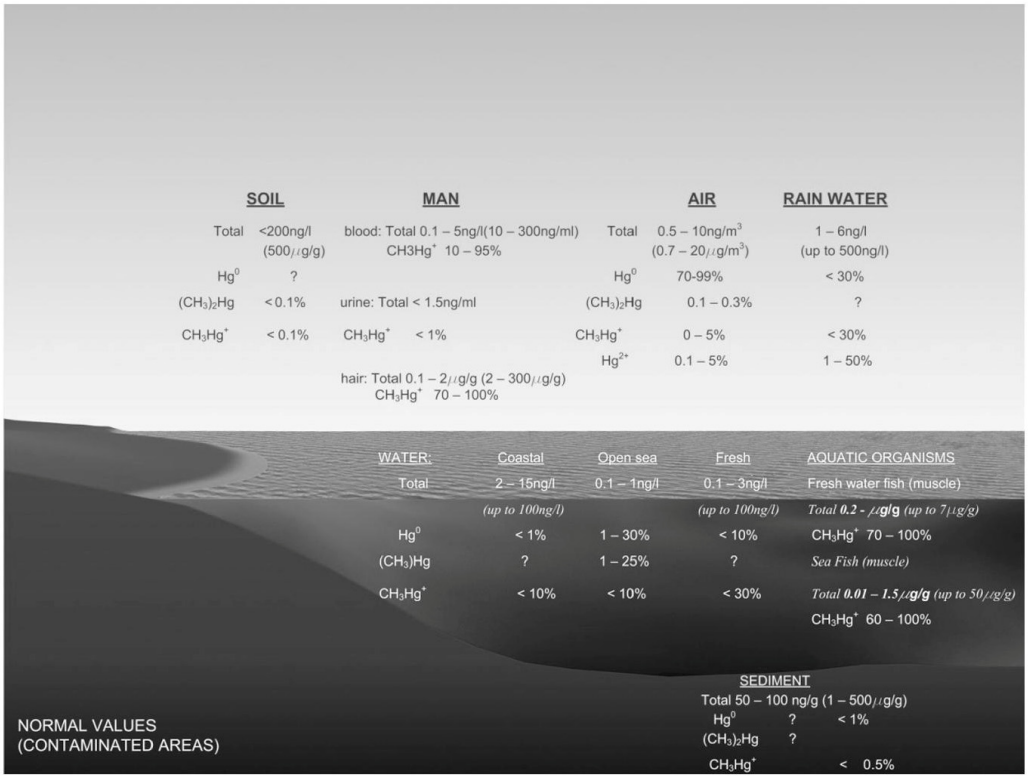


Figure 3H.1 Concentration ranges of mercury and methyl mercury in the bio- and geospheres.

waste, from the production of cement, and from uncontrolled releases in factories that use mercury (see Figure 3H.2). Metallic mercury is a liquid at room temperature, but some of the metal will evaporate into the air and can be carried long distances. In air, mercury vapour can be changed into other forms of mercury, and can be further transported to water or soil in rain or snow. Inorganic mercury may also enter *water or soil* from the weathering of rocks that contain mercury, from factories or water treatment facilities that release water contaminated with mercury, and from incineration of municipal waste that contains mercury (for example, in discarded thermometers, electrical switches or batteries). Inorganic or organic compounds of mercury may be released to water or soil if mercury-containing fungicides are used.

Microorganisms (bacteria, phytoplankton in the ocean, and fungi) convert inorganic mercury to methyl mercury. Methyl mercury released from microorganisms can enter water or soil and remain there for a long time, particularly if the methyl mercury becomes attached to small particles in the soil or water. Mercury usually stays on the surface of sediments or soil and does not move through the soil to underground water. If mercury enters the water in any form, it is likely to settle to the bottom where it can remain for a long time.

Mercury can enter and *accumulate in the food chain*. The form of mercury that accumulates in the food chain is methyl mercury. Inorganic mercury accumulates up the food chain to a much smaller degree. When small fish eat

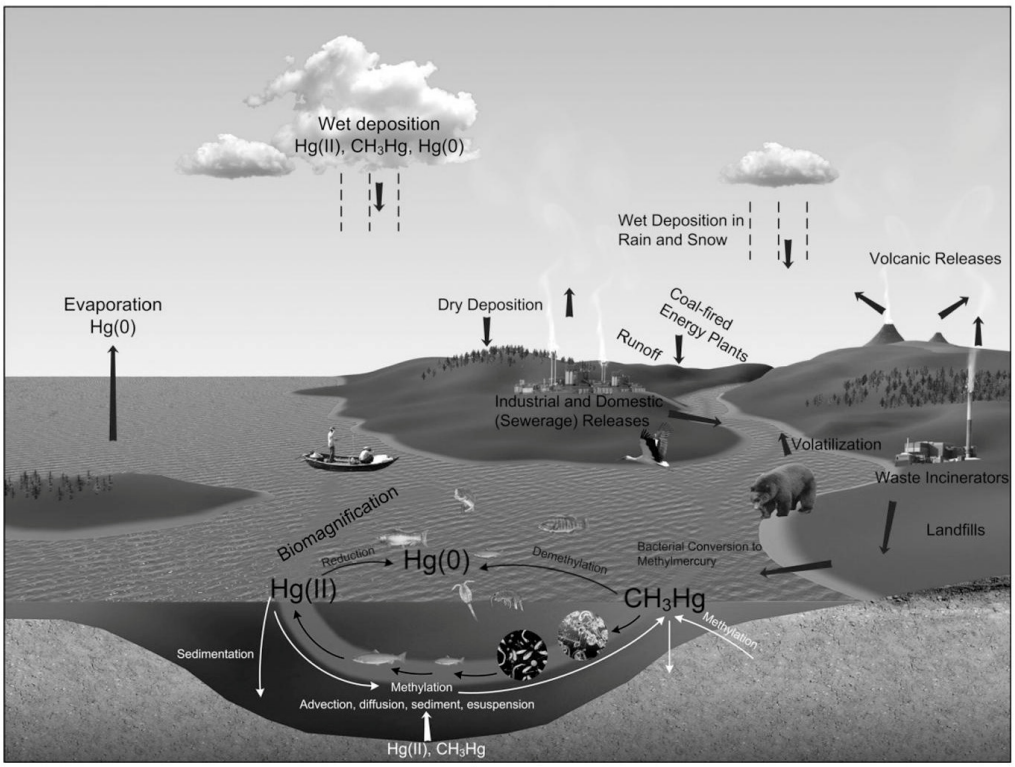


Figure 3H.2 Mercury cycling in the environment.

the methyl mercury in food, it accumulates in their tissues. When larger fish eat smaller fish or other organisms that contain methyl mercury, most of the methyl mercury originally present in the small fish will be stored in the tissues of the larger fish (bio-magnification). As a result, the larger and older fish living in contaminated waters build up the highest amounts of methyl mercury in their bodies. Seawater fish (especially sharks and swordfish) and marine mammals (*e.g.* dolphins, whales, seals) that live a long time and can grow to a very large size tend to have the highest levels of mercury in their tissues.

Plants (such as corn, wheat, and peas) have very low levels of mercury, even if grown in soils containing mercury at significantly higher than background levels. Rice, however, grown in contaminated sites can contribute a large portion of the daily dose of inorganic and methyl mercury in countries dependent on a diet rich in rice.[1] Also, mushrooms can accumulate high levels if grown in contaminated soils.

3H.2 Exposure Pathways

This global cycling of mercury results in its distribution in the most remote regions of the planet such as Arctic waters.[2,3] Microorganisms in the aquatic environment are capable of converting inorganic mercury to methyl mercury, which bio-accumulates and biomagnifies in food webs. As a consequence,

higher organisms such as sharks and fish-eating marine mammals contain the highest mercury concentrations. This implies that humans must have consumed methyl mercury in fish dating back to times before *Homo sapiens* evolved.[4]

Because mercury is mostly deposited in the environment in its inorganic form of Hg (II) and methyl mercury is more toxic than other stable forms of mercury, the biogeochemical cycling of mercury in the environment plays a key role in modulating mercury toxicity. Direct transformation processes involving methyl mercury in the environment are the methylation of inorganic mercury and the degradation of methyl mercury. The reduction of inorganic mercury to elemental mercury and the oxidation of elemental mercury to inorganic mercury affect methyl mercury formation indirectly by controlling levels of inorganic mercury, the substrate for methylation.[5]

3H.2.1 Exposure to Elemental or Inorganic Mercury

People may be exposed to elemental or inorganic mercury from dental amalgams and through inhalation of ambient air during occupational activities where mercury and mercury compounds are produced.[6] Occupational exposures have been reported from chlor-alkali plants, mercury mines, mercury-based small-scale gold and silver mining, refineries, thermometer factories, dental clinics with poor mercury handling practices and production of mercury-based chemicals. Furthermore, small scale or artisanal mining, using gold–mercury amalgamation to extract gold from ore, is a significant source of exposure for the workers and nearby populations.[7]

Amalgams release mercury vapour that can be inhaled. Concentrations of mercury vapour in the air in the oral cavity were shown to exceed occupational health standards. However, the quantity of vapour is small because the volume of the cavity is small. Furthermore, the vapour retained is much less than that inhaled under conditions of occupational exposure. Levels of mercury vapour in ambient air are low and the intake from this source is negligible. Therefore, with the exception of certain occupational exposures, dental amalgam is the main source of human exposure to mercury vapour.[3]

Exposures to elemental mercury or inorganic mercury can also occur as a result of the use of some skin-lightening creams and soaps, the presence of mercury in some traditional medicines, use of mercury in cultural practices, and various accidental mercury spills in homes, schools or other locations. Moreover the use of mercuric compounds as fungicides in latex paint and to disinfect grain seeds can result in exposure to inorganic mercury, but such use is prohibited in many countries.[7]

3H.2.2 Exposure to Organic Mercury

People are exposed to methyl mercury mainly through their diet, especially through the consumption of freshwater and marine fish and consumption of other animals that consume fish (such as marine mammals). The highest levels

are found in fish of older ages that are apical predators such as the king mackerel, pike, shark, swordfish, walleye, barracuda, large tuna, scabbard and marlin, and fish-consuming mammals such as seals and toothed whales.[8–12] Trimming, skinning and cooking mercury-contaminated fish does not reduce the mercury content of the fillet portion. However people that consume moderate amounts of a variety of fish are not at risk.[11] For example, based on the levels of methyl mercury in fish available on the Slovenian market and the JECFA provisional tolerable weekly intake (PTWI) for methyl mercury, a 70 kg man can eat a portion (150 g) of fish at the top of the food chain approximately once per week, or approximately three portions (150 g) of fish lower on the food chain.[12]

Minor exposure to other forms of mercury may result from the use of thimerosal (ethyl mercury thiosalicylate) as a preservative in some vaccines and other pharmaceuticals. However, the use of thimerosal in vaccines is being discontinued, or significantly reduced, in many countries, especially in vaccines intended for children.[7]

3H.3 Toxicokinetics

The toxicokinetics of mercury is dependent on the route of exposure (inhalation exposure, oral exposure or dermal exposure) and on the form of mercury to which the person has been exposed.

3H.3.1 Absorption

Metallic mercury is highly lipophilic, and absorption of the inhaled vapour, followed by rapid diffusion across the alveolar membranes of the lungs into the blood, has been reported to be substantial. Exposure to 0.1–0.4 mg/m^3 elemental mercury vapour can result in approximately 70–80% of inhaled mercury vapour.[13,14]

Ingesting small amounts of metallic mercury such as contained in a standard thermometer does not produce symptoms of intoxication, because the absorption of ingested metallic mercury is negligible.[15,16] Animal studies indicate that the absorption of inorganic mercury compounds such as mercuric chloride is approximately 10–30%.[17,18] The rate of oral absorption of inorganic mercury compounds in rats is dependent on intestinal pH, compound dissociation, age and diet.[19]

Organic mercury compounds are more readily absorbed in the gastrointestinal tract than inorganic mercury compounds. About 95% of the methyl mercury ingested is thought to be absorbed by the oral route. However, the absorption and bioavailability of methyl mercury may be affected by dietary components in food such as dietary fibre found in cereal products or selenium in fish.[20,21] Depending on the species of fish a low percentage of mercury in the fish is bioavailable (less than 20%) in both simulated stomach and intestinal digestion. The low absorption of methyl mercury could be attributed to the low ability of enzymes in the *in vitro* method to release the mercury contained in the

samples, perhaps due to the fact that mercury is complexed by selenium, rather than a lack of bioavailability of methyl mercury itself. In fact, a recovery of 89% from *in vitro* enzymolysis of a sample spiked with methyl mercury was obtained.[22] Higher faecal extraction and lower tissue accumulation of mercury in rats from contaminated fish than from methyl mercury chloride added to fish has been demonstrated.[23]

3H.3.2 Distribution

Because of its lipophilic nature, metallic mercury is distributed throughout the body and it can cross the blood–brain and placental barriers very easily. It is distributed in all tissues and reaches peak levels within 24 hours, except in the brain where peak levels are achieved within 2–3 days.[13] The mercury concentration in red blood cells in humans is twice that measured in the plasma.[24] The kidney is the major organ of mercury deposition after inhalation exposure of rats to metallic mercury vapour.[25,26] In chronic long-term occupational exposure to mercury vapour by mercury miners, the organs that accumulated and retained the highest concentrations were thyroid and brain, and this was associated with a molar mercury : selenium ratio of 1 : 1.[27]

In contrast to metallic mercury, inorganic mercury hardly crosses the placental barrier. In the plasma the mercuric ion binds to albumin and globulins. After administration of mercuric salts, levels of mercuric ions in the plasma are similar to mercuric ions in red blood cells. Binding also occurs in tissues, with the brain retaining mercury the longest.[20] After oral exposure to mercuric chloride, animal studies showed the highest level in the kidneys.[28,29] Renal uptake of mercury salts occurs from luminal membranes in the renal proximal tubule in the form of the cysteine S-conjugates (Cys-S-Hg-S-Cys), or from the basolateral membrane through organic anion transporters.[30]

In humans, the distribution of methyl mercury is similar to that of metallic mercury, because it is readily transferred to all tissues, including the brain and fetus. Methyl mercury is bound to thiol-containing molecules such as cysteine, which mimic methionine to cross the blood–brain barrier and placenta through the neutral amino acid carrier.[30] Approximately 90% of methyl mercury has been found in red blood cells.[31,32] The highest levels of organic mercury are found in the kidneys.[33–35] Concentrations of methyl mercury in hair are proportional to simultaneous concentrations in blood and are on average 250 times higher. They are also proportional to concentrations in the target tissue, the brain. Levels in cord blood are proportional to but slightly higher than levels in maternal blood.[36] Methyl mercury is also excreted in breast milk. However, unlike the placenta, where methyl mercury moves more easily across the placental barrier than inorganic mercury, inorganic mercury is more readily eliminated from breast milk than methyl mercury.[37]

The distribution, metabolism, media or possible media for biological monitoring in the case of exposure to elemental mercury, inorganic mercury and methyl mercury are shown in Figures 3H.3 and 3H.4.[38]

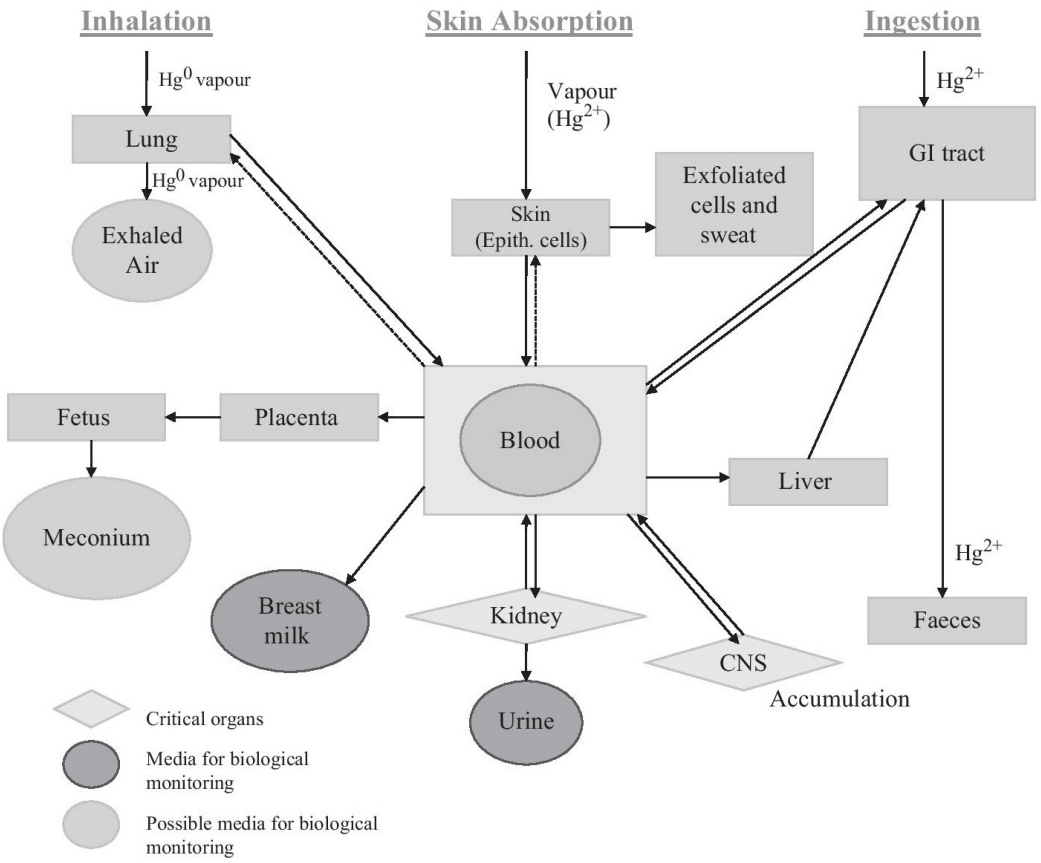


Figure 3H.3 Absorption, distribution, metabolism, and media for biological monitoring in the case of exposure to elemental and inorganic mercury.[38] GI, gastrointestinal; CNS, central nervous system.

3H.3.3 Metabolism

When metallic mercury vapour is inhaled through the lungs and rapidly enters the bloodstream, the dissolved vapour can undergo rapid oxidation to its inorganic divalent form by hydrogen peroxide catalase.[39] Because ethanol is a competitive substrate for the hydrogen peroxide catalase, the oxidation of metallic mercury can be inhibited by ethanol.[40] The oxidation of metallic mercury may occur in the brain, liver, lungs and probably other tissues to some degree.[41–43] Some studies suggest that the divalent inorganic mercury cation might be reduced by mammalian tissue to metallic mercury after its oxidation.[44,45]

After absorption, methyl mercury can be converted into inorganic mercury in tissues, especially the divalent cation.[45] After methyl mercury exposure several studies have reported high levels of inorganic mercury in tissues and faeces.[46–48] The intestinal flora can also convert methyl mercury into inorganic mercury.[49,50]

3H.3.4 Elimination and Excretion

The main excretory pathways of metallic and inorganic mercury in humans are the urine and faeces, with a half-life of approximately 1–2 months.[41] In a study

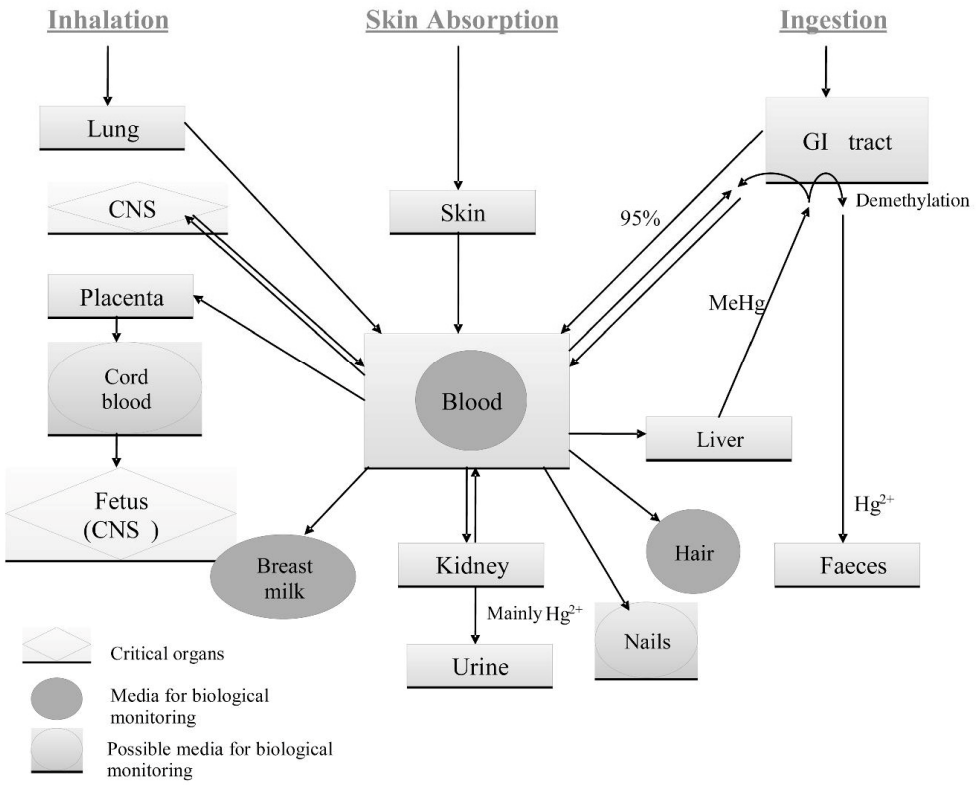


Figure 3H.4 Absorption, distribution, metabolism, and media for biological monitoring in the case of exposure to methyl mercury.[38] GI, gastrointestinal; CNS, central nervous system.

of former chlor-alkali workers with long term exposure to metallic mercury, the elimination of mercury in urine was well characterized by a one-compartment model, with an estimated half-life of 55 days. A tendency towards longer half-lives with shorter duration exposure compared to long term exposure was also observed.[51] Therefore the excretion of mercury depends on the duration of exposure. Furthermore, after acute mercury exposure in humans, urinary excretion accounts for 13% of the total body burden, while after long term exposure urinary excretion increases to 58%. Elimination of metallic mercury also occurs through expired air. After human exposure to mercury vapour for less than an hour, excretion through expired air accounted for 7%. The half-life for this elimination pathway was estimated to be 14–25 hours.[13,24] In a group of chlor-alkali workers with long term exposure to metallic mercury, a two-compartment model was used to estimate the half-lives in whole blood and plasma. In whole blood the half-lives were 3.8 and 45 days for the fast and slow phase, respectively, while for plasma the half lives were 2 and 36 days for the fast and slow phase, respectively.

After an acute exposure to a high level of mercuric chloride, the elimination half-life in urine was estimated to be 25.9 days.[52] The overall half-life of inorganic mercury from the body was estimated to be 60 days and it is eliminated at the same rate as from the kidneys, where most of the body burden is localized.[20]

The predominant excretory route for methyl mercury is the faecal pathway, with less than one-third of total mercury excretion occurring through the urine.[53] In a study that included four Japanese people, the extraction of methyl mercury into faeces was confirmed, but they found similar concentrations of methyl mercury in the urine compared to concentrations in faeces.[54]

Animal studies showed that methyl mercury is secreted in the bile and can be reabsorbed in the intestine. Clearance half-times are longer with methyl mercury than with inorganic compounds. The half-time in the blood is estimated to be 50 days.[20] Elimination of methyl mercury compounds generally follows first order kinetics because excretion is directly proportional to the body burden and independent of the route of administration.[55] Duration of exposure may affect the extraction process of mercury. A two-compartment model was established for a single oral dose in monkeys, while following repeated dosing for 2 years a one-compartment model was considered a more reasonable fit for the data. Therefore the average steady-state blood levels of mercury after chronic-duration exposure should not be estimated on the basis of short-term exposure data.[33]

3H.4 Sampling, Sample Handling and Analysis

Hundreds of papers have been published in recent years on the development and validation of sensitive analytical methods for total mercury and mercury speciation. Several review papers have also been published summarizing the advantages and disadvantages of various analytical protocols,[56] therefore only brief notes are provided in this chapter. While much has been done on the measurement techniques, relatively little is known about the effects of storage on the stability of methyl mercury in biological samples. Significant external contamination of samples with methyl mercury is unlikely to occur, however extreme precautions are necessary to avoid contamination by inorganic mercury.

Blood and hair samples are often analysed in order to estimate exposure of humans to mercury and its compounds. Blood should be taken by venepuncture. Given that some commercial containers may contain mercury compounds added as preservatives, it is advisable to check each commercial batch before use. Samples should be refrigerated but not frozen, because it is sometimes useful to measure mercury in plasma and red blood cells separately. The separation of plasma and red blood cells should be performed as soon as possible to avoid haemolysis of the sample. If extensive haemolysis has occurred, the sample should be homogenized before an aliquot is taken for analysis. Blood samples may also be heparinized for total blood, serum, and red blood cell analyses. If unavoidable, samples may be stored deep frozen. However, repeatedly frozen and thawed blood samples showed a remarkable decrease in methyl mercury concentrations.[57] There is some evidence that methyl mercury may be destroyed during lyophilization of blood samples.

Analysis of human hair offers several advantages over analysis of blood samples, for example ease of sampling and sample storage, the concentration of

methyl mercury is approximately 250 times higher than in blood, and analysis of different longitudinal sections of hair can give information on the history of exposure to methyl mercury ingested through food. Adherent dust and grease should be removed by one of the following solvents: hexane, alcohol, acetone, water, diethylether, or detergents. The International Atomic Energy Agency (IAEA) and the World Health Organization (WHO) recommend the use of only water and acetone.[58] Long-term storage of human hair samples has shown that methyl mercury is stable for a period of a few years if stored dry and in darkness at room temperature.

Biological samples are preferably analysed fresh or after lyophilization. Deep-freezing of fresh samples, especially with long storage, should be avoided, because it has been noticed that in some organisms methyl mercury may decompose with repeated freezing and thawing (particularly in bivalves and blood).[57] Methyl mercury and total mercury in lyophilized biological samples, such as biological certified reference materials (CRMs), are stable for years. CRMs are, however, sterilized either by autoclaving or by gamma ray-irradiation. This important step prevents bacteriological activity, which may otherwise lead to methylation/demethylation processes. In general, very little is known about the effects of sterilization on the stability of methyl mercury compounds. More studies are needed to investigate the stability of organic mercury compounds in biological samples, particularly under various conditions of sample preparation and long-term storage.

3H.4.1 Analytical Methods

3H.4.1.1 Total Mercury

Most methods for the determination of total mercury in solid samples require preliminary digestion of the sample. They are classified as wet (oxidizing digestion) and dry (combustion/pyrolysis) decomposition methods.

For the determination of low level mercury concentrations, a number of instrumental analytical methods can be used. Among the most frequently employed are the following: cold vapour atomic absorption spectrometry (CV AAS), cold vapour atomic fluorescence spectrometry (CV AFS), inductively coupled mass spectrometry (ICP-MS), electrochemical methods, and neutron activation analyses (NAA). During the last two decades CV AAS and CV AFS have replaced most of other techniques. The relative detection limits of some techniques are presented in Table 3H.1. It should be noted, however, that the detection limits reported are dependent on the overall analytical procedure, including sample preparation, prior to the final quantification step.

3H.4.1.2 Speciation of Mercury

In general, methods are classified according to the isolation technique and the detection system. Most methods for the isolation/separation of organic

Table 3H.1 The methods used most frequently for quantification of mercury and their relative detection limits.[55]

Method		*Detection limits*
	Colorimetric methods	0.01–0.1 μg g^{-1}
AAS	Graphite furnace (GF AAS)	1 ng g^{-1}
	Cold vapour (CV AAS)	0.01–1 ng g^{-1}
AFS	Cold vapour (CV AFS)	0.001–0.01 ng g^{-1}
NAA	Instrumental (INAA)	1–10 ng g^{-1}
	Radiochemical (RNAA)	0.01–1 ng g^{-1}
GC	Electron capture detector	0.01–0.05 ng g^{-1}
	Atomic emission detector	0.05 ng g^{-1}
	Mass spectrometer	0.1 ng g^{-1}
	CV AAS/AFS	0.01–0.05 ng g^{-1}
HPLC	UV	1 ng mL^{-1}
	CV AAS	0.5 ng mL^{-1}
	CV AFS	0.08 ng mL^{-1}
	Electrochemical detectors	0.1–1 ng mL^{-1}
ICP MS		0.01 ng mL^{-1}
ICP		2 ng mL^{-1}
AES		
Photo-acoustic spectroscopy		0.05 ng
X-ray fluorescence		5 ng g^{-1} to 1 ng g^{-1}
Gold-film analyzer		0.05 μg g^{-1}

mercury compounds are based on solvent extraction, differential reduction, difference calculations between "total" and "ionic" mercury, derivatization, or on paper and thin layer chromatography. The most common approaches to organic mercury separation and detection are presented schematically in Figure 3H.5.

There are also a few methods that are based on differential reduction. In the method developed by Magos,[59] the inorganic mercury in an alkaline digested sample is selectively reduced by stannous chloride, while organic mercury compounds are reduced to elemental mercury by a stannous chloride–cadmium chloride combination. The elemental mercury released can be measured by CV AAS.

Quality assurance refers to those procedures that ensure that analytical results are valid, traceable, reproducible, representative, complete and accurate, *i.e.* close to the "true value". It also includes measures developed to assess performance. It is generally accepted that mercury analysis and speciation must be done by well trained staff who, in principle, should be involved in the measurement process from sampling to the production of final results, particularly if speciation of mercury is intended. The use of reference materials certified for mercury and its compounds plays an important role in method validation and demonstration of traceability. At present there are many reference materials certified for total mercury concentrations in various matrices (sediment, soil, ash, water, plants, and tissues) of different origin. Unfortunately, only a few reference materials are certified for methyl mercury compounds.[56,60]

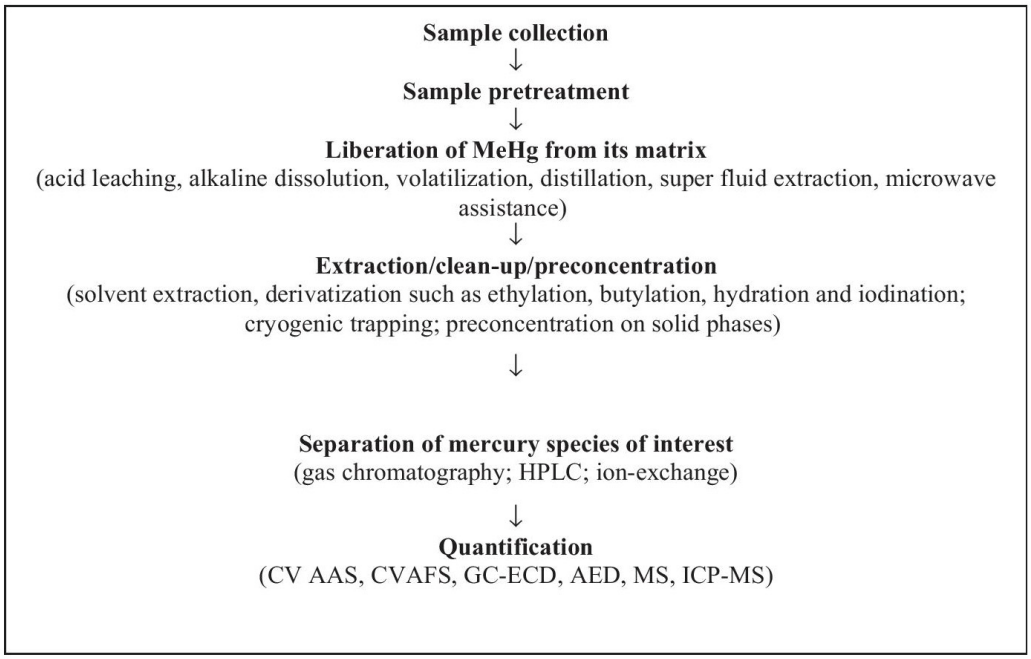


Figure 3H.5 Steps for the determination of organic mercury compounds. AED, atomic emission detector; CV AAS, cold vapour atomic absorption spectrometry; CV AFS, cold vapour atomic fluorescence spectrometry; GC-ECD, gas chromatography–electron capture detector; HPLC, high performance liquid chromatography; ICP-MS, inductively coupled mass spectrometry.

3H.5 Human Biomonitoring of Mercury Exposure

Exposure to mercury is estimated by measuring mercury in human tissues (hair, blood, urine), which are considered forms of biomonitoring. These measurements are also known as biomarkers of exposure. The selection of media depends on the mercury compounds, exposure pattern (*e.g.* chronic, acute) and time of sampling after exposure.[61] The presence of mercury in the blood indicates recent or current exposure to mercury and does not give information on the historical exposure and seasonal (or other peak) variations. In the general population, methyl mercury from fish and mercury vapour from dental amalgam fillings influence mercury concentrations measured in blood, but this is not true for occupationally exposed people – in this case mercury vapour is the major contributor to blood mercury concentration.[62,63] Exposure to elemental mercury is well represented by the presence of mercury in urine. Given that inorganic mercury is slowly excreted from kidneys through the urine, urinary mercury reflects not only recent exposure to elemental mercury, but also exposure that occurred at some time in the past.[62,64] In the non-occupationally exposed population, the number of amalgam surfaces was found to be the best predictor for urinary mercury. Interestingly, in previously exposed chlor-alkali workers, more than 1 year after the exposure ceased no association between the number of amalgam surfaces and urinary mercury concentration

was found.[65] Given that the concentration of waste products in urine can vary significantly owing to dilution with water, mercury in urine is preferably adjusted for creatinine or specific gravity.

Scalp hair is a matrix of choice in studies assessing environmental mercury exposure, because it provides a simple, integrative, and non-invasive sample for estimating long-term average exposure to methyl mercury. Assessment of methyl mercury exposure through measuring total mercury in hair is based on the assumption that methyl mercury in hair is proportional to the blood concentration of methyl mercury and that almost 100% of the total mercury in hair is methyl mercury.[62,66] However, the exact relationship between mercury exposure and mercury excretion in hair follicles is not known, and it can vary greatly between individuals and ethnic groups.

3H.5.1 Human Biomonitoring of Occupational Exposure

In certain working environments workers are exposed to mercury by inhaling metallic mercury vapour. Exposures in the working environment are monitored by measuring inorganic mercury in urine or blood. Urine has been used as the prefered matrix because metallic mercury is mostly excreted through urine (see Section 3H.3.4) and also because the collection of urine is non-invasive. The maximum urine mercury concentration set by WHO (1991)[67] is 50 μg/g creatinine. Mercury urine levels rarely exceed 5 μg/g creatinine in persons who are not occupationally exposed to mercury.[61] Individual human biomonitoring studies in eight Swedish chlor-alkali plants showed that urinary mercury excretion in workers has declined from about 200 μg/L in the 1950s to 150 μg/L in the 1960s and less than 50 μg/L in the 1990s.[62] Decrease in exposure has also been observed in the UK since the 1980s and 1990s.[64] In the 1990s, workers from chlor-alkali plants had in general an approximately 10 times higher Hg concentration in urine, as well as in blood, than non-exposed controls.[63,68] The occupational mercury contribution from dental work is generally low; however, individuals can show urinary mercury concentrations close to a level of 15 μg/L.[69] Peak exposures have been reported to occur mainly during the insertion and removal of amalgams and during polishing of amalgam surfaces.[64]

However, as mentioned above, urinary mercury reflects average long-term exposure, while blood mercury is a good indicator of recent exposure peaks.[62,64] Urinary mercury is an integrating marker of exposure to metallic and inorganic mercury over many previous months. In long-term workers, 60–70% of mercury was found in urine, while in workers exposed for less than 6 months, only 20–25% of mercury contributed to urinary mercury. Moreover, previous weeks of exposure contributed only 10% to urinary mercury.[64] When monitoring occupational exposure, it should be borne in mind that peak urinary mercury may occur a few weeks after exposure. It has been reported that there was a delay of 2–3 weeks in peak urinary mercury in people without previous exposure, which was explained by the time taken for renal uptake and subsequent excretion.[64,70] In contrast to urinary mercury, blood mercury

increased immediately after exposure – urine-to-blood ratios for acutely exposed workers (maintenance engineers) were <0.02 compared with ~ 0.4 usually found in those chronically exposed.[64]

When total blood mercury is used for monitoring exposure to mercury vapour, methyl mercury from fish consumption can be a disturbing factor.[63] The results of the studies described above[63,68] have demonstrated clearly that inorganic mercury in blood is a better marker for occupational exposure than total or organic mercury. Furthermore, in the case of measuring total mercury in blood, significantly higher differences between exposed and non-exposed groups were observed in serum samples than in whole blood samples (3-fold *vs.* 11-fold).

If the exposure is stable, natural intra-individual variation for mercury in blood is moderate (about 10%), but rapid changes caused by changes in exposure can occur. In urine, the day-to-day variation is relatively high, even when 24-hour samples are used under stable exposure conditions.[62] Variability in individual urinary mercury results was reported to be due to different methods of correction, diurnal variation of mercury excretion and levels of laboratory analytical precision.[64] A consistent diurnal variation of urinary mercury concentration (creatinine corrected) has been demonstrated in 36 occupationally exposed workers, the concentration being highest in the morning and lowest in the late evening.[71] A fixed time point for sample collection has been suggested to remove the effects of diurnal variation in excretion of mercury.[64] Creatinine correction of mercury concentration significantly reduced the mean intra-individual variation, both between and within days, to $\sim 50\%$ of the variation in uncorrected urine values.[64] When a correction for specific gravity or creatinine is made, typical coefficients of variation are 15–20%, and the levels on two consecutive days often differ by 25–50%.[62]

3H.5.2 Human Biomonitoring of Environmental Exposure

In most epidemiological studies, total mercury in hair is used as a biomarker of methyl mercury exposure, because it provides a long-term biomarker of exposure to methyl mercury through fish consumption. Mercury concentrations in the hair of populations that consume no fish are normally below 0.5 μg/g; in populations with low to moderate fish consumption, hair total mercury varies from below 1 to 2 μg/g, while people with frequent consumption (once or more per day) may have total mercury levels in hair exceeding 10 μg/g.[61]

It is usually assumed that total mercury in hair is about 250 times higher than the blood mercury concentration at the moment hair is formed and that almost 100% of total Hg in hair is methyl mercury.[61,66] The latter is only true in populations with high fish consumption, whereas in populations with relatively low fish consumption, inorganic mercury might constitute a larger fraction of hair mercury.[66] Relatively high inter-individual variability in the hair-to-blood MeHg ratio has been reported, ranging from 140 to 370[32] and from

150 to 690,[72] related to low to medium exposure levels. Furthermore, high variability in the relationship between mercury levels in hair and methyl mercury exposure through fish consumption was reported by Canuel *et al.*[73] Therefore, it is suggested that in the general population hair mercury is to be used as an approximate indicator of long-term MeHg exposure from fish consumption, while blood mercury is a more accurate indicator of recent exposure to mercury (organic and/or inorganic).

Mercury in blood is present as methyl mercury and inorganic mercury, therefore it is important to perform speciation analyses for accurate assessment of specific exposure. Especially in cases of a low to moderate level of exposure to methyl mercury from fish consumption, inorganic mercury from dental amalgam fillings or other sources (vaccines, food, *etc.*) might constitute a substantial proportion of total mercury – from 0 to 80%.[72,74] However, there is no need to perform mercury speciation at very low exposure levels, as such exposure presents little risk. The normal mean total mercury concentration for populations with little or no fish consumption in the USA is 2 μg/L.[66] It should also be mentioned that speciation of mercury at levels below $\sim$1 μg/L is demanding and requires additional clean-up steps. Therefore, in terms of cost-effectiveness, it is sufficient to measure total mercury. Speciation analysis is required when total mercury levels above 1 μg/L are detected. Levels of mercury found in biological samples of various populations worldwide are presented in Table 3H.2.

3H.5.2.1 Mixed Occupational and Environmental Exposure

A good example of mixed occupational and environmental exposure is the gold mining area in the Tapajos river basin of the Amazon, where inhabitants are exposed to metallic mercury by direct inhalation of Hg vapour (gold miners and gold shop workers) and to methyl mercury in fish from rivers downstream of the gold-mining sites. Akagi *et al.*[84] reported that total mercury levels in blood were not correlated with total mercury levels in urine, but inorganic mercury levels were. On average 73% of the total mercury in blood was in the form of methyl mercury, not associated with occupational exposure. Abnormally high levels of mercury, mostly in inorganic form, were found in the urine of gold shop workers (162 ± 95 μg/g creatinine), while hair total mercury in gold shop workers was significantly lower (4.1 ± 1.3 mg/kg) than in the inhabitants of the fishing villages (around 30 mg/kg).

3H.5.3 Human Biomonitoring of Prenatal Exposure

Methyl mercury and metallic mercury move freely across the placenta from maternal to fetal blood, while inorganic mercury is prevented from being transferred to the fetus and accumulates in the placenta.[52,72,75,85] Various studies have reported cord blood methyl mercury to be almost twice as high as maternal blood methyl mercury,[72,74,86,87] which is a consequence of higher

Table 3H.2 Environmentally relevant levels of total mercury in biological samples from different populations worldwide as reported in the studies indicated.

Population	*Country /Region*	*Biological samples*	*Total Hg, mean (range)*	*Units*	*Reference*
Pregnant women, *not consuming fish*	Slovenia	blood	1.02 (0.42–1.63)	ng/mL	Horvat *et al.*, 1988[72]
		cord blood	1.16 (0.29–3.80)	ng/mL	Horvat *et al.*, 1988[72]
		hair	0.17 (0.09–0.35)	μg/g	Horvat *et al.*, 1988[72]
Pregnant women, *seafood consumers*	Central Adriatic, Yugoslavia	blood	3.7 (1.2–9.6)	ng/mL	Horvat *et al.*, 1988[75]
		hair	1.48 (0.42–3.28)	μg/g	Horvat *et al.*, 1988[75]
Pregnant women; *general*	Slovenia	hair	0.39 (0.02–2.08)	μg/g	Miklavčič *et al.*, 2010[12]
Adults, *not consuming fish*	Sweden	blood	0.28 (0.11–1.4)	ng/mL	Lindberg *et al.*, 2004[76]
		hair	0.06 (0.04–0.32)	μg/g	Lindberg *et al.*, 2004[76]
Pregnant women, *frequent fish consumption*	Seychelles	hair	6.8	μg/g	NRC, 2000[66]
Pregnant women, *frequent consumption of pilot whale*	Faroe Islands	hair	4.3 (2.8–14.6)	μg/g	Grandjean *et al*, 2005[77]
Pregnant women, *frequent fish consumption*	Faroe Islands	cord blood	22.4 (0.90–351)	ng/mL	Grandjean *et al.*, 2005[77]
Pregnant women, *frequent fish consumption*	Greece, Aegean islands	hair	1.36 (0.046–17.5)	μg/g	Gibičar *et al.*, 2006[78]
Pregnant women, *general*	Turkey	blood	0.38 (0–2.34)	ng/mL	Unuvar *et al.*, 2007[79]
		cord blood	0.50 (0–2.36)	ng/mL	Unuvar *et al.*, 2007[79]

Table 3H.2 (*continued*)

Population	*Country /Region*	*Biological samples*	*Total Hg, mean (range)*	*Units*	*Reference*
Women; *general*	Czech Republic	blood	0.94	ng/mL	Batariova *et al.*, 2006[80]
Men, *general*	Czech Republic	blood	0.86	ng/mL	Batariova *et al.*, 2006[80]
Adults 18–69; *general*	Germany	blood	0.6 (0.20–12.3)	ng/mL	GerES III[81]
Adults 18–69; *general*	Germany	urine	0.34 (0.1–0.69)	μg/g crea	GerES III[81]
Children 3–14; *up to 3 fish meals per month*	Germany	blood	<0.2 (<0.2–0.27)	ng/mL	GerES IV, Becker *et al.*, 2008[82]
Children 3–14; *more than 3 fish meals per month*	Germany	blood	0.29 (<0.2–0.42)	ng/mL	GerES IV, Becker *et al.*, 2008[82]
Children 3–14, *without amalgam fillings*	Germany	urine	<0.1 (<0.1–0.18)	μg/L	GerES IV, Becker *et al.*, 2008[82]
Children 3–14, *with amalgam fillings*	Germany	urine	0.2 (<0.1–0.51)	μg/L	GerES IV, Becker *et al.*, 2008[82]
Children 6–11; *general*	USA	blood	0.42 (0.36–0.48)	ng/mL	CDC, 2009[83]
		urine	0.30 (0.25–0.36)	μg/g crea.	CDC, 2009[83]
Adults, 20 years and above; *general*	USA	blood	0.98 (0.86–1.12)	ng/mL	CDC, 2009[83]
		urine	0.51 (0.46–0.57)	μg/g crea.	CDC, 2009[83]

haematocrit levels[88] and also higher haemoglobin concentrations in cord red blood cells.

As a biomarker of prenatal exposure, mercury in umbilical cord blood and not maternal blood mercury is measured preferentially. Despite the strong association between cord blood methyl mercury concentration and maternal blood methyl mercury concentration, high variation in the cord blood-to-maternal blood ratio of methyl mercury was found in several studies: 1.8 (1.0–3.5),[72] 1.8 (0.88–3.1, 95% CI)[74], 1.7 ± 0.56[86] and 1.72 (1.44–2.01, 95% CI).[87] As concluded by Murata *et al.*,[87] the high variation indicates that cord blood mercury should be considered a more useful and valid biomarker of prenatal exposure to methyl mercury than maternal blood mercury.

In addition to cord blood mercury, which was demonstrated to be the best biomarker of prenatal methyl mercury exposure,[89] the suitability of umbilical cord tissue mercury was assessed and discussed. These studies showed a strong correlation between cord blood and cord tissue mercury, while the correlation between maternal hair and cord tissue mercury was not as strong. In addition, cord tissue mercury was almost as good a predictor of methyl mercury-associated neuropsychological deficits at 7 years of age as was cord blood mercury.[89]

Infant exposure to methyl mercury and inorganic mercury *via* breast feeding is low compared with late fetal exposure.[90] Based on the reported milk-to-plasma ratios of about 0.2 for methyl mercury and 0.6–1.0 for inorganic mercury, it seems that inorganic mercury is transported more easily to breast milk than is methyl mercury. The association between inorganic mercury in maternal blood and mercury in breast milk indicates transport of inorganic mercury into milk as well.[90]

3H.6 Predictors and Identification of High Risk Populations

There are two general types of susceptible subpopulations: (1) those that are more sensitive to the effects of mercury and (2) those that are exposed to higher levels of mercury, such as those that frequently consume locally caught fish from mercury-contaminated water bodies or long-lived predatory oceanic species, individuals with dental amalgams, and occupationally exposed workers (miners, workers in chlor-alkali plants, dentistry and industries for the production of batteries, thermometers and fluorescent lamps).[61]

A sensitive population is a group that may experience more severe adverse effects at comparable exposure levels or adverse effects at lower exposure levels than the general population. For mercury, the most sensitive subpopulations are developing organisms, particularly the fetus, because of the sensitivity of the developing nervous system.[20] Among mercury species, methyl mercury and metallic mercury reach the fetus during pregnancy by crossing the placenta.[91] Trans-placental passage may occur by mimicry of essential compounds or by binding to such compounds for which a transport mechanism exists. Prenatal

exposures to environmental chemicals interfere with the above-described processes, adversely affecting the structural integrity of the nervous system, which results in altered functioning.[92,93] Exposure to methyl mercury and metallic mercury during early fetal development can cause subclinical brain dysfunction at doses much lower than those affecting adult brain functions. Although these chemicals might have caused impaired brain development in millions of children worldwide, these latent impairments could not be unmasked until the natural neuronal attrition associated with ageing occurred. Numerous studies have shown that Parkinson's disease and Alzheimer's disease are of purely genetic origin in only a minority of cases and appear in most instances to arise through interactions among genetic and environmental factors. Exposure of the developing brain to environmental toxic agents during windows of vulnerability in early life may be an important contribution to causation of these diseases.[94]

The susceptibility of infants and children is further enhanced by their increased exposures, increased absorption rates and diminished ability to detoxify many exogenous compounds relative to those of adults.[95] Selective susceptibility may be due to differences in metabolizing enzymes, rates of excretion and binding affinity to target proteins. Partial lack of a blood–brain barrier in the fetus[91] is also of great importance, because the barrier fails to prevent some selected molecules from entering the nervous system.[93] Small lipophilic particles (*e.g.* metallic mercury) can be easily passed transcellularly,[93] while methyl mercury can enter the brain by conjugating with L-cysteine (methyl mercury–L-cysteine complex), and thus exploiting the L-methionine uptake pathway (mimicry) to traverse the protective blood–brain barrier.[95] A methyl mercury–glutathione complex has been proposed, although it is expected that this complex would be water soluble.[66] The blood–brain barrier itself can be subject to damage by mercury – it accumulates in endothelial cells and damages their membranes, leading to brain haemorrhage and oedema.[93] It has been observed that high-dose exposure disrupts the blood–brain barrier.

Individuals with diseases of the liver, kidneys, nervous system and lungs are at higher risk of suffering from the toxic effects of mercury. Individuals with a dietary insufficiency of zinc, glutathione, antioxidants or selenium and those who are malnourished may be more sensitive to the toxic effects of mercury because of the diminished ability of these substances to protect against mercury toxicity. There is also limited population variability with regard to elimination of methyl mercury.[61,66]

The sensitivity of individuals is further influenced by alterations in chromosomal structure and *polymorphism of enzyme activities* involved in the metabolism of mercury.[96] Glutathione S-transferase is a gene family involved in the detoxification of electrophilic compounds by conjugation with glutathione, therefore enhancing the excretion of mercury via the bile or urine. In higher organisms at least five glutathione S-transferases gene classes have been well studied. It was shown that glutathione deficiency is associated with sensitivity to both mercuric chloride and methyl mercury.[97] As was observed in two tribes living in the same area of the Amazonian region subjected to intensive gold

prospecting, and with the same dietary habits based on fish, the GSTM1 gene could be involved in mercury metabolism or could be associated with reduced mercury levels: in the study an association between a high frequency of the GSTM1 + allele and lower mercury contamination was found.[97]

Furthermore, the processes of cysteine and glutathione synthesis have been shown to be reduced in autistic children.[98] Autistic individuals have 20% lower plasma levels of cysteine and 54% lower levels of glutathione, which, among other things, adversely affects their ability to detoxify and excrete metals such as mercury. A decreased capacity for mercury excretion in autistic children was supported by the finding that mercury levels in the hair of the first haircut of autistic children were about 8-fold lower than in normal children, although autistic children had been exposed to significantly higher mercury levels through maternal dental amalgams and thiomersal.[98]

3H.7 Associations of Biomonitoring Data and Adverse Effects

A number of adverse health effects associated with environmental exposure to mercury have been identified in humans and in animal studies. Most extensive are the data on neurotoxicity, particularly in developing organisms. At high exposures, mercury can cause cerebral palsy, seizures, microcephaly, mental retardation and also death. The most severe effects were seen in Minamata and Niigata, Japan, where children were born with severe cerebral palsy in a population that consumed seafood contaminated with methyl mercury from industrial discharges.[99] Neurological disorders were also observed in populations exposed chronically to low doses of methyl mercury, especially in children of mothers whose diet included substantial quantities of fish and other seafood.

Given that most toxicological studies aim at deriving information that may be used to establish a virtually safe dose for regulatory purposes, the US EPA derived a Reference Dose (RfD) in 2001[21] on the basis of an analysis of the health effects of methyl mercury performed by the NRC.[66] The RfD is the dose below which no unacceptable risk of adverse effect is expected.[100] It was evaluated based on data from three epidemiological studies that were suitable for quantitative analysis. These longitudinal prospective developmental studies were conducted in the Seychelles, the Faroe Islands and New Zealand. Mother–child pairs, 779, 900 and 237, respectively, were selected from fish-eating populations living in these islands. Children were assessed at different ages on a number of standardized neuropsychological endpoints, which were compared with maternal hair mercury concentration or cord blood mercury concentration (Faroe Islands) as the biomarker of exposure. Investigators in the Seychelles study reported no evidence of impairments related to prenatal methyl mercury exposure, whereas the other two studies found exposure-related effects in a number of neuropsychological endpoints.[101] In the assessment described by the NRC, emphasis was placed on the results of the Faroe Islands study, the larger of the two studies that identified methyl mercury-related developmental neurotoxicity.

To identify a biomarker concentration that is associated with a dose affecting a certain proportion of a selected population, the relationship between the neuropsychological performance (endpoint) and exposure has to be modelled. The NRC used a linear dose–effect relationship using five endpoints that had been reported as significantly affected by methyl mercury exposure.[102] The benchmark dose ($BMDL_{05}$) was established, which represents an estimate of the lower confidence limit (5th percentile) of the dose that affects a small percentage of the population compared to the control group.[103] That methyl mercury concentration was than translated into the RfD. This determination requires the back-calculation of dose using a pharmacokinetic model. A one-compartment pharmacokinetic model was used to convert cord blood (or maternal hair) mercury to maternal intake. A one-compartment model is a significant simplification of the pharmacokinetics of methyl mercury in the maternal body and maternal–fetal unit, and it does not address the variance in the parameters (*e.g.* body weight, blood volume, hair–blood partition coefficients). Each of the model parameters is a variable whose possible values in a population can be described by a probability distribution. The ingested dose of methyl mercury corresponding to a measured biomarker concentration, therefore, should also be described by a probability distribution. The ingested dose used as the basis for the RfD should be at the lower range of doses that could result in a given biomarker concentration. In order to address inter-individual toxicokinetic variability in the derivation of the RfD, an uncertainty factor of the central-tendency estimate of the ingested dose was applied. The dose was divided by an uncertainty factor of 10, which was derived from probabilistic analysis of the distributions of the parameters of the one-compartment pharmacokinetic model.[66,101]

On the basis of integrative analysis of all three studies and all endpoints, a $BMDL_{05}$ of 32 ng/g methyl mercury in maternal whole blood was calculated, giving an RfD of 0.1 μg/kg body weight per day as an exposure without recognized adverse effects.[21] The cord blood total mercury level equivalent to the RfD is 5.8 ng/mL. The corresponding maternal hair total mercury concentration would then be about 1 μg/g.

Reassessing each of the input parameters in the one-compartment model, taking into account cord blood-to-maternal blood ratio and reducing the uncertainty in the estimate of the central tendency of the maternal dose, Stern[104] has revised the US EPA reference dose. The cord blood-to-maternal blood ratio had a significant influence on the estimate of maternal dose; the maternal intake dose corresponding to the fetal cord blood mercury concentration of 58 ng/mL was assessed to be half the value previously reported by the US EPA.

Other levels of daily or weekly methyl mercury or mercury intakes estimated to be safe have also been established by several countries and international organizations based on the available information. All levels represent levels of exposure that protect the most susceptible part of the population. The estimates are given in Table 3H.3.

The expected constant and linear relationship between the oral dose of methyl mercury and body burden is used by government officials to establish

Table 3H.3 Examples of estimated safe levels of daily or weekly methyl mercury or mercury intakes. LOAEL, lowest-observed-adverse-effect-level; PTWI, provisional tolerable weekly intake.

Exposure	*Estimate*	*Estimated intake*	*Unit*	*Established by*	*Reference*
Total mercury	PTWI	5	μg/kg body weight/week	JECFA	WHO, 1987[105]
Methyl mercury		1.6			WHO, 2004[106] WHO, 2007[107]
Elemental mercury	RfD, based on LOAEL	0.3	$\mu g/m^3$ air	US EPA	US EPA 1995[108]
Inorganic mercury	RfD, based on LOAEL	0.3	μg/kg body weight/day	US EPA	US EPA 1995[108]

guidelines on safe levels of methyl mercury exposure. However, the mercury level in hair, which is used in most epidemiological studies as the only indicator of human exposure, does not always reflect the reported level of methyl mercury intake *via* fish consumption. As reported by Canuel *et al.*,[73] the expected constant and linear relationship between the oral dose of methyl mercury and body burden seemed to vary among ethnic groups, most probably owing to differences in specific genetic characteristics and/or interactive effects of other dietary components. The reported difference between expected and actual hair concentration was up to 14-fold.[73] Moreover, Budtz-Jørgensen *et al.*[103] have demonstrated that imprecision in the exposure variable is likely to lead to underestimation of the exposure effect.

To summarize, all these studies lead us to the conclusion that the accurate assessment of exposure itself is crucial in risk assessment studies. For example, hair mercury can provide a good assessment of exposure to methyl mercury, but excretion of methyl mercury from blood to hair depends significantly on the metabolism of individuals. In this case blood methyl mercury is the preferred matrix of choice. Also, a considerable body of literature exists focusing on the effects of prenatal mercury exposure through fish consumption on neuro-development. Yet, findings have been inconsistent, particularly when assessing the effects of exposures to low mercury levels.

3H.8 Geographical Variations: Identification of "Hot Spots"

Depending on the mercury source and how it was released into the environment, mercury may be present in concentrated "hot spots" or dispersed over extensive areas. "Hot spots" are defined as sites that contain high mercury concentrations relative to the local levels in soils and sediments. Hot spots may result from a single well-delimited pollution source. The area concerned may be small – a few hundred square metres – but the potential consequences in terms

of human exposure are significant for the local population. Hot spots may also result from several associated single-source releases, in which case the area concerned is much more extensive than in the previous case, and the associated pollution impact can be regional with a radius exceeding the tens of kilometres scale. The contamination is more diffuse and may affect the whole population living in the area.

Altogether, more than 1200 sites have currently been identified[109] where mercury ore was mined and/or processed, 220 sites where mercury has been used in the chlor-alkali industry (both active and converted plants were taken into account), approximately 500 locations where precious metals (gold and silver) are processed in large-scale mining activities, and more than 600 locations where non-ferrous metal ore is processed (including zinc, copper, lead and nickel) (Figure 3H.6). As artisanal small-scale gold mining (ASGM) activities are conducted at hundreds of small sites they are considered as point sources, and cannot be identified individually. Estimates of mercury releases from ASGM activities based on country data were made in the report of Telmer and Veiga.[110]

From a global perspective, most mercury-contaminated sites identified (>70%) are concentrated in industrial regions of Europe and North America that are adjacent to the Atlantic Ocean and Mediterranean Sea. In contrast to Europe and North America, the number and extent of mercury contaminated sites in other parts of the world (especially Asian countries and India) are increasing as a result of the rising use of mercury in various products and processes.

The recent UNEP/WHO publication "*Guidance for Identifying Populations at Risk from Mercury Exposure*"[61] summarizes the risks of mercury exposure to public health in general and provides guidelines for the identification of populations at risk.

People living in contaminated sites are exposed to elevated levels of mercury, frequently exceeding safe levels. The release of mercury to the environment from mineral deposits enriched in mercury can have an impact on humans and biota through direct and indirect pathways. Direct pathways include ingestion of tailings and soils contaminated with mercury and respiration of mercury vapour and enriched particles. Ingestion is of concern primarily for young children who may eat soil directly, or be exposed by a high level of hand–mouth activity. Indirect pathways that have an impact on humans are more important and include consumption of fish and, more rarely, edible plants that have been contaminated with methyl mercury.

The factors that determine the occurrence and severity of adverse health effects include the chemical form of mercury, the dose, the age or developmental stage of the person exposed (the fetus is considered to be the most susceptible), the duration of exposure, and the route of exposure (inhalation, ingestion, or dermal contact). Dietary patterns can increase exposure, *e.g.* in a fish-eating population when fish and seafood are contaminated with mercury.

The primary targets of toxicity of mercury and mercury compounds are the nervous system, the kidneys and the cardiovascular system. It is generally accepted that developing organ systems (such as the fetal nervous system) are



Figure 3H.6 Distribution of contaminated sites in the world.[109]

the most sensitive to the toxic effects of mercury. At the same time, studies have indicated that levels of mercury in the fetal brain appear to be significantly higher than those in maternal blood. Other systems that may be affected include the respiratory, gastrointestinal, haematological, immune and reproductive systems.

Although the toxic nature of mercury is well acknowledged, little is known of the effects of mercury on humans as a consequence of long-term exposure to low concentrations, which are often seen in contaminated sites. In many cases, the use of biomarkers, such as mercury concentrations in blood and urine, are not sufficient to assess the internal doses and potential effects on the central nervous system, kidney, the immune system, and other possible effects. Therefore, better scientific understanding of the risks to human health, especially to those people living close to potentially polluted sites, is needed.

In addition, there are many important gaps in knowledge defining the benchmark dose for significant effects owing to uncertainties associated with epidemiological studies carried out so far.

- Exposure assessment is imprecise. In the case of methyl mercury, calculation of the intake is complex because it is based on the conversion of biomarker data such as hair levels into daily intake.
- The effect of a single factor has been assumed (in this case a single form of mercury) in a situation in which many covariates may affect the final impact. There are a large number of potential confounding factors in major epidemiological studies on methyl mercury, such as the source and pattern of methyl mercury exposure (or elemental mercury exposure), the nature of the populations, the influence of nutrition, and the presence of other pollutants such as polychlorinated biphenols (PCBs), which make comparison of studies and interpretation of the data difficult.
- In numerous contaminated sites/regions, humans are exposed simultaneously to elemental mercury through inhalation, and to inorganic mercury and methyl mercury through food consumption. So far, very little is known of the effects of these combined exposures on adults and children. Further studies are needed to develop safe limits of exposure for the most vulnerable groups.

Most human health-related studies in contaminated sites were carried out in mercury mining areas,[111] artisanal gold mining sites (GEF-UNIDO project)[112,113] and chlor-alkali sites (Kazakhstan, Albania, Italy and Sweden).[114–116] Unfortunately, these studies mainly addressed exposure. Risk characterization, however, is the integration of the hazard identification, dose–response assessment, and exposure assessments to describe the nature and magnitude of the health risk. Risk characterization of the relevant populations in the above-mentioned studies have not been addressed adequately. This has prevented the development and implementation of effective programmes to protect the populations.

Only limited information on exposure to mercury in contaminated sites is available. Exposure to methyl mercury as a consequence of long-range

transport, deposition and availability has recently been addressed by numerous groups. A few studies implemented in contaminated sites indicate that direct exposure to elemental, inorganic and methyl mercury in contaminated sites significantly exceeds that in background areas and contributes to the exposure of wildlife. It is documented that wild fish, mammals and birds that live on fish may be at risk from elevated dietary methyl mercury intake and toxicity. In controlled feeding studies, a diet that contained mercury (as methyl mercury) at environmentally realistic concentrations resulted in a range of toxic effects in fish, birds and mammals, including behavioural, neurochemical, hormonal and reproductive changes. A limited number of field-based studies support laboratory-based results that demonstrate significant relationships between methyl mercury exposure and various indicators of methyl mercury toxicity, including reproductive impairment.[117]

Very limited information is available on the effects of mercury released from contaminated sites on candidate wildlife species. Limited (but useful) guidelines for an ecosystem response to mercury contamination are available,[118] but these are commonly not included and implemented in the monitoring strategies, particularly for existing contaminated sites. Further development of monitoring strategies is needed to improve our knowledge and prevent negative impacts.

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3.2 Analytical Quality Requirements in Human Biomonitoring Programs: Trace Elements in Human Blood

This section is a manuscript authored by J. Snoj Tratnik, D. Mazej and M. Horvat. It has been accepted for publication in the International Journal of Environmental Research and Public Health.

The study deals with the quality of HBM measurement results, which is of great importance to assure metrological comparability of the measurements obtained in HBM or epidemiological studies.

It is based on the results of the inter-laboratory comparison exercise for Hg, Cd and Pb in blood samples, which are most commonly used biomarkers of exposure to potentially toxic metals. The results were used to quantify the contribution to the required sample size arising from analytical measurement variability. Besides the inter-laboratory comparison data, we used measurement uncertainty, which was calculated for the measurement procedures used in our laboratory, and population variability, which was obtained from the study on 6-11 years old children partly described in the Kobal et al. (2017) and Hrubá et al. (2012), and was conducted at roughly the same time as the inter-laboratory comparison. Based on the data, sample size calculations were performed.

The calculations demonstrated that the required sample size increased the most on account of variability arising from the measurements performed in different laboratories. To reduce the effect of the measurement variability, the manuscript proposes a single-laboratory approach as a cost-effective compromise.

The outcome of this work is crucial for planning of HBM and related population-based studies (e.g. epidemiological). It sets the framework for observing spatial and temporal trends of exposure to environmental pollutants with statistical significance, being one of the aims of a HBM study, but also to allow precise exposure assessment for the purpose of association studies linking exposure and health outcomes (epidemiological studies). It therefore complies with the first aim of the thesis:

- Set the methodological framework for the assessment of low-level Hg exposure and its link to neurodevelopment: evaluation of analytical quality requirements.

Based on the outcome of this work, all analyses done for the purpose of the present thesis are based on the single-laboratory measurements.

I analyzed Hg in the inter-laboratory exercise samples and samples of school-age children and calculated measurement uncertainty for the analytical procedure used in our laboratory (CVAAS). For the purpose of this section, I performed sample size power calculations and drafted the manuscript.

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Analytical Quality Requirements in Human Biomonitoring Programs: Trace Elements in Human Blood

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Abstract: Human biomonitoring (HBM) programs consist of several interrelated and equally important steps. Of these steps, the study design must answer a specific question: How many individuals must be recruited in order to define the spatial or temporal trends of exposure to environmental pollutants in a given HBM study? Two components must be considered at this stage: the population variability of the expected exposure and the performance characteristics of the analytical methods used. The objective of the present study was to quantify the contribution to the required sample size arising from (i) measurement uncertainty and (ii) inter-laboratory measurement variability. For this purpose, the sample size was calculated using the measurement uncertainty of one laboratory, inter-laboratory comparison exercise data, and population variability for commonly studied metals (mercury, cadmium, and lead) in blood. Measurement uncertainty within one laboratory proved to have little influence on the sample size requirements, while the inter-laboratory variability of the three metals increased the requirements considerably, particularly in cases of low population variability. The multiple laboratories approach requires that laboratory variability be considered as early as the planning stage; a single-laboratory approach is thus a cost-effective compromise in HBM to reduce variability due to the participation of different laboratories.

Keywords: human biomonitoring; inter-laboratory comparison; measurement uncertainty; quality control; sample size; trace elements

1. Introduction

Human biomonitoring (HBM) studies are an effective tool to assess human exposure to environmental pollutants and the potential health effects of such pollutants, and are therefore applied worldwide at the national and regional levels. Only recently has it been recognized that harmonized approaches are needed, including support activities, to guarantee the acquisition of comparable results. HBM activities are, in general, fragmented, and are carried out for various purposes. Different population groups are recruited using different protocols and recruitment strategies, making it impossible to compare results or conduct comparable health risk assessments. The recent DEMOCOPHES study was the first attempt to perform harmonized HBM on an European scale, from the study design and protocols to the laboratory measurements and data analysis [1,2]. Furthermore, the ongoing European HBM Initiative HBM4EU is developing harmonized approaches for HBM in Europe, and has recognized trace elements cadmium (Cd), lead (Pb), and mercury (Hg) as priority substances in these HBM activities (https://www.hbm4eu.eu/). Similarly, the Minamata Convention on Mercury was recently ratified, with the aim of protecting human health and the environment from the adverse effects of Hg (www.mercuryconvention.org). Evaluating the effectiveness of this convention

includes HBM. In such cases, "*metrology support is of great importance to allow sustainable implementation of the program(s) and provide quality data to assess time and spatial trends*" [3]. The concepts and principles of metrological traceability, which is a prerequisite for obtaining the metrological comparability of measurement results [3], are well elaborated in the *International Vocabulary of Basic and General Terms in Metrology* [4]. However, their practical implementation in various programs has yet to be elaborated.

In summary, two aspects of assuring the appropriate quality of HBM measurement results are critically important: The first is to formulate an appropriate design for protocols and recruitment strategies, and the second is to assess the metrological comparability of the measurement results obtained by participating laboratories. The present paper deals primarily with the second requirement. To demonstrate the equivalence of the measurement results obtained by different laboratories, a case study, including inter-laboratory comparison (ILC) exercises, was implemented in the framework of the EU-funded project PHIME (6th FP, *Public health impact of long-term, low-level mixed element exposure in susceptible population strata*, 2006–2011). Different laboratories in the participating countries within and outside Europe were involved in four ILC exercises, with the aim of determining the total levels of Hg, Cd, and Pb in both freeze-dried and fresh blood human samples.

The overall aim of the PHIME project was to establish associations between low-level exposure to trace elements and critical health effects in susceptible population groups. One of the specific aims was to assess the geographical variability of exposure to such elements in Europe [5]. Within this framework, the present case study was designed to quantify the contribution to the sample size arising from the analytical performance of the methods used. For this purpose, sample sizes were calculated using (1) population variability, (2) measurement uncertainty within a single laboratory, and (3) inter-laboratory variability based on the ILC exercises. Population variability in the blood levels of total Hg, Cd, and Pb was assessed in blood samples from 6–11-year-old children [5]. The collection of samples obtained as part of the PHIME case study and for use in inter-laboratory exercises was conducted in accordance with the Declaration of Helsinki and was approved by the National Medical Ethics Committee of Republic of Slovenia, Ministry of Health, Ljubljana (no. of accordance 98/05/06). All participants and the parents of participating children signed an informed consent form prior taking part in the study.

2. Methods

2.1. ILC Sample Preparation

Four sets of ILC samples were prepared. Set PT-WB1 included lyophilized human blood from 12 non-exposed persons (each of whom provided 300–400 mL of fresh blood); set PT-WB2 included lyophilized blood from a person occupationally exposed to elemental Hg (this individual provided about 500 mL of fresh blood); and set PT-WB3 included lyophilized human blood from three fish eaters (each of whom provided 250 mL of fresh blood). All samples were collected from voluntary blood donors using the standard blood bag system. The last sample set included three samples of fresh blood from three healthy voluntary donors (samples FF 3613, FF 3614 and GG 0461), collected into several 4 mL lithium heparinized sample tubes (Vacuette, Greiner Bio-One, Kreismünster, Austria).

Blood samples PT-WB1, 2, and 3 were frozen at −20 °C and lyophilized at −47 °C and 0.030 mbar in a freeze dryer (Alpha 1-4, Martin Christ, Osterode am Harz, Germany). Samples were ground in a planetary micro mill (Pulverisette 7, Fritsch Milling and Sizing, Idar-Oberstein, Germany) using agate balls and jars. Particle size was <250 µm. The samples were then sterilized by gamma irradiation at a dose of 5 Mrad using ^{60}Co source at the Ruđer Bošković Institute in Croatia to ensure the long-term stability of the material by inhibiting microbial growth. Homogenization was performed by mixing each sample in a rotating plastic container for 48 h. The total weights of the resulting samples were approximately 900 g (PT-WB1), 110 g (PT-WB2), and 150 g (PT-WB3), respectively. Blood samples FF 3613, FF 3614, and GG 0461 were stored at −20 °C before shipping them to the recipient laboratories.

For the purpose of bulk homogeneity testing, 10 aliquots of the PT-WB1 sample and 6 aliquots of the PT-WB2 and PT-WB3 samples, each of ~200 mg, were taken randomly from a rotating plastic container.

Based on the demonstrated homogeneity of the bulk material, the material from the rotating plastic container was then aliquoted into 20 mL glass bottles, each containing 4–5 g of the sample. Between- and within-bottle homogeneity was tested using 6 random bottles of the PT-WB1 sample and 3 bottles each of the samples PT-WB2 and PT-WB3. ILC samples were analyzed at the Jožef Stefan Institute (JSI). Three independent sample aliquots were analyzed. Approximately 500 mg of the sample was dried at 85 °C until it reached a constant weight to determine the dry weight.

In order to guarantee the stability of the samples during the implementation of the ILC, samples from three different bottles per sample set were re-analyzed. The results of the homogeneity and stability tests were obtained using the regular quality system of the JSI laboratory, and are presented in Section 3.1 below.

2.2. Analytical Methods Used to Characterize the ILC Samples

To characterize the ILC samples (in terms of homogeneity and stability), the selected elements were determined using: (1) acid digestion and inductively coupled plasma mass spectroscopy; (2) acid digestion and cold vapor atomic absorption spectrometry (CVAAS); and (3) thermal desorption and CVAAS. The measurement procedures are described elsewhere [6–10] and in detail in the following paragraphs. The quality of the described procedures was assured using the reference material Seronorm Whole Blood Level 1 (Sero AS, Billingstad, Norway).

2.2.1. Acid Digestion and Inductively Coupled Plasma Mass Spectroscopy

Approximately 0.2 g of each lyophilized blood sample was weighed into a 50 mL Teflon tube. Six mL 65% HNO_3 and 2 mL 30% H_2O_2 were added and the tube was heated in an aluminum block at 80 °C overnight and then at 130 °C for 2 h. After cooling 2 mL 30% H_2O_2 was added two times and the tube was heated each time for 15 min at 90 °C. Finally, the solution was diluted to 40 g with Milli-Q water. Measurements were taken using an Octapole Reaction System Inductively Coupled Plasma Mass Spectrometer (7500ce, Agilent Technologies, Santa Clara, CA, United States) equipped with an ASX-510 Autosampler (Cetac Technologies, Omaha, NE, United States). Helium mode of the Octapole Reaction System (Agilent Technologies) was used. All isotopes were quantified using three central points of the spectral peaks. The instrumental conditions were as follows: nebulizer, Micro Mist; spray chamber, Scott-type; spray chamber temperature, 5 °C; plasma gas flow rate, 15 L/min; carrier gas flow rate, 0.82 L/min; make-up gas flow rate, 0.11 L/min; nebulizer pump, 0.1 rps; RF power, 1500 W; reaction cell gas He 4.1 mL/min; isotopes monitored, ^{111}Cd, ^{206}Pb, ^{207}Pb, and ^{208}Pb. The instrument was tuned daily using a solution containing 10 ng/g each of lithium, magnesium, yttrium, calcium, thallium, and cobalt. The detection limit was 0.03 ng/g for Cd and 1 ng/g for Pb.

2.2.2. Acid Digestion and CVAAS

Approximately 0.5 g of blood was weighed in a 50 mL volumetric flask, and after the addition of 1 mL of distilled water, 1 mL of HNO_3, 1 mL of $HClO_4$, and 5 mL of H_2SO_4 it was heated at 200 °C on a hotplate for 20 min. After cooling, the digested sample was filled up with distilled water. The measurement of total Hg is based on CVAAS using a semi-automated Hg vapor introduction system. A known volume of the sample was introduced into the reaction vessel. After reduction with $SnCl_2$, Hg vapor was swept from the solution by aeration and purged into the absorption cell, where Hg absorbance was measured at 253.7 nm. This method is described in greater detail elsewhere [9]. A standard solution of inorganic mercury (Hg^{2+}) prepared from pure elemental Hg (certificate of purity no. 13/2005) was used to calibrate the instrument and check its accuracy. The limit of detection was 0.05 ng/g.

2.2.3. Thermal Desorption and CVAAS

The DMA-80 Direct Mercury Analyzer (Milestone, Shelton, CT, USA) was used. Approximately 0.05 g of sample was weighed in quartz boats and placed in an auto-sampler. The samples were initially dried by an oxygen stream passing through a quartz tube located inside a controlled heating coil. It was then thermally decomposed at 650 °C and the released Hg was reduced on a catalytic column. Hg vapor was collected in a gold amalgamation trap and subsequently desorbed. The Hg content was determined using atomic absorption spectrometry at 254 nm. This method is described in detail elsewhere [11]. A standard solution of inorganic mercury (Hg^{2+}) prepared from pure elemental Hg (certificate of purity no. 13/2005) was used to calibrate the instrument and check its accuracy. The limit of detection was 0.08 ng/g.

2.3. Measurement Uncertainty

Measurement uncertainty was calculated for the measurement procedures described above. The measurement uncertainty for the total amount of Hg in the blood by thermal desorption and CVAAS was estimated based on the ISO 21748:2010 "Guidance for the use of repeatability, reproducibility and trueness estimates in measurement uncertainty estimation," using the reproducibility and recovery data from the validation study. The estimated measurement uncertainty for the determination of the total Hg in blood was 6.2% and the expanded uncertainty (k = 2) was 12.5%. The estimation is valid for the "normal" exposure range (i.e., below 5 μg/L).

The measurement uncertainty of Cd and Pb was evaluated using the Nordtest approach from the measurements of the reference materials Seronorm Whole Blood Levels 1 and 2 (Sero AS). The estimated measurement uncertainty for determining the level of Cd in blood was 11.7% (k = 1) at a concentration of approximately 0.3 μg/L (Level 1) and 6.3% (k = 1) at a concentration of approximately 6 μg/L (Level 2). The estimated measurement uncertainty for determining Pb in blood was 10.8% (k = 1) at a concentration of approximately 10 μg/L (Level 1) and 5.1% (k = 1) at a concentration of approximately 310 μg/L (Level 2).

2.4. Shipment of Samples

The lyophilized blood samples were shipped in 20 mL bottles at room temperature and frozen fresh blood samples were shipped in 4 mL sampling tubes with cold packs by an accredited courier using high-speed delivery. Deliveries were confirmed by the receiving laboratory.

2.5. Analytical Methods Used by the Participating Laboratories

The participants in the ILC exercises were asked to analyze all elements using their validated methodology intended for the HBM case study. They were also asked to make three separate determinations of each element and to report the results, together with a short description of the method used. Using the quality controls applied routinely within the laboratory was a condition of participation. The results for the lyophilized material were reported on a dry weight basis.

The sample pre-treatments and detection procedures used by the participating laboratories are presented in Table 1. The sample pre-treatments included mostly acid digestions (heating or microwave) in various combinations or sole use of perchloric, nitric, or sulfuric acids or hydrogen peroxide; in a minority of cases other oxidizing agents were added. One laboratory used thermal combustion on the samples (DMA). Detection procedures included CVAAS and fluorescence spectrometry (CVAFS), inductively coupled plasma mass spectroscopy (ICPMS), electrothermal atomic absorption spectrometry (ETAAS), flame atomic absorption spectrometry (FAAS), graphite furnace atomic absorption spectrometry (GFAAS), inductively coupled plasma atomic emission spectrometry (ICPAES), and neutron activation analysis (NAA).

Table 1. List of sample pre-treatments and measurement methods used by the participating laboratories. ILC, inter-laboratory comparison.

Lab ID	Pre-Treatment (Acids/Alkalines Used, T and Duration of Digestion)	Reported Analytes/Detection			Quality Control (Reference Materials and ILC)	Accreditation
		Hg	Cd	Pb		
1	$HClO_4/HNO_3$ (5:1), 70 °C, 16 h, open-lose caps	CVAFS	ICP MS	ICP MS	no data	
2	HNO_3/H_2O_2, 120–130 °C in closed vessels; none for DMA	CVAAS, DMA	ICPMS	ICP MS	Seronorm WB1, NIST 1547, NIST 966, IAEA-086	no
3	Ultraclave, 250 °C, 1 h	ICPMS	ICPMS	ICPMS	CRM NIST 966, Seronorm WB1	no
4	Pb, Cd: HNO_3/H_2O_2, Milestone Mega1200+FAM 40, 20 min; Hg: none	CVAAS	ETAAS (Zeeman)	ETAAS (Zeeman)	Seronorm WB1	yes
5	HNO_3/H_2O_2 in closed vessels, Milestone MLS 1200 MEGA	-	-	ICPMS	Dorm2, Dolt3, BCR 184, NIST 1577b	yes
6	HNO_3/H_2O_2 in closed vessels, FAR IR elect. oven, <140 °C, 4 h	-	ETAAS (Zeeman)	ETAAS (Zeeman)	ClinChek (whole blood), internal reference material	
7	HNO_3 in closed vessels, A.Parr Multiwave	CVAAS	-	-	Seronorm WB1; WB2, WB3	no
8	Hg: HNO_3, 100 °C 4 h in closed teflon vessel, Pb, Cd: deproteinization after dissolution in water	CVAAS	ETAAS (Zeeman)	ETAAS (Zeeman)	BCR 194, 195; ILC: CDC, USA; Instituto Superiore di Sanita, Italy	no
9	Hg: HNO_3/H_2SO_4; Pb and Cd: 10 × dilution with solution containing Triton X-100, EDTA, ammonia	CVAAS	ICPAES	ICPAES	no data	yes
10	HNO_3/H_2O_2, 160 °C, 10 h; closed vessels	ICPMS	ICPMS	ICPMS	Dorm-2, Dolt-3, ClinCheck (serum)	no
11	Three procedures were used: (1) HNO_3, 95 °C, 3 h; (2) Aqua regia, 95 °C, 30 min; (3) KOH/BrCl, 95 °C, 3 h	CVAFS	-	-	SRM 1566b, Dorm-2	yes
12	$HNO_3/HClO_4$ in open vessels, 130 °C, 4–5 h	NAA	FAAS Deuterium	FAAS Deuterium	IAEA A-13	yes
13	$HNO_3/H_2SO_4/HClO_4$ (1:5:1) in open flasks, 30 min, 200–230 °C	CVAAS	-	-	no data	
14	HNO_3 in closed vessels, Microwave oven, 160 °C 15 min for Hg, 190 °C 10 min for Pb, Zn	CVAAS Deuterium	-	GFAAS Deuterium	no data	yes
15	dilution in H_2O, Magos method	CVAAS	-	-	Seronorm WB1; ILC: International comparison program Centre de toxicoiogie du Quebec	no

All laboratories claimed to have experience in analyzing the selected metals in human blood samples and compliance with the ISO IEC 17025 quality standard; six laboratories were also accredited for trace elements analysis in biological and/or environmental samples. The reference materials used for quality control and assurance and participation in external ILC are listed in Table 1.

2.6. Statistical Methods

Between- and within-bottle homogeneity in the ILC exercise was tested using analysis of variance (ANOVA). The coefficient of variation (C_v = standard deviation/mean of measurements) was determined to measure the total Hg in bulk homogeneity testing in the ILC exercise.

Descriptive statistics (mean, standard deviation [SD], geometric mean, geometric SD, CI 95%) of the total Hg, Cd, and Pb concentrations in the blood of children were obtained. The geometric SD was calculated as the SD of log-transformed data.

The sample size required to detect significant differences in the mean concentrations of the selected metals between populations was calculated using STATA 12 software (StataCorp LP, Texas, U.S.). Population means, together with SDs, were used in sample size calculations as an example of population variability. T-tests (one-sample comparison of mean to hypothesized value; H0: Mean $_{\text{hypothesized}}$ = Mean $_{\text{postulated}}$) were employed to compare population means to postulated means, which differed by 5%, 10%, or 20%, taking into account the SD of the selected population (SD_{pop}). The statistical powers of 0.80, 0.90, and 0.95 and a significance level $\alpha = 0.05$ (one-sided) were employed. The predicted ratio of the sample sizes was 1:1.

In order to account for the variability due to measurement uncertainty within one lab and among multiple laboratories, the measurement uncertainty estimated for each element at the JSI lab (SD_{lab}) and the SD of measurements from the ILC exercises ($SD_{inter\text{-}lab}$) were used. The combined variability was calculated as:

$$SD_{comb1} = \sqrt{SD_{lab}{}^2 + SD_{pop}{}^2} \quad (1)$$

where SD_{lab} is a measurement of uncertainty (variability within one laboratory) and SD_{pop} is the standard deviation of values among the selected population; and

$$SD_{comb2} = \sqrt{SD_{inter-lab}{}^2 + SD_{lab}^2 + SD_{pop}{}^2} \quad (2)$$

where $SD_{inter\text{-}lab}$ is the standard deviation of measurements among different laboratories (inter-laboratory variability).

3. Results

3.1. The Homogeneity and Stability of the ILC Samples

According to the results of the bulk homogeneity testing, which yielded coefficients of variation of 7.3%, 0.9%, and 0.8% for total Hg in PT-WB1, 2, and 3, respectively, the bulk materials were homogenous. Between- (s_{bb}) and within-bottle (s_{wb}) homogeneity testing revealed no statistically significant variability. The maximum within-bottle homogeneity was 12%, while the between-bottle homogeneity was 4.5%; both were observed in the case of Pb. The homogeneities for Hg and Cd were lower (within-bottle < 7%, between-bottle < 2%). These values indicate that the materials were confirmed to be homogenous for all tested elements and were therefore suitable for distribution to laboratories. Stability testing showed no significant differences between the three testing periods within 6 months and confirmed the stability of selected elements for the duration of the ILC exercises. The residual moisture content (expressed as the mean ± SD, n = 4–6) was 3.4 ± 0.4%, 4.4 ± 0.2%, and 4.8 ± 0.2% for PT-WB1, 2, and 3, respectively.

3.2. Total Hg, Cd and Pb in Blood: Results of the ILC Exercise

Among the three selected metals, the measurement variability observed between the participating laboratories was the highest in the case of Cd (Figure 1). The fresh blood samples, representing a concentration range considered normal for the general adult population (means of 0.54, 0.46, and 0.48 ng/g), showed somewhat higher variability in the Cd concentrations (26%, 34%, and 31%, respectively) compared to the lyophilized samples with respective mean concentrations of 3.3 (27%), 3.1 (27%), and 9.6 ng/g (18%) (Figure 1). One laboratory reported inadequate levels in PT-WB1 (11.3 ng/g), which was therefore excluded from the total mean and SD calculation.

Total Hg measurements showed inter-laboratory variabilities of 12% (mean 8 ng/g), 15% (mean 54 ng/g), and 16% (mean 103 ng/g) in the lyophilized material (Figure 1), and, similarly, between 9% and 18% in the fresh blood samples at levels that are typical for people who consume fish three or more times a month (means 1.09, 1.39, and 1.80 ng/g, respectively). In the latter, the variability depended on the concentration level, the highest being in the lowest concentration range. Three laboratories reported inadequate levels for PT-WB1, with inadequate limits of detection (199 ng/g, <50 ng/g, <50 ng/g). These results were excluded from the total mean and SD calculations.

The respective inter-laboratory variabilities for Pb measurements were 10% (mean 159 ng/g), 16% (mean 158 ng/g), and 20% (mean 71 ng/g) for lyophilized material (Figure 1) and between 4% and 22% in the fresh blood samples, the latter representing concentrations found in non-exposed populations (mean levels between 25 and 31 ng/g). Four laboratories reported inadequate levels in the PT-WB1 (55, 315, 573, and 781 ng/g, respectively), which were therefore excluded from the total mean and SD calculations.

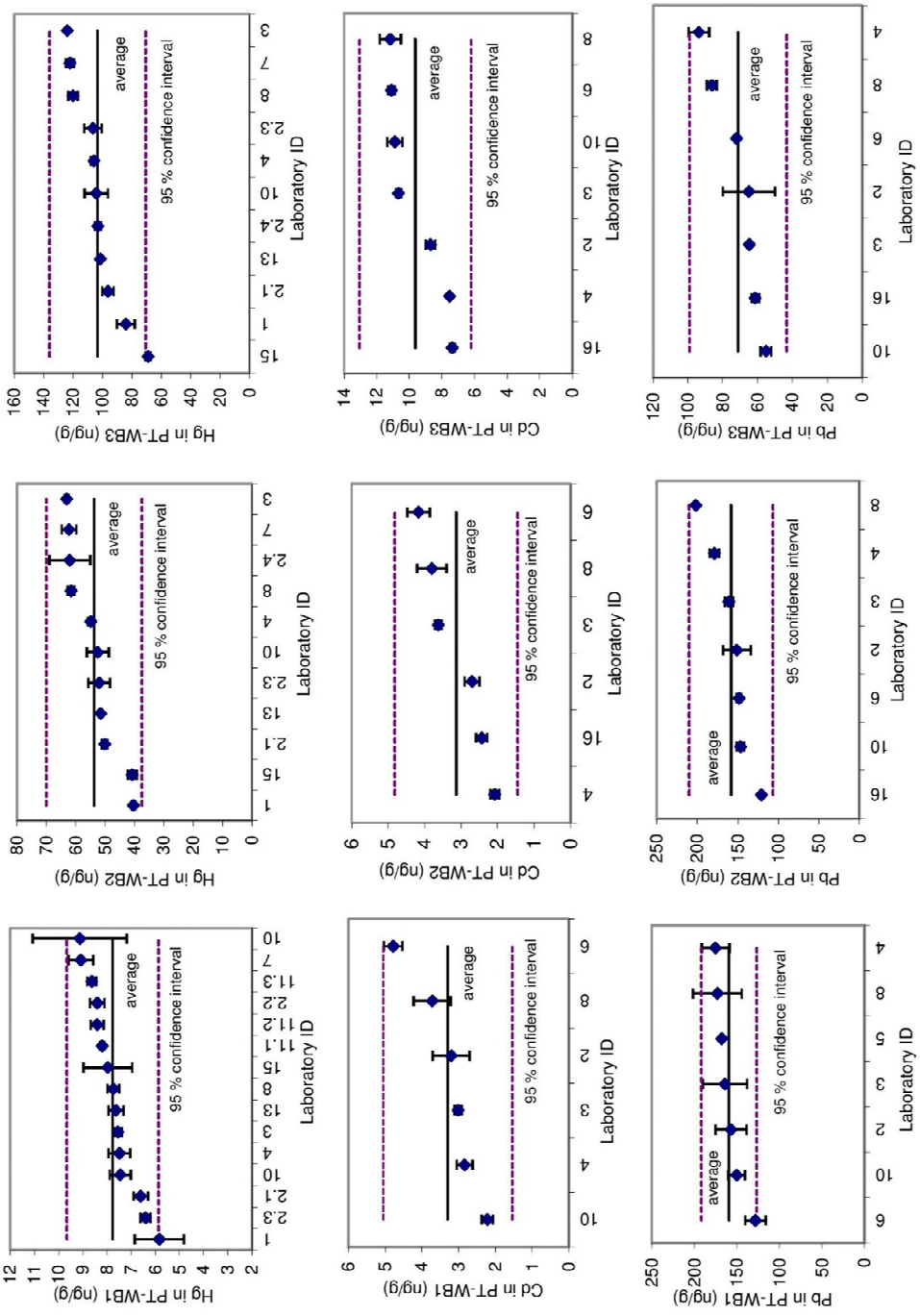


Figure 1. Total Hg, Cd, and Pb concentrations in the lyophilized ILC samples from the participating laboratories.

3.3. Total Hg, Cd and Pb in Blood: Population Variability

Population variability was estimated based on the concentrations of total Hg, Cd, and Pb measured in the blood of children from Slovenia, a case study implemented in the framework of the PHIME project. The Slovenian study population consisted of residents of urban, rural, and Hg-contaminated (the town of Idrija) areas. The results of the urban population group were presented along with other participating regions by Hrubá et al. [5]; however, for the purposes of the present study, children from the rural and contaminated areas were also included. The Slovenian study population relied on a population group of 6- to 11-year-old schoolchildren, among whom both genders and all age groups were equally represented. The participating children came from different socioeconomic backgrounds. All children were recruited, sampled, and analyzed simultaneously, following the same protocol described in Hrubá et al. [5]. The descriptive statistics for the Hg, Cd, and Pb levels in the blood of the Slovenian study population are presented in Table 2.

Table 2. Population variability: total levels of Hg, Cd, and Pb in the blood (μg/L) of 6- to 11-year-old children from three study areas in Slovenia. GM, geometric mean; Mean, arithmetic mean; min-max, range; *n*, sample size; 95% CI, 95% confidence intervals; SD, standard deviation.

Analyte Study Area	*n*	Min-Max	Mean (SD)	GM (SD)	95% CI
Total Hg					
All	174	0.35–4.39	0.97 (0.64)	0.84 (0.55)	0.78–0.91
Urban	45	0.35–3.05	1.05 (0.53)	0.94 (0.54)	0.82–1.08
Rural	66	0.35–3.72	0.82 (0.57)	0.71 (0.44)	0.63–0.80
Hg-contaminated	63	0.41–4.39	1.08 (0.75)	0.92 (0.63)	0.81–1.05
Cd					
All	150	0.13–0.69	0.23 (0.12)	0.20 (0.12)	0.19–0.22
Urban	42	0.09–0.28	0.15 (0.04)	0.14 (0.04)	0.13–0.16
Rural	65	0.13–0.54	0.25 (0.11)	0.23 (0.12)	0.20–0.25
Hg-contaminated	43	0.13–0.69	0.27 (0.14)	0.24 (0.15)	0.20–0.28
Pb					
All	165	5.33–56.8	16.7 (7.27)	15.4 (7.15)	14.6–16.4
Urban	42	6.90–23.7	14.0 (4.08)	13.4 (4.68)	12.3–14.8
Rural	64	5.33–56.8	17.8 (9.29)	16.1 (8.84)	14.4–16.5
Hg-contaminated	59	7.38–36.9	17.3 (6.08)	16.3 (6.81)	14.9–17.9

The international variability reported by the authors [5] was considerable for blood Hg, and varied by a factor of 8 between European cities, with the highest levels observed in the Slovenian children. Cd and Pb were only marginally different between the European cities, with the highest geometric mean being 1.5 times that of the lowest [5]. Within the Slovenian study population, Hg blood levels were lower in the rural population than in the urban group ($p = 0.011$), while no significant difference was observed between the urban and Hg-contaminated areas ($p = 0.355$). However, Cd levels were higher in the rural and contaminated area populations than in the urban study group (both $p < 0.001$); the same trend was observed for Pb ($p = 0.055$ and 0.035, respectively) (Table 2).

For the purpose of the power size calculations presented in this paper, the levels in the urban population were considered, as this particular area type was used in the international comparison conducted by Hrubá et al. [5]. All three variables were distributed log-normally, so geometric means and geometric SDs were considered in the sample size calculations.

3.4. Sample Size Calculation

Based on the observed variability within the urban study population group, the sample size required to observe a significant difference in blood Hg level was calculated and is presented in Figure 2. For this calculation it was assumed that all measurements were performed in one laboratory using the CVAAS method (described in Section 2.2). In order to observe a 20% difference in a mean Hg level between two populations or population groups, a minimum of 71 participants is required at

statistical power of 0.90 and a significance level of 0.05. Lower detected differences in mean levels require a considerably higher number of participants—e.g., 283 participants are required to observe a 10% difference and 1131 participants are required to observe a 5% difference (Figure 2).

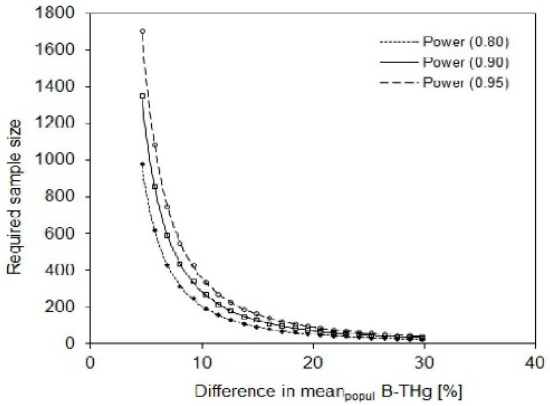


Figure 2. The sample size required to reveal a significant difference in blood Hg levels between two population groups, assuming that all analyses are performed in one laboratory. Statistical power = 0.80, 0.90, and 0.95; $\alpha = 0.05$; one-sided.

The population variability observed in evaluating the blood Cd levels in urban Slovenian children necessitated a sample size of 18 participants to reveal 20% difference in means between two populations or population groups, assuming that all measurements are performed in one laboratory (described in Section 2.2). Seventy participants would be required to reveal a significant difference of 10% and 280 participants would be required to reveal a 5% difference, with statistical power of 0.90 and a significance level of 0.05 (Figure 3).

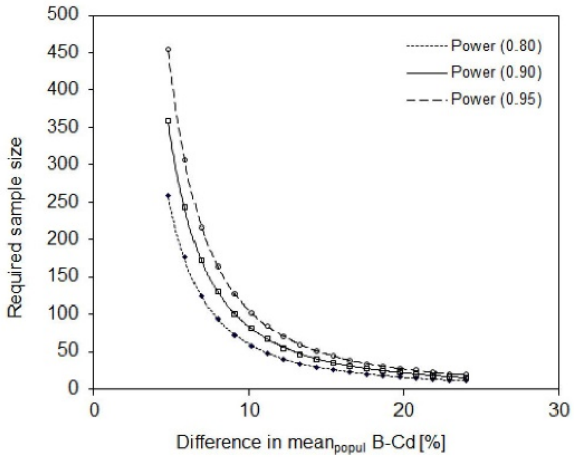


Figure 3. The sample size required to reveal a significant difference in mean blood Cd between two population groups, assuming that all analyses are performed in one laboratory. Statistical power = 0.80, 0.90, and 0.95; $\alpha = 0.05$; one-sided.

The population variability observed in evaluating blood Pb in urban Slovenian children necessitated a sample size of 27 participants to reveal a 20% difference in means between two populations or population groups, assuming that all measurements were performed in one laboratory (described in Section 2.2). To reveal significant differences of 10% and 5%, the sample size would have

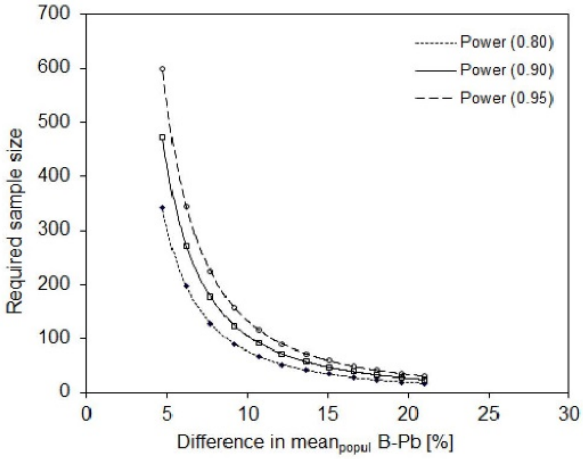


Figure 4. The sample size required to reveal a significant difference in mean blood Pb between two population groups, assuming that all analyses are performed in one laboratory. Statistical power = 0.80, 0.90 and 0.95; $\alpha = 0.05$; one-sided.

3.4.1. Measurement Uncertainty within a Single Laboratory

Measurement uncertainty at relevant concentration ranges was calculated to account for analytical variability within a single laboratory (SD_{lab}). Considering the measurement uncertainty calculated for the analytical procedures within the JSI lab (Section 2.4), the required sample sizes would need to be increased from 283 to 286 (Hg), 70 to 81 (Cd), and 105 to 115 (Pb) (Table 3).

Table 3. The sample size (n) required to observe 5, 10, and 20% differences in blood levels of Hg, Cd, and Pb between population groups (effect size). Combined SDs include SD_{lab}, $SD_{inter\text{-}lab}$, and SD_{pop}. Statistical power = 0.90; $\alpha = 0.05$; one-sided. GM, geometric mean of the study population; SD, standard deviation.

				n According to the Effect Size					*n* According to the Effect Size		
	GM (SD)	SD_{lab}	$SD_{pop} + SD_{lab}$	5%	10%	20%	$SD_{inter\text{-}lab}$	$SD_{pop} + SD_{lab} + SD_{inter\text{-}lab}$	5%	10%	20%
Hg	0.94 (0.54)	0.06	0.543	1144	286	72	0.113–0.170	0.555–0.569	1195–1256	299–314	75–79
Cd	0.14 (0.04)	0.02	0.043	324	81	21	0.036–0.048	0.057–0.064	568–716	142–179	36–45
Pb	13.4 (4.68)	2.95	4.91	460	115	18	0.536–2.95	4.94–5.72	466–625	117–157	30–40

3.4.2. Measurement Variability among Multiple Laboratories

Accounting for the inter-laboratory variability ($SD_{inter\text{-}lab}$) observed in the ILC exercise (Figure 1), the required sample sizes would need to be increased by an additional 5–10% in the case of Hg, 75–220% in the case of Cd, and 1–37% in the case of Pb blood measurements (Table 3). The range represents minimal to maximal increases in the sample sizes, depending on the ILC sample set used. Only the sample sets of the relevant concentration range were considered in the calculations.

4. Discussion

With the aim of evaluating the significance of measurement variability among multiple laboratories, relative to the variability within a single laboratory and to the study population, the prerequisite in this study was to prepare a sufficient quantity of uniform sample material for distribution among the different laboratories. The material was prepared from fresh blood samples collected from healthy volunteers. Lyophilization and homogenization of the collected blood samples provided homogenous materials for all tested elements, which were suitable for distribution to the participating laboratories, and were confirmed to be stable for the duration of the ILC exercises. Because lyophilized samples

contained higher concentrations of elements than fresh blood samples, they were not representative of routine samples. The presented ILC exercise therefore also included a limited number of fresh blood samples with the purpose of validating the variability of the relevant concentration ranges. They showed concentration ranges of the studied metals that are typically observed in general population groups within Europe. Although the inter-laboratory variability of the fresh blood samples was somewhat higher than in the lyophilized ILC samples, they did not differ considerably. The exposure levels observed in the study population that were used to assess population variability were more or less similar to other studies that have reported on the aforementioned elements in children's blood. Certain (although not marked) differences include undetected Cd levels in the German and US surveys [12,13] and somewhat higher Pb levels identified in the Czech HBM study [14] while lower ones were found in the US [12]. Compared to the Slovenian study population, Hg levels were lower in the German, US, and Czech studies [12,14,15], which is in line with the international comparison conducted by Hrubá et al. [5], which found that Slovenian children had the highest Hg levels among the participating European countries. Overall, the levels observed in our study population were within exposure levels that do not present an elevated health risk according to the reference values established for the European population [14,16].

Based on the variability within the study population, the highest required sample size was calculated for Hg measurements. The latter had the highest population variability (57%) among the three metals studied. Lead and Cd in the blood showed variabilities of 35% and 29%, respectively. Accordingly, the lowest required sample size was observed for Cd (Figures 2–4).

The sample size calculation results showed a negligible increase (1%) in the required sample size if the measurement uncertainty within one laboratory was in the range of 10% of the population variability (Hg), while an increase of 10% was observed in the case of Pb with an uncertainty of one-third of the population variability. The highest increase occurred in the case of Cd (16%), with its measurement uncertainty representing 40% of the population variability. If the hypothetical uncertainty was equal to population variability, the required sample size would double.

If the measurement variability among multiple laboratories participating in the ILC was added, the increase in the required sample size would be considerable, particularly in the case of Cd (Table 3, Figure 5). Cadmium blood levels, their low variability among the urban study group, and high inter-laboratory variability reflect the great significance of centralized analysis. However, as observed in the case of blood Hg measurements, high population variability decreased the influence of multiple laboratory analyses.

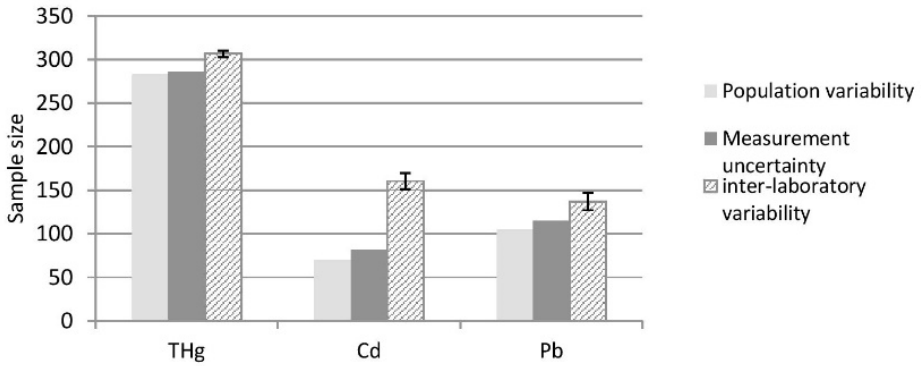


Figure 5. The sample size required to detect a 10% difference in mean values between two population groups depending on the (1) population variability, (2) population variability and measurement uncertainty within one lab, and (3) variability among different laboratories (inter-laboratory variability). Bars represent variation due to the use of different ILC samples. ILC, inter-laboratory comparison.

The calculated sample size is in line with the Slovenian case study, including three different population groups (urban, rural, and Hg-contaminated; see Table 2). The observed differences in blood

Hg concentrations between rural and urban (25%) and between rural and contaminated areas (23%) were in the range of the calculated sample size requirements and was shown to be significant, while that between urban and Hg-contaminated was not (2% difference in means). The sample size used in the study of children [5], with the number of participating children being between 21 and 57, depending on the country, was also sufficient to observe significant differences between the participating European countries, with the means ranging from 0.21 μg/L in Poland to 0.94 μg/L in Slovenia. In this study Hg blood levels were determined by two laboratories, which showed excellent agreement [5].

The between-group difference of more than 60% in mean blood Cd levels in the Slovenian case study was clearly significant (Table 2). The international levels reported by Hrubá et al. [5] showed very little variation in blood Cd between the European countries, but due to the centralized laboratory analysis, the differences observed between the participating cities were statistically significant.

In the case of blood Pb, the difference of 20% between urban and rural populations was revealed to be only marginally significant, which was due to the variability within the rural study group being almost double that of the urban group used in the sample size calculations. In this particular case we would need 118 participants in each group. Consistent with the calculations presented in this paper, the difference of 22% observed between urban and industrial population was revealed to be statistically significant.

From the sample size calculations using three commonly studied metals, it is obvious that requirements for sample size in HBM studies vary considerably. They depend on the selected analyte(s) and its/their variability within study groups, as well as the number of laboratories involved in sample analysis and the degree to which they agree with the reported results. As the HBM studies usually aim to measure different toxicants or their metabolites in the same subjects, the sample size requirements should be based on the one with the highest population variability, which in our case was Hg. Depending on the desired effect size to be observed, sample size increases following a square function. The effect size a particular study aims to observe is a matter of exposure range in a certain population group and toxicological relevance. In the case study described, an effect size with a minimum 20% difference was observed to be statistically significant for all selected elements and seemed relevant from the exposure perspective. Such an effect size requires at least 79 participants in each study group, taking into account inter-laboratory variability. A difference of 10% increased the sample size to approximately 300; a further reduction in effect size to 5% requires a sample size exceeding 1200 participants, which is regularly achieved in national HBM surveys such as those conducted in Germany, France, the Czech Republic, Slovenia, the United States, etc. [12,14,17,18]. Although health effects could occur at relatively low levels of exposure, as has been debated for methyl Hg in children [19], the toxicological relevance of such a small effect size is still to be clarified for many of the environmental pollutants, with an emphasis on susceptible population groups. Nevertheless, its importance in time-trend studies and exposure pathway identification, as the main objectives in HBM, remains. In spite of the low levels observed in children living in the selected European countries, strict analytical quality control and centralized analyses conducted by Hrubá et al. [5] revealed an association of blood Hg with amalgam fillings and fish intake; it was also found that blood Cd was associated with living close to dense traffic. Small changes can be especially relevant in assessing micro-elements (e.g., Zn, Cu, Se) with a rather narrow concentration range.

The present study has certain limitations, including the type of the ILC samples used (lyophilized blood), the corresponding concentration ranges, and the number of laboratories analyzing the ILC samples, which differed between the elements selected for the study. However, the measurement variability obtained from the analysis of lyophilized blood was validated by the analysis of fresh blood containing the relevant concentration ranges. Moreover, for each element, measurement variability within a single laboratory was compared to the variability among multiple laboratories and their relevant contributions to the required sample sized were assessed.

Despite the limitations, the data collected in this study clearly demonstrate that analyses of biological samples in an HBM study should preferably be performed in a single laboratory (i.e.,

a centralized analysis should be done) in order to minimize measurement variability. Laboratories must assure quality control using a regular quality system and participate in ILC exercises to achieve a relevant concentration range and matrices. The laboratory should also prove adequate measurement uncertainty in the range, as demonstrated in our case study. Uncertainty that constitutes 10% of the population variability contributes negligibly to the required samples size, while uncertainty constituting one-third of the population variability increases sample size requirements for 10%.

However, the choice of a single laboratory is not feasible in a practical sense, as "cross-European" and other international studies are usually beyond the capacity of one laboratory. This increases the importance of regular and strict quality controls, including the use of appropriate certified reference materials (CRMs) and the participation in international laboratory comparisons. This is where the support of national metrology institutes and regional metrology organizations, such as the European Association of National Metrology Institutes, in quality control procedures is required [20]. The development of harmonized approaches for HBM, such as the recently implemented COPHES (Consortium to Perform Human biomonitoring on a European Scale) project and the currently ongoing European HBM Initiative (HBM4EU) should also provide the means for sustainable metrology support in order to guarantee the comparability of the results over time and throughout geographical regions, be that Europe or worldwide.

Metrological comparability is required not only for measurements of chemical substances, but must extend to the application of new biomarkers that can be applied in molecular epidemiology, genetic toxicology, "omics-technology", epigenetics, and bioinformatics. These new biomarkers are crucial to the identification of toxicity pathways in humans, and will provide new insights and applications in HBM and environmental health studies. Metrology support will strengthen these initiatives with improved quality, reliability, and comparability of measurements to ensure and strengthen the position of HBM at the European level.

5. Conclusions

The present work evaluated the significance of measurement variability within a single laboratory or among multiple laboratories and its influence on HBM design, particularly sample size, compared to population variability. Measurement uncertainty within one laboratory for Hg, Cd, and Pb in blood showed little influence on the sample size requirements, while the inter-laboratory variability of the three metals increased the requirements considerably, particularly in the case of low population variability, as was observed for Cd in blood. Such low variability is common in general populations, where no contamination sites ("hot spots") are present but the potency of the substances analyzed are still within the range capable of causing health effects in susceptible population groups. In such cases, the effect of the measurement variability can be reduced using a single laboratory approach (centralized analysis). The approach used in this study should also be applied in planning proficiency testing schemes where z-scores are used to measure the performance of a particular laboratory. The z-scores require predefined quality standards, which should be carefully defined prior to the proficiency study, and should take into account the objectives of the HBM in terms of time and spatial trends.

Author Contributions: Conceptualization, J.S.T., D.M. and M.H.; data curation, J.S.T. and D.M.; formal analysis, J.S.T.; methodology, J.S.T., D.M., and M.H.; resources, M.H.; supervision, M.H.; visualization, J.S.T.; writing (original draft), J.S.T.; writing (review and editing), J.S.T., D.M., and M.H.

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3.3 Results of the First National Human Biomonitoring in Slovenia: Trace Elements in Men and Lactating Women, Predictors of Exposure and Reference Values

The paper is authored by J. Snoj Tratnik, I. Falnoga, D. Mazej, D. Kocman, V. Fajon, M. Jagodic, A. Stajnko, A. Trdin, Z. Šlejkovec, Z. Jeran and M. Horvat from the JSI; J. Osredkar, A. Sešek-Briški, M. Krsnik, A. B. Kobal from the University Medical Center, Ljubljana; and L. Kononenko from the Ministry of Health, Chemicals Office of the Republic of Slovenia who financed the study. It has been published in the International Journal of Hygiene and Environmental Health.

The paper provides an important contribution to the HBM framework in Slovenia. The first national HBM survey enabled to assess the background exposure to Hg and other potentially toxic elements (Pb, Cd, As) and concomitant status of essential elements (Se, Cu, Zn, Mn) in the Slovenian general population. For this purpose, a reference population was established, representative for the population of lactating women and men of the childbearing age. The reference population was selected as a vulnerable population, because it presents an exposure that can be eventually passed to their children through *prenatal* or *perinatal* periods. The study covered all geographic areas of the country with equivalent number of participants in each study area and equal distribution between the sexes. The study areas were classified as rural (Kočevje and Cerknica, Savinjsko-Posavsko, Pomurje), urban (Ljubljana, Koper, Maribor) or potentially contaminated (Bela krajina, Jesenice, Posočje and Idrija, Mežica Valley, Celje, Zasavje). The latter were selected from past or current industrial activities.

Recruitment of study participants followed strict inclusion and exclusion criteria which defined the reference population: residency in one of the selected areas in the same place for at least five years; childbearing age (defined by WHO as 18-49 years); mother's first and singleton pregnancy; absence of any health problems; the mother was breastfeeding; the mother was available for sampling 2–8 weeks after birth; living within a 100–200-m radius from active waste dumps, mines, industrial objects or crematories (refers to the participants in urban and rural areas). Occupational exposure to chemicals was excluded from the study.

The reference values have been derived for the first time in Slovenia, and present the background exposure for the selected population. Because of the known controversy of the concentrations in urine and their normalization for dilution (see Section 1.4), the reference values in urine are provided separately for volume expressed, creatinine and specific gravity normalized concentrations. The different expressions were evaluated in the statistical models. In case of blood, normalization of concentration per hematocrit was evaluated. The paper fulfilled two aims of the thesis:

- set the methodological framework for the assessment of low-level Hg exposure and its link to neurodevelopment: definition of the reference population living in Slovenia to assess the background exposure and evaluation of biomarkers of exposure including confounding factors;
- assessment of exposure to Hg and other potentially toxic elements in the Slovenian population of adults in childbearing age.

I conducted a part of the Hg analysis in urine, blood and hair, compiled the database to include data from questionnaires and laboratory analyses, conducted statistical analysis, derived the reference values, and wrote the manuscript.

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Results of the first national human biomonitoring in Slovenia: Trace elements in men and lactating women, predictors of exposure and reference values

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ABSTRACT

The first national human biomonitoring in Slovenia surveyed cadmium (Cd), lead (Pb), mercury (Hg), arsenic (As), manganese (Mn), selenium (Se), copper (Cu) and zinc (Zn) in a childbearing population (18–49 years) selected from lactating *primiparous* women and men (N = 1084). The overall aim was to estimate trace elements' levels and geographical variations in order to identify sources of possible exposures and set the national reference values. The study population was selected evenly from 12 study areas across Slovenia, including rural, urban and known or potentially contaminated environments. Within 6–8 weeks after delivery, venous blood, spot urine, scalp hair and breast milk samples were collected to determine the selected elements. The data analysis included descriptive statistics and multiple linear regression using elemental concentrations in biological matrices, questionnaire data and environmental datasets. Essential elements showed no significant deficiencies or excessive levels in the study population and were largely determined by sex and/or the participating women's physiological status (*postpartum*, lactation), as well as by certain dietary sources. Toxic elements' levels were mainly below the levels considered to present increased health risk. Lifestyle and nutritional habits appeared as significant determinants of exposure to Cd (smoking and game meat consumption), Hg (seafood and amalgam fillings), As (seafood) and Pb (alcohol consumption, smoking, game meat consumption and type of water supply). A distinctive geographical pattern was confirmed, due to past mining activities combined with naturally elevated background levels in the cases of Pb (Mežica Valley), Hg (Idrija and Posočje) and As exposure (Zasavje). Increased seafood consumption in the coastal study area contributed to higher Hg and As (arsenobetaine) levels. Extensive sample size database accompanied with life-style and environmental data improved the prediction of exposure patterns, set the reference values for the childbearing population living in Slovenia, and provided a strong basis for evaluating spatial and temporal trends in exposure. To our best knowledge, this is the first study to establish reference values for lactating *primiparous* women.

1. Introduction

Based on the legislation for the implementation of human biomonitoring (HBM) in Slovenia (Act for Chemicals, No. 110/03), a protocol to start the first national HBM survey was established in 2007, mainly triggered by the absence of an overall exposure data about chemicals originating from the environment or food intake. The methodology to asses exposure to chemicals in the national survey was selected based on the initiatives and the recommendations of the World Health Organization (WHO) - the International Programme on Chemical Safety (IPCS, 2004), and the European Environment and Health Action Plan 2004–2010 (CEC, 2004). The selection criteria for the chemicals, study areas and the study population were based on national air and soil monitoring results; the chemicals' toxicological hazards, persistence

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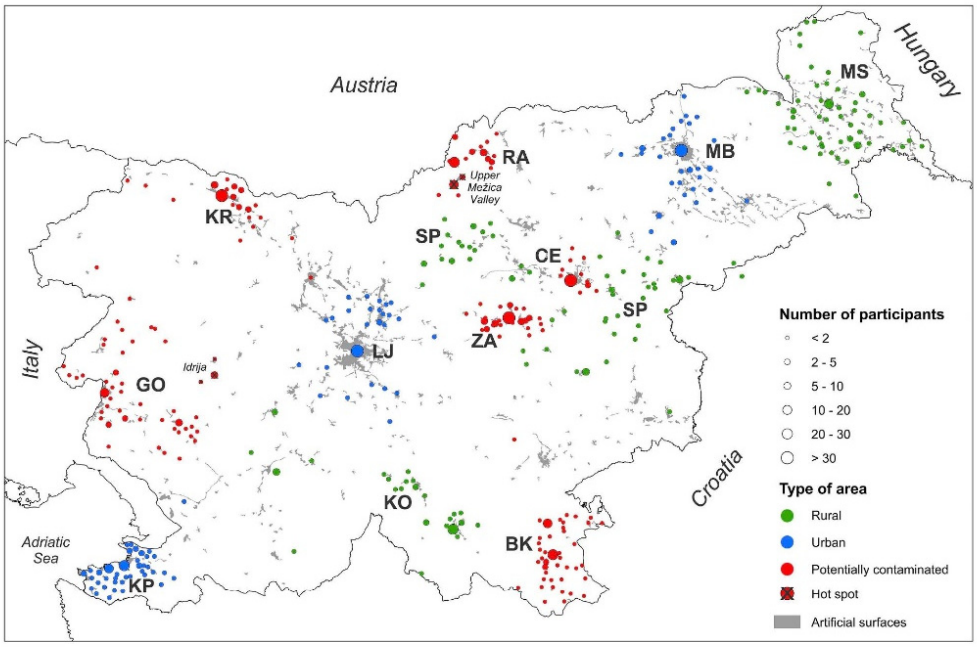


Fig. 1. Geographical distribution of participants in the Slovenian HBM survey. Circles depict total number of people recruited in an individual settlement. Study areas: BK = Bela Krajina, CE = Celje, GO = Idrija and Posočje, KO = Kočevje and Cerknica, KP = Koper, KR = Jesenice, LJ = Ljubljana, MB = Maribor, MS = Pomurje, RA = Mežica Valley, SP = Savinjsko-Posavska, ZA = Zasavje.

and bioaccumulation potential; the estimated size of exposed populations, analytical capacity, certain public concerns, and trends in other countries. Slovenia has three well-known heavily polluted areas ('hotspots'), where increased exposure of non-occupationally exposed inhabitants has been reported. These sites include the Upper Mežica Valley in the north, with residual pollution from a closed lead (Pb) and zinc (Zn) mine with a smelting plant (Eržen and Janet, 2005; Finžgar and Leštan, 2008; Miler and Gosar, 2012); the former mercury (Hg) mine town of Idrija in the west (Kobal et al., 2017; Kocman et al., 2011; Kotnik et al., 2005); and the area of Krupa River (southwest), contaminated with polychlorinated biphenyls (PCBs) from improper waste disposal from a former transformer and capacitor factory (Jan and Tratnik, 1988). In this paper, we do not deal with exposure to PCBs and other organic pollutants.

Along with the Upper Mežica Valley, other potentially Pb-contaminated areas in Slovenia include Šaleška Valley with its thermo-power plant Šoštanj and the industrial area of Celje (Eržen, 2011; Eržen and Janet, 2005). Other potentially polluted areas from past and current industrial activities are more or less spread across the country, including Lower Mežica Valley and Jesenice (northwest) with an ironworks industry; Central Sava Valley (central) with its coal-mining activities, thermo-power plant, cement plant, chemical company and glassware; and Celje (east) with its metallurgy, chemical-processing company and ironworks. Moderate arsenic (As) contamination due to other industrial/mining activities was reported in Mežica Valley, Sava Valley (Zagorje, Zasavje area), Idrija, Podljubelj and Litija, Celje and Jesenice (Gosar and Šajn, 2005; Perharič, 2013). However, a recent study on 3–12-year-old children in Sava Valley, specifically in the vicinity of the former Znojile antimony mine, did not show elevated As levels in their urine samples (Perharič et al., 2017).

For all these areas with various or even mixed industrial activities (past and/or current), complete population datasets on exposure to potentially toxic elements, accompanied by essential elements, are scarce. Therefore, several elements – Pb, Hg, cadmium (Cd), As, Zn, copper (Cu), manganese (Mn) and selenium (Se) – were selected for the first national HBM survey. Along with the potentially toxic elements, monitoring of endogenous essential elements enables a more realistic estimate of the health risks from low to moderate exposures. The elements Zn, Cu and Se can interact with toxic metals (and vice versa), influencing their absorption, distribution, metabolism and elimination in the human body. For example, Zn and Cu deficiency can increase the manifestation of the toxic effect of non-essential metals (Fitzgerald et al., 2008; Goyer, 1995), while the micronutrient Se can protect against the harmful effects of Hg by preventing damage from free radicals or forming biologically inactive Se-Hg complexes (Goyer, 1995; Suzuki et al., 1998) and similarly protect against As (Zeng et al., 2005) and also other non-essential elements (Cd, Pb) toxicity (Rahman et al., 2019). Various other mechanisms are proposed as well (Rahman et al., 2019). Of course, it should be noted that essential metals can also be toxic in cases of excessive exposure. The latter is well recognised for Mn, an essential element, which may act as a neurotoxicant in case of excessive exposure, reported mainly in adults' occupational exposures (Dobson et al., 2004; Horning et al., 2015).

In this paper, we summarise the results of the first national HBM survey that monitored Cd, Pb, Hg, As, Mn, Se, Cu and Zn levels in adults of childbearing age, selected from lactating *primiparous* women and men of the same age range. Participants from 12 study areas across Slovenia were recruited, including rural, urban and presumably metal-contaminated areas, and biological matrices were used to assess internal exposure. The main aims were to (1) determine trace elements levels and their geographical variations, (2) identify sources of possible exposures, (3) estimate babies' exposure via maternal milk and (4) establish the reference values for selected elements in the selected population. The latter is particularly important in relation to the lactating

primiparous women, for whom the reference levels are not available. *Postpartum* period is considered as the most critical period in the lives of mother and babies; whereat the drop of oestrogen after birth occurs between second and fifth month (Romano et al., 2010). The sources of potential exposure, tested in relation to the concentrations in the biological matrices, were selected from available data, starting from the general knowledge that exposure routes in non-occupationally exposed humans may be direct, through environmental compartments (air, soil and water) or food and lifestyle habits.

2. Methods

2.1. Study population and study areas

The study population included lactating *primiparous* mothers (mainly 6–8 weeks after delivery) and male participants of the same age range recruited from 12 geographically separate areas of Slovenia. The areas were classified into rural (n = 3), urban (n = 3) and potentially contaminated environments (n = 6) (Fig. 1). Kočevje and Cerknica (KO), Pomurje (MS) and Savinjsko-Posavska (SP) were selected as rural study areas; Ljubljana (LJ), Koper (KP) and Maribor (MB) as urban areas; and Bela Krajina (BK), Celje (CE), Jesenice (KR), Mežica Valley (RA), Posočje and Idrija (GO) and Zasavje (ZA) as potentially contaminated due to geological presence of metals, past and/or recent industrial activities (smelting plants, cement factory, power plant, glass factory, former Hg and Pb mines, ironworks, and former transformer and capacitor factory). The known 'hotspots' are within the GO (former Hg mine) and RA (former Pb mine) areas (Fig. 1). The selected rural areas have mainly agricultural activities, while the urban areas have multiple sources of current pollution, mainly from traffic. Potentially contaminated areas of CE and KR are at the same time also urban environments.

2.2. Recruitment and sampling

Recruitment and sampling spanned six years in two separate periods. The first period (2008–2009) covered KO, LJ and BK; the second (2011–2014) covered the rest of the study areas. Women were recruited in the third trimester of their pregnancies via gynaecologists, maternity hospitals or maternity schools in the selected study areas. Their partners were invited to participate in the study at any point. In each study area, 50 women and 50 men were planned for inclusion. In case a mother's partner was unwilling or ineligible to participate, another male participant was selected among family members or friends. The inclusion criteria for the participant were as follows: (a) residency in one of the selected areas in the same place for at least five years; (b) childbearing age; (c) mother's first and singleton pregnancy; (d) both mother and child should be healthy, without any problems in pregnancy; (e) the mother was breastfeeding; and (f) the mother was available for sampling 2–8 weeks after birth. Occupational exposure to chemicals was excluded from the study. For the participants in urban and rural areas, an additional exclusion criteria was (g) living within a 100–200-m radius from active waste dumps, mines, industrial objects or crematories.

The eligible participants donated venous blood (women 16 mL, men 23 mL), spot urine (50 mL), 3 cm of scalp hair close to the scalp from the occipital area (1 g) and women a maximum of 80 mL of breast milk (collected from multiple feeds in max 6 days). Sampling was carried out by regional health centres mainly 6–8 weeks after each mother's delivery (the actual range was 2 weeks–7 months, but 73% of women were within 6–8 weeks). The samples were transported to the central laboratory within 4–6 h after the sampling, where they were aliquoted into subsamples for specific analysis. In case some samples were transported to the central laboratory the next day, partial aliquoting was done in the regional laboratory. Sample cooling at 2–8 °C during transport and storage before aliquoting was guaranteed. Blood, urine and milk samples were stored long-term at −20 °C. Prior to the sampling, urine collection containers, as well as sample storage material (cryo-vials), were checked for trace elements content. Blood was collected in specialty tubes for trace element testing (Becton Dickinson Vacutainer® No. 368381). Standard needles to withdraw venous blood were used, pre-screening was not performed. Individual hair samples were stored in clean zip-lock polypropylene bags and an additional paper envelope. They were transported away from any Hg sources and stored in a clean room (< 10 ng Hg/m^3). Hair samples were not washed prior to analysis. All materials used in the laboratory analysis were checked for contamination through blank sample measurements. The study was approved by the Republic of Slovenia National Medical Ethics Committee, with numbers of accordance 42/12/07 and 53/07/09. Informed written consent was obtained from all participants. Participation was voluntary, and the participants could withdraw from the study at any stage.

2.3. Questionnaire data

The mothers and the male participants filled in questionnaires composed of (a) general information (age, body weight - for women pre-pregnancy weight, body height, education level and occupation), (b) basic home characteristics (residential environment - rural, urban, suburban; type of heating; type of water supply - public, private, bottled; vicinity of industry; age of residence), (c) health conditions (medicine, dietary supplements and number of dental amalgam fillings) and (d) lifestyle (smoking and hobbies) and nutritional habits (food consumption frequency, daily water intake, alcohol and coffee/tea intake and use of glazed pot). The participants reported their intake separately for fresh, canned and frozen seafood, which was set as relevant for Hg exposure. Due to the relatively high uncertainty of the self-reported consumption data, the intake of specific types of seafood was not summed up for the purpose of determining exposure sources in the cases of other elements. An additional set of questions regarding the pregnancy and lactation period was included in the maternal questionnaire (birth weight, birth length, intake of alcohol and smoking during pregnancy, and feeding).

2.4. Chemical analysis

2.4.1. Trace elements

Trace element analysis were performed in blood, milk, urine and hair samples using validated analytical methods. Both analytical and quality control procedures of element analysis used are described in detail in Supplemental material.

Briefly, measurements of Cd, Pb, As, Mn, Se, Cu and Zn in previously digested or diluted samples were performed using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) equipped with an Octapole Reaction System (ORS) (Jagodic et al., 2017; Potočnik et al., 2016; Stajnko et al., 2017). Total mercury was determined by cold vapour atomic absorption and fluorescence spectrometry (CVAAS and CVAFS) using three different analytical procedures (Akagi, 1997; Horvat et al., 1991; Miklavčič et al., 2013a, 2011), depending on the matrix type and the sampling period. All measurements were made under strict quality control procedures. Blank samples, control samples and reference materials were tested together with the samples on a daily basis. The limits of detection (LOD) were 0.2, 0.4, 0.1, 0,4, 8, 7, 30, 0.1 ng/mL for Cd, Pb, As, Mn, Se, Cu, Zn, and Hg in blood, respectively. In urine the respective LODs were 0.03, 0.3, 0.1, 2, 3, 3, 0.1 ng/mL and in milk 0.1, 0.2, 0.05, 2, 6, 35, 0.5 ng/mL. The LOD of the procedure to determine Hg in hair was 1 ng/g of hair.

Concentrations of elements in urine were normalised for differences in diuresis among samples using (1) creatinine (crt) concentration and (2) specific gravity (SG). Although it is known that crt-normalised levels predict the daily excretion of trace elements better than non-normalised levels for most of the elements due to systematic variation in urinary

flow rates (Hays et al., 2015), there is the risk of underestimating the exposure due to creatinine over-compensation, particularly for Cd and As (Hoet et al., 2016). Besides, creatinine varies depending on sex, age, body mass index, fat-free mass and race/ethnicity (Barr et al., 2005). Specific gravity appears to be a more reliable alternative in the context of environmental exposures, without the risk of over-adjustment and with fewer uncertainties associated with its use (Hoet et al., 2016). This finding has been confirmed in our recent study (Stajnko et al., 2017); moreover, SG normalisation has been used in As methylation studies due to the common nutritional factors influencing both creatinine and As levels (Balakrishnan et al., 2018; Drobná et al., 2016; Skröder et al., 2018). However, to enable comparability, we have decided to present urine concentrations expressed per volume, as well as crt- and SG-normalised.

2.4.2. Arsenic speciation

Arsenite (AsIII), arsenate (AsV), monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) were determined in 1:1 diluted and thawed urine samples using high-performance liquid chromatography (HPLC) separation on a Hamilton PRP-X100 column, followed online by hydride generation and CVAFS, as described earlier (Šlejkovec et al., 2008). The LODs ranged from 0.1 (AsIII, MMA) to 0.2 µg/L of urine (AsV, DMA). Speciation was performed in a subset of samples (n = 161) containing elevated total As (> 15 µg/L of urine). In selected samples, arsenobetaine (AsB) was determined using the same method with the Zorbax SCX chromatographic column and with additional online UV digestion before the hydride generation step.

2.5. Biochemical analysis

Haematocrit (%) in blood samples was determined as part of haemogram obtained using standard routine method at the University Medical Centre Ljubljana.

Creatinine in urine was determined by a standardised routine method (compensated Jaffé reaction) on the biochemical analyser Dimension (Dade Behring). Repeatability was assured by the reference material supplied by the analyser/reagent producer. The method's comparability and accuracy were verified by the inter-laboratory comparison certificate.

The SG was determined using a PAL-10S refractometer (Atago®, Japan), with a measurement range of 1.000–1.060. As the standards in SG normalisation calculations (Suwazono et al., 2005), SG means of 1.013 for females and 1.019 for males were used.

2.6. Acquisition of environmental and other ancillary data

The database on the drinking water comprised the concentration levels of the selected elements measured at 108 locations, evenly distributed across Slovenia. The available data was part of the radon (Rn) survey results about tap water in Slovenia in 2014 (Vaupotič and Gregorič, 2014). Information on actual concentrations in tap water for the individual subjects was not available, therefore drinking water concentrations from the closest location were assigned to each subject.

The database on the bio-indicators of air quality and deposition comprised the results of measurements of As, Cd, Cu, Hg, Pb, Zn, Mn and Se performed in moss *Hypnum cupressiforme* at 102 locations, evenly distributed across Slovenia, in 2010 (Harmens, 2010). The measured values from the closest location were assigned to each subject.

The geochemical composition of soil (Cd, Pb, Hg, Mn, Cu and Zn) at the address of each subject was obtained from an interpolated geochemical map of Slovenia (Geological Survey of Slovenia, 2006), available in raster format with a 500-m resolution.

Concentrations of selected elements in drinking water, moss and soil are presented by specific study areas (Tables S39–46 in the Supplementary data).

2.7. Statistical analysis

The descriptive statistics included the determination of median and mean values; standard deviation; 95% CI (CI_{95}) of the mean; 5^{th}, 10^{th}, 50^{th}, 90^{th} and 95^{th} percentiles (P5, P10, P50, P90 and P95). To derive reference values, the CI_{95} of P95 was determined for toxic elements as defined by the German HBM Commission and P2.5 and P97.5 for essential elements according to the International Federation of Clinical Chemistry (IFCC). The corresponding CI_{95} were calculated using a parametric method on ln-transformed data. The data below the LOD was assigned values of LOD/2. In cases > 10% samples were below the LOD (Cd in blood and milk, Pb in urine and milk, Cu in urine), the actual measured levels below the LOD were used in data analysis. In cases > 50% of the samples were below the LOD, only the percentile distribution was provided. Elemental concentrations were further stratified by sex and separate study areas (provided in the Supplementary tables). To compare concentrations of elements among the study areas or defined population groups, a one-way ANOVA was performed. Multiple linear regression models were used to evaluate the association between the elemental levels, separately in blood and urine (dependent variables), and potential predictor variables (independent variables) which were obtained through (a) questionnaires and (b) environmental databases. The models were omitted for all elements in breast milk and essential elements in urine. The first because the levels in this matrix could vary largely depending on the water/fat content (Gidrewicz and Fenton, 2014), and no normalisation method was used; and the second because levels in urine do not reliably reflect the status of essential elements. Confounding variables (sex and age) were fixed into the models. The sampling period (first versus second) was additionally fixed into the model if a significant difference was found between the two periods. In the blood models, haematocrit level was used as a confounder as well. In the urine models, Se concentration in blood was used as a confounder to account for a possible increased excretion of toxic elements. Because of the explained risk of misinterpretation using crt-normalised levels (Barr et al., 2005; Hoet et al., 2016), urinary models for specific elements were constructed based on SG normalised levels. In the sex-specific models for women, time between delivery and sampling (days) was included as a confounder due to gradual return to pre-pregnancy state. A separate set of variables was used for each model, they were selected from the bivariate correlations (ANOVA, Pearson's correlation) with probability values (p-values) < 0.25 and based on the predictions from the literature or previous studies. Highly correlated variables were not included in the same model to avoid the multi-collinearity effect. Log-normally distributed data was normalised using a natural logarithm. P-values < 0.05 were considered statistically significant, and p-values < 0.1 were marginally significant. Statistical analyses were performed using STATA 12 (USA).

3. Results and discussion

3.1. General characteristics of the study population

The study population included the adult childbearing population living in Slovenia, which was diverse in terms of the type of living environment, sex, education (although skewed towards higher levels) and occupation (Table 1). Among the women invited to participate in the study through gynaecologists, 17% responded positively. The number of women recruited through maternity schools and maternity hospitals was small and their response could not be estimated, as well could not be the response rate of the men. Sex was equally distributed among the study areas; so was the participants' mean age. Less than half (42%) of the male participants were female's partners. The majority of the participants had finished secondary or high school; more women (49%) were university degree holders compared with men (30%). A university degree (or higher) was the most frequent in urban areas; the primary level or apprenticeship was the least represented in most study

Table 1
General characteristics of the study population of men and lactating women from Slovenia.

Population group	n	Living environment (%) *			Age (years)	Level of education (%)			BMI (kg/m^2)	Smoking (%)**		Occupation (%)		
		R	U	Sub	Mean (min-max)	I	II	III	Mean (min-max)	YES	NO	I	II	III
Total	*1084*	*38*	*29*	*33*	*30 (18–49)*	*12.7*	*47.8*	*39.5*	*24.6 (16.6–44.8)*	*11.0*	*89.0*	*27*	*51*	*13*
Female	536	39	28	33	29 (19–39)	7.2	43.4	49.4	23.2 16.6–44.8)	13.3	86.7	34	51	2
Male	548	37	29	34	31 (18–49)	18.1	52.1	29.8	26.0 (17.5–37.6)	8.8	91.2	20	50	24
Study area														
Celje (CE)[c]	79	14	49	37	31 (20–40)	11.5	43.6	44.9	24.5 (18.3–34.9)	6.3	93.7	34	50	6
Posočje and Idrija (GO)[c]	96	44	28	28	30 (21–49)	12.2	51.1	36.7	24.3 (16.6–35.5)	11.7	88.3	30	39	20
Koper (KP)[b]	102	40	22	38	30 (20–42)	12.4	37.1	50.5	24.2 (17.2–36.8)	6.0	94.0	27	58	8
Jesenice (KR)[c]	83	30	38	32	29 (19–49)	12.2	60.8	27.0	24.4 (17.5–35.6)	14.5	85.5	20	62	12
Maribor (MB)[b]	98	19	35	46	31 (23–39)	3.0	50.0	47.0	24.7 (17.7–37.6)	14.0	86.0	29	56	3
Pomurje (MS)[a]	90	74	15	11	29 (20–48)	15.3	54.1	30.6	24.7 (17.7–35.1)	12.6	87.4	26	51	12
Mežica Valley (RA)[c]	79	42	30	28	33 (18–42)	16.0	48.0	36.0	24.7 (17.6–37.9)	3.8	96.2	30	44	11
Savinjsko-Posavska (SP)[a]	81	67	7	26	29 (21–40)	16.7	55.1	28.2	25.0 (18.0–36.9)	8.5	91.5	23	53	14
Zasavje (ZA)[c]	104	20	40	40	29 (21–44)	7.1	55.1	37.8	25.3 (18.2–35.9)	4.9	95.1	45	84	16
Ljubljana (LJ)[b]	100	16	40	43	30 (23–44)	4.1	25.8	70.1	23.7 (16.6–40.4)	5.7	94.3	26	63	6
Bela Krajina (BK)[c]	101	60	11	29	29 (21–41)	19.4	52.0	28.6	25.3 (18.8–44.8)	32.3	67.7	16	53	23
Kočevje and Cerknica (KO)[a]	71	37	25	37	28 (19–38)	30.3	43.9	25.8	24.4 (18.6–37.1)	10.5	89.5	25	34	31

Notes: Type of study area: **a** – rural, **b** – urban, **c** – potentially contaminated; type of living environment (*independent of the type of study area): **R** – rural, **U** – urban, **Sub** – suburban; Min – minimal value, Max – maximum value; BMI – body mass index; Level of education: **I** – primary or apprenticeship, **II** – secondary or high school, **III** – university or higher. **The women's smoking status refers to the pre-pregnancy period. Occupation type: **I** – managers, professionals; **II** – technicians, clerical support workers, service and sales workers; **III** – agricultural, forestry, fishery, craft and related trade workers, plant and machine operators and assemblers, elementary occupations and armed forces. Unemployed and students are omitted from the classification.

areas. Occupation Group II (see Table 1 for details) was the most represented in all study areas; the urban areas had the lowest proportion of Group III workers. The mean body mass index (BMI) levels were within the range considered normal in all study areas; 10% of the participants (7% women and 12% men) were obese (BMI $>$ 30 kg/m^2). Among the participants, 11% were smokers (the women's smoking status before pregnancy).

3.2. Levels of trace elements

Tables 2 and 3 present the concentrations of selected trace elements in biological samples. The levels of potentially toxic elements in the study population were generally low and, with minor differences, similar to those reported in the general populations studied in Europe (Baeyens et al., 2014; Becker et al., 2003, 2002; Castaño et al., 2012; Černá et al., 2012; Nisse et al., 2017; Puklová et al., 2010) and North America (CDC, 2018a; Haines et al., 2017) over the last two decades. The essential element status in our study population was assessed using the same matrices as those for toxic elements, except in hair. The observed variability was mainly due to the lactating status of the female study group, which was associated with decreased Se and increased Mn, Cu and Zn levels in the blood. Further explanations with national and international comparisons are provided in the specific sections that follow.

The percentages of the study population exceeding the reference levels or not complying with the reference intervals are specified in the Supplementary data (Tables S1–S37). In summary, population percentages exceeding the health- or population-based reference levels for potentially toxic elements (Schulz et al., 2011) or not complying with the essential elements ranges relevant for European populations (Barceloux, 1999; Iyengar, 1998; Wilhelm et al., 2004) were observed to a small extent. However, it should be stressed that for metals which transfer placenta at least to a certain extent (e.g. Cd, Pb, Hg), it is believed that there are no safe limits for maternal blood levels. There is a lack of international levels of concern specifically for pregnancy (or lactating period) and comparisons can only be made with national reference values (Taylor et al., 2014), as was done in the present study.

Table 4 presents predictors of exposure for individual element as obtained by multiple linear regression for the total population and separately for men and lactating women. In summary, life style and/or nutritional habits appeared as significant predictors ($p < 0.05$) of exposure to Cd (smoking and consumption of vegetable and game meat), Hg (consumption of seafood and amalgam fillings), As (consumption of seafood), and also Pb (alcohol intake, smoking, private water supply and consumption of game meat) for the total population. The geochemical data for top soil across Slovenia appeared as a significant environmental predictor in the cases of Hg and Pb exposure. In comparison to soil, Pb concentrations in moss showed stronger correlations with concentrations in biological samples. Blood Hg concentrations were also associated with tap water Hg concentrations. Apart from seafood, As exposure was also associated with tap water As (as indicated by As levels in blood). Exposure patterns for toxic elements noticeably depended on the sex and/or physiological status and the biomarker of exposure. The influence of sex could not be distinguished from the physiological (*postpartum*) status of the women. The models confirmed the sex/physiological difference in Cd concentration (higher in women's blood). Concentrations of Cd and Pb in blood increased with age, but Hg decreased. The most important determinant of all essential elements concentrations was sex/physiological status; men had higher Se levels, while women had higher Mn, Cu and Zn levels, which is consistent with the lactating status of female participants. In general, when comparing the elements according to the type of study area, higher Se and Zn levels were observed in contaminated areas than in rural areas, but did not differ from urban areas. The Cu levels were higher in contaminated areas than in urban areas, and even higher in rural areas. Similarly, Mn and Cd levels (in urine, but not in blood) prevailed in rural areas, presumably as a result of agricultural activities. Nutritional habits showed associations with Mn (consumption of game meat and bottled water), Se and Zn levels (consumption of seafood). Intake of dietary supplements was associated with increased Mn and Se blood levels in men, but decreased Mn and Zn levels in women. The observed predictors are discussed in detail in the specific sections that follow.

3.3. Cadmium

3.3.1. Basic exposure levels

Eight percent of men and two percent of women in our study population exceeded the population-based reference Cd level of 1 µg/L in blood (Schulz et al., 2011), while two and less than one percent,

Table 2
Levels of toxic elements in blood, urine and hair (and breast milk of females) of the study population of men and lactating *primiparious* women from Slovenia. crt – creatinine, SG – specific gravity.

Biomarker	Unit	Population group	N	N < LOD	GM [95% CI]	min-max	P5	P10	P50	P90	P95
Cd in blood	***µg/L***	*Total*	1083	301	**0.28** [0.27–0.30]	< LOD-4.80	< LOD	< LOD	0.29	0.72	1.01
		Men[a]	548	230	**0.23** [0.22–0.25]	< LOD-4.80	< LOD	< LOD	0.23	0.80	1.22
		Women[b]	535	71	**0.35** [0.33–0.37]	< LOD-3.08	< LOD	0.14	0.36	0.71	0.87
Cd in urine	***µg/L***	*Total*	1001	14	**0.19** [0.18–0.20]	< LOD-3.83	0.05	0.07	0.20	0.47	0.67
		Men[a]	506	2	**0.21** [0.19–0.22]	< LOD-1.75	0.07	0.09	0.22	0.49	0.62
		Women[b]	495	12	**0.17** [0.16–0.19]	< LOD-3.83	0.04	0.06	0.17	0.46	0.70
	µg/g crt	*Total*	989	14	**0.20** [0.19–0.21]	< LOD-2.79	0.07	0.09	0.19	0.47	0.61
		Men[a]	500	2	**0.16** [0.15–0.16]	< LOD-1.14	0.06	0.07	0.15	0.32	0.42
		Women[b]	489	12	**0.26** [0.25–0.28]	< LOD-2.79	0.10	0.12	0.25	0.58	0.79
	µg/L SG	*Total*	951	14	**0.21** [0.21–0.22]	< LOD-3.65	0.08	0.10	0.20	0.48	0.57
		Men[a]	479	2	**0.22** [0.21–0.23]	< LOD-1.11	0.08	0.11	0.22	0.46	0.56
		Women[a]	472	12	**0.21** [0.20–0.22]	< LOD-3.65	0.07	0.10	0.19	0.49	0.61
Cd in milk	***µg/L***		470	272	–	< LOD-0.34	< LOD	< LOD	< LOD	0.14	0.18
Pb in blood	***µg/L***	Total	1084	0	**18.0** [17.5–18.5]	3.86–116	9.13	10.3	17.5	32.5	41.5
		Men[a]	548	0	**19.3** [18.5–20.1]	3.86–116	9.16	10.4	18.7	37.4	46.1
		Women[b]	536	0	**16.7** [16.2–17.3]	4.25–71.9	8.82	10.3	16.3	28.0	33.2
Pb in urine	***µg/L***	*Total*	812	247	**0.46** [0.44–0.49]	< LOD-4.41	< LOD	< LOD	0.49	1.43	1.91
		Men[a]	402	65	**0.66** [0.61–0.71]	< LOD-4.40	< LOD	< LOD	0.71	2.31	2.31
		Women[b]	410	182	**0.33** [0.31–0.36]	< LOD-4.41	< LOD	< LOD	0.34	0.97	1.19
	µg/g crt	*Total*	812	247	**0.49** [0.46–0.51]	< LOD-5.31	< LOD	< LOD	0.50	1.19	1.49
		Men[a]	402	65	**0.49** [0.46–0.52]	< LOD-5.31	< LOD	< LOD	0.50	1.12	1.42
		Women[a]	410	182	**0.49** [0.45–0.52]	< LOD-3.44	< LOD	< LOD	0.51	1.27	1.57
	µg/L SG	Total	804	246	**0.53** [0.50–0.55]	< LOD-6.11	< LOD	< LOD	0.55	1.28	1.62
		Men[a]	397	64	**0.70** [0.65–0.75]	< LOD-5.21	< LOD	< LOD	0.75	1.51	2.07
		Women[b]	407	181	**0.40** [0.37–0.43]	< LOD-6.11	< LOD	< LOD	0.39	0.98	1.14
*Pb in milk**	***µg/L***		353	161	**0.23** [0.21–0.25]	< LOD-5.42	< LOD	< LOD	0.21	0.72	0.99
Hg in blood	***µg/L***	Total	1083	2	**1.18** [1.12–1.24]	< LOD-31.0	0.30	0.41	1.20	3.42	4.78
		Men[a]	548	1	**1.25** [1.15–1.34]	< LOD-31.0	0.26	0.38	1.28	3.72	5.13
		Women[b]	535	1	**1.11** [1.04–1.19]	< LOD-10.2	0.31	0.43	1.11	3.09	4.06
Hg in urine	***µg/L***	*Total*	1055	12	**0.44** [0.41–0.48]	< LOD-12.3	0.05	0.11	0.45	2.22	3.47
		Men[a]	527	2	**0.56** [0.50–0.62]	< LOD-12.3	0.08	0.12	0.56	2.67	3.80
		Women[b]	528	10	**0.35** [0.31–0.39]	< LOD-7.05	0.04	0.08	0.36	1.74	2.93
	µg/g crt	*Total*	1020	12	**0.47** [0.43–0.50]	< LOD-8.67	0.06	0.11	0.51	1.78	2.48
		Men[a]	516	2	**0.42** [0.38–0.46]	< LOD-7.92	0.06	0.09	0.44	1.50	2.19
		Women[b]	504	10	**0.52** [0.47–0.58]	< LOD-8.67	0.07	0.11	0.59	1.92	2.64
	µg/L SG	*Total*	989	12	**0.49** [0.46–0.53]	< LOD-10.2	0.07	0.13	0.53	1.98	2.83
		Men[a]	495	2	**0.59** [0.54–0.65]	< LOD-10.2	0.10	0.15	0.61	2.30	3.30
		Women[b]	494	10	**0.41** [0.37–0.46]	< LOD-7.88	0.05	0.10	0.46	1.66	2.25
Hg in hair	***ng/g***	*Total*	947	0	**275** [258–292]	10.0–7068	48.0	79.5	303	867	1203
		Men[a]	444	0	**282** [256–311]	11.0–7068	46.0	77.0	306	984	1396
		Women[a]	503	0	**268** [248–290]	10.0–1947	52.3	82.0	300	731	993
Hg in milk	***µg/L***		470	18	**0.14** [0.13–0.16]	< LOD-3.39	0.02	0.04	0.16	0.51	0.64
As in blood	***µg/L***	Total	1084	1	**0.89** [0.85–0.94]	< LOD-28.9	0.35	0.40	0.77	2.62	3.74
		Men[a]	548	1	**0.83** [0.79–0.88]	< LOD-22.4	0.36	0.40	0.72	2.12	3.40
		Women[b]	536	0	**0.96** [0.89–1.03]	0.20–28.9	0.32	0.39	0.81	2.90	5.16
As in urine	***µg/L***	*Total*	812	0	**6.37** [5.89–6.90]	0.26–1026	1.28	1.68	5.63	30.7	54.2
		Men[a]	402	0	**7.79** [6.94–8.74]	0.48–1026	1.57	2.09	6.40	37.0	71.6
		Women[b]	410	0	**5.24** [4.72–5.82]	0.26–266	1.06	1.45	4.85	23.1	35.1
	µg/g crt	*Total*	812	0	**6.70** [6.24–7.19]	0.37–499	1.71	2.17	5.84	26.6	50.5
		Men[a]	402	0	**5.80** [5.22–6.43]	0.76–499	1.52	1.85	4.80	24.5	52.9
		Women[b]	410	0	**7.71** [7.02–8.48]	0.37–219	2.15	2.53	6.79	28.6	48.8
	µg/L SG	*Total*	804	0	**7.22** [6.72–7.75]	0.71–672	1.87	2.37	6.24	28.6	55.7
		Men[a]	397	0	**8.27** [7.44–9.21]	0.97–672	2.27	2.58	6.69	37.6	84.5
		Women[b]	407	0	**6.31** [5.75–6.93]	0.71–269	1.79	2.15	5.81	21.6	37.1
As in milk	***µg/L***		470	9	**0.18** [0.17–0.19]	< LOD-3.70	0.07	0.08	0.16	0.49	0.79

Notes: Different letters ([a,b]) indicate that the study groups differ statistically significantly. *The first sampling period's results are excluded. The sex-related difference may (also) be on account of the lactating status of women.

respectively, exceeded the HBM I Cd value of µg/L in urine (Schulz et al., 2011). The Cd levels in the study population were generally low and similar to those reported in the general populations studied in Europe (Baeyens et al., 2014; Becker et al., 2003, 2002; Castaño et al., 2012; Černá et al., 2012; Nisse et al., 2017) and North America (CDC, 2018a; Haines et al., 2017). The levels in the blood of the male study group were lower than those reported for the male military recruits examined in 2001 (median = 0.5 µg/L, n = 463, age = 18–27 years, non-smoking) who were living in nine health regions (Eržen and Zaletel Kragelj, 2004), which mostly overlap with the present study areas. In the absence of analytical issue (LODs between the studies were equivalent), higher levels in the non-smoking military recruits than in the present male study group could hypothetically be due to longer periods spent outdoors by the first group, increasing their contact time with dust.

3.3.2. Geographic variability within the country

Cadmium levels in blood and urine were more or less comparable

Table 3
Levels of essential elements in blood and urine (and breast milk of females) of the study population of men and lactating *primiparous* women from Slovenia. crt – creatinine, SG – specific gravity.

Biomarker	Unit	Population group	N	< LOD	GM [95% CI]	min-max	P5	P10	P50	P90	P95
Mn in blood	***µg/L***	Total	811	0	**13.8** [13.5–14.2]	5.69–40.5	8.18	9.02	13.3	22.1	26.2
		Men[a]	402	0	**11.1** [10.8–11.4]	5.69–29.8	7.64	8.26	11.0	15.0	16.4
		Women[b]	409	0	**17.2** [16.7–17.7]	5.83–40.5	10.4	11.5	17.3	25.9	28.0
Se in blood	***µg/L***	Total	1084	0	**105** [103–106]	53.9–226	74.2	80.5	104	138	152
		Men[a]	548	0	**115** [114–117]	60.3–226	87.3	91.7	113	151	166
		Women[b]	536	0	**94.6** [93.1–96.1]	53.9–176	70.7	75.1	94.5	119	127
Se in urine	***µg/L***	*Total*	812	0	**13.5** [12.7–14.2]	0.50–121	2.92	4.61	14.3	35.7	46.0
		Men[a]	402	0	**19.5** [18.3–20.8]	2.81–121	6.05	8.01	20.1	45.8	52.0
		Women[b]	410	0	**9.36** [8.68–10.1]	0.50–51.9	2.40	2.95	10.2	24.7	28.9
	µg/g crt	*Total*	812	0	**14.1** [13.8–14.5]	1.00–134	8.26	9.42	14.1	21.4	24.0
		Men[a]	402	0	**14.5** [14.0–15.0]	1.57–54.3	8.32	9.93	14.5	22.4	24.3
		Women[b]	410	0	**13.8** [13.3–14.3]	1.00–134	8.10	9.13	13.6	20.6	23.6
	µg/L SG	*Total*	804	0	**15.2** [14.7–15.7]	2.17–138	6.90	8.11	14.8	29.3	34.1
		Men[a]	397	0	**20.7** [19.9–21.6]	3.13–64.7	10.6	12.4	21.0	34.0	38.0
		Women[b]	407	0	**11.2** [10.8–11.7]	2.17–138	6.01	7.16	11.5	17.4	20.5
Se in milk	***µg/L***		470	0	**12.6** [12.3–13.0]	5.36–38.1	8.13	9.11	12.6	17.6	19.3
Cu in blood	***µg/L***	Total	1084	0	**951** [941–961]	532–2004	737	766	940	1196	1262
		Men[a]	548	0	**847** [839–856]	532–1404	708	738	844	976	1041
		Women[b]	536	0	**1070** [1057–1083]	657–2004	857	908	1062	1262	1364
Cu in urine	***µg/L***	*Total*	812	223	**5.39** [5.06–5.75]	< LOD-67.3	< LOD	< LOD	6.27	17.2	22.3
		Men[a]	402	81	**6.26** [5.74–6.83]	< LOD-64.5	< LOD	< LOD	7.07	17.7	21.6
		Women[b]	410	142	**4.66** [4.24–5.12]	< LOD-67.3	< LOD	< LOD	5.04	16.8	22.5
	µg/g crt	*Total*	812	223	**5.66** [5.37–5.97]	< LOD-86.2	< LOD	< LOD	5.84	14.2	19.6
		Men[a]	402	81	**4.66** [4.34–5.00]	< LOD-41.1	< LOD	< LOD	4.94	9.36	13.5
		Women[b]	410	142	**6.86** [6.37–7.40]	< LOD-86.2	< LOD	< LOD	7.09	17.3	24.9
	µg/L SG	*Total*	804	223	**6.07** [5.75–6.40]	< LOD-80.2	< LOD	< LOD	6.50	15.2	19.4
		Men[a]	397	81	**6.64** [6.16–7.15]	< LOD-43.9	< LOD	< LOD	7.40	15.8	19.3
		Women[b]	407	142	**5.56** [5.15–6.00]	< LOD-80.2	< LOD	< LOD	5.45	15.1	19.5
Cu in milk	***µg/L***		470	0	**355** [346–365]	99–954	221	250	360	525	578
Zn in blood	***µg/L***	Total	1084	0	**6606** [6549–6663]	3010–11733	5150	5518	6636	7806	8285
		Men[a]	548	0	**6494** [6419–6571]	3400–10301	5082	5460	6553	7646	8050
		Women[b]	536	0	**6721** [6636–6807]	3010–11733	5274	5647	6768	8011	8494
Zn in urine	***µg/L***	*Total*	812	0	**276** [261–291]	13.0–2213	71.9	98.4	287	708	923
		Men[a]	402	0	**297** [275–322]	17.3–2213	78.2	104	317	774	978
		Women[b]	410	0	**256** [237–277]	13.0–1921	66.1	93.9	267	661	824
	µg/g crt	*Total*	812	0	**290** [277–239]	19.7–3371	96.6	128	307	619	754
		Men[a]	402	0	**221** [208–235]	19.7–1283	75.0	112	232	454	528
		Women[b]	410	0	**377** [356–400]	23.1–3371	131	181	401	742	972
	µg/L SG	*Total*	804	0	**311** [297–325]	15.8–3458	109	148	323	663	816
		Men[a]	397	0	**315** [295–336]	15.8–1400	93.1	148	334	689	887
		Women[a]	407	0	**307** [289–325]	45.8–3457	110	147	313	639	771
Zn in milk	***µg/L***		470	0	**1935** [1842–2032]	207–7904	741	1005	2063	3708	4404

Notes: Different letters ([a,b]) indicate that the study groups differ statistically significantly. The sex-related difference may (also) be on account of the lactating status of women.

among the study areas, with some minor but significant differences (Table S1, S9—S11). Although the highest Cd levels in soil existed in the industrially contaminated area of KR and were two to six times higher than in other areas (Table S39), this was not reflected in the HBM levels. As the Cd levels in breast milk were mostly below the LOD and the latter differed between the study periods, comparison among the study areas was not performed (Table S30). According to the type of study area, there were no significant differences in Cd levels in blood, while higher urinary Cd levels were observed in rural than in urban and/or contaminated areas (Table 4), which could hypothetically be associated with increased levels in crops due to the application of artificial phosphate fertilisers (Roberts, 2014).

3.3.3. Predictors of exposure

In line with tobacco smoking and the diet, generally recognised as the main sources of Cd exposure (ATSDR, 2012), our study showed the association mainly with smoking and to a minor extent, with the consumption of certain food items (i.e., game) (Table 4). This finding was particularly evident in blood, the matrix which was confirmed as a superior exposure biomarker versus urinary Cd at low exposure levels in cases when 24-h urine is unattainable (Stajnko et al., 2017). Male smokers had on average 5 times higher Cd levels in their blood than non-smokers, while female smokers had 1.2 times higher levels than non-smoking women. Urinary levels (SG normalised) did not show association with smoking (Table 4). The highest two values observed in the blood (> 4 µg/L) were represented by male heavy smokers (both 20 cigarettes/day) (Table S1), while the highest urine value, regardless of the normalisation method (3.65 µg/L, 2.79 µg/g crt or 3.65 µg/L SG) was found in the contaminated area of ZA and was not associated with smoking (Tables S9–10). Association between blood Cd and smoking is evident from various national or regional HBM studies on a general adult populations (Becker et al., 2002; Černá et al., 2012; Nisse et al., 2017; Stajnko et al., 2017), and in contrast to our study also for crt-normalised urine levels (Becker et al., 2003; Castaño et al., 2012; CDC, 2018a; Den Hond et al., 2015; Nisse et al., 2017) or non-normalised levels (Becker et al., 2003; Hoet et al., 2013).

The dietary source associated with Cd levels in blood or urine was vegetable but was found significant only in the rural area of SP ($p = 0.041$, model $R^2 = 0.36$, $p < 0.001$, $n = 78$) and the industrially contaminated area of ZA ($p = 0.008$, model $R^2 = 0.26$, $p < 0.001$, $n = 94$). On the contrary, we found no increased Cd exposure due to vegetable consumption in the industrially contaminated area of CE,

Table 4
Multiple linear regression: predictors of elements exposure in the Slovenian study population.

		Estimate of change (95% CI), multiplicative factor		
		Total population	Women, lactating	Men
Cd in blood (μg/L)	*Model R*[b]	*0.17 (p < 0.001)*	*0.04 (p = 0.003)*	*0.30 (p < 0.001)*
		N = 973	*n = 471*	*n = 487*
Type of study area		ns	ns	ns
Sex[a]	Men	1.00	–	–
	Women	**1.58 (1.39–1.79)****	–	–
Age (years)	≤25	1.00	1.00	
	25–35	**1.14 (1.02–1.28)***	**1.19 (1.02–1.38)***	ns
	> 35	**1.28 (1.08–1.51)***	**1.34 (1.01–1.77)***	1.22 (0.98–1.50)#
Smoking	No	1.00	1.00	1.00
	Yes	**2.16 (1.88–2.48)****	**1.21 (1.03–1.43)***	**5.05 (4.03–6.32)****
Consumption of game	No		1.00	
	Yes	ns	**1.22 (1.02–1.45)***	ns
Cd in urine (μg/L SG)	*Model R*[b]	*0.02 (p = 0.002)*	*0.008 (p = 0.214)*	*0.02 (p = 0.017)*
		N = 892	*n = 424*	*n = 455*
Type of study area	Rural	1.00		1.00
	Urban	0.90 (0.80–1.01)#	ns	0.88 (0.76–1.02)#
	Contaminated	**0.88 (0.79–0.97)***	ns	0.89 (0.78–1.01)#
Sex[a]		ns	–	–
Age (years)	≤25	1.00		1.00
	25–35	**1.14 (1.03–1.27)***	ns	ns
	> 35	**1.28 (1.11–1.49)***	ns	**1.27 (1.07–1.51)***
Smoking		ns	ns	ns
Pb in blood (μg/L)	*Model R*[b]	*0.18 (p < 0.001)*	*0.11 (p < 0.001)*	*0.19 (p < 0.001)*
		n=958	*n=463*	*n=480*
Sex[a]	Men	1.00	–	–
	Women	**1.10 (1.01–1.19)***	–	–
Age (years)	≤25	1.00		1.00
	25–35	**1.11 (1.03–1.19)***	1.09 (0.99–1.20)#	1.12 (1.00–1.25)#
	> 35	**1.19 (1.07–1.32)***	1.18 (0.99–1.41)#	**1.20 (1.05–1.38)***
Game consumption	No	1.00		1.00
	Yes	**1.11 (1.03–1.20)***	ns	**1.11 (1.00–1.23)***
Smoking	No	1.00	1.00	1.00
	Yes	**1.15 (1.05–1.25)***	**1.12 (1.00–1.24)***	**1.17 (1.01–1.36)***
Alcohol consumption	No	1.00	1.00	1.00
	Yes	**1.18 (1.11–1.25)****	**1.13 (1.04–1.22)***	**1.23 (1.12–1.36)****
Type of water supply	Public	1.00		1.00
	Bottled	ns	ns	ns
	Private	**1.24 (1.08–1.43)***	ns	**1.35 (1.08–1.69)***
Pb concentration in moss (twofold change)		**1.12 (1.09–1.14)****	**1.15 (1.09–1.21)****	**1.12 (1.09–1.16)****
Pb in urine (μg/L SG)	*Model R*[b]	*0.22 (p < 0.001)*	*0.11 (p < 0.000)*	*0.07 (p < 0.001)*
		n=733	*n=351*	*n=369*
Sex[a]	Men	1.00	–	–
	Women	**0.61 (0.70–0.54)****	–	–
Age (years)	≤25	1.00	1.00	1.00
	25–35	ns	ns	ns
	> 35	**1.32 (1.11–1.57)***	**1.69 (1.23–2.31)***	1.22 (0.97–1.53)#
Smoking	No	1.00	1.00	1.00
	Yes	**1.36 (1.15–1.61)****	**1.30 (1.06–1.59)***	**1.51 (1.11–2.04)***
Alcohol consumption	< once/month	1.00	1.00	1.00
	≥once/month	**1.24 (1.11–1.39)****	**1.23 (1.05–1.44)***	**1.26 (1.06–1.49)***
Pb concentration in moss (twofold change)		**1.11 (1.07–1.16)****	**1.18 (1.10–1.26)****	**1.12 (1.04–1.21)***
Hg in blood (μg/L)	*Model R*[b]	*0.18 (p < 0.001)*	*0.14 (p < 0.001)*	*0.20 (p < 0.001)*
		N = 734	*n = 352*	*n = 370*
Age (years)	≤25	1.00	1.00	1.00
	25–35	ns	ns	ns
	> 35	**0.72 (0.58–0.91)***	0.69 (0.47–1.03)#	**0.71 (0.53–0.97)***
Consumption of fresh seafood	Less than once/month	1.00	1.00	1.00
	1-3 times/month	**1.34 (1.17–1.54)****	**1.28 (1.06–1.55)***	**1.42 (1.16–1.74)****
	At least once/week	**1.89 (1.56–2.28)****	**1.82 (1.42–2.33)****	**2.06 (1.50–2.83)****
Consumption of canned seafood	Less than once/month	1.00	1.00	1.00
	1-3 times/month	**1.40 (1.22–1.61)****	**1.41 (1.16–1.71)****	**1.41 (1.14–1.75)***
	At least once/week	**1.65 (1.43–1.91)****	**1.39 (1.13–1.70)***	**1.91 (1.53–2.38)****
No. of amalgam fillings	0–3	1.00	1.00	1.00
	4–9	ns	1.17 (0.98–1.39)#	ns
	> 9	**1.35 (1.10–1.65)***	1.29 (0.98–1.70)#	**1.48 (1.09–2.01)***
Hg concentration in soil (twofold change)		**1.10 (1.05–1.14)****	**1.09 (1.01–1.18)***	**1.10 (1.05–1.17)****
Hg concentration in water (twofold change)		**1.10 (1.01–1.21)***	**1.13 (1.00–1.28)***	ns

(*continued on next page*)

Table 4 (*continued*)

		Estimate of change (95% CI), multiplicative factor		
		Total population	Women, lactating	Men
Hg in urine (μg/g SG)	*Model R*[b]	*0.17 (p < 0.001)*	*0.13 (p < 0.001)*	*0.18 (p < 0.001)*
		N = 699	*n = 331*	*n = 358*
Sex[a]	Men	1.00	–	–
	Women	**0.68 (0.58–0.80)****	–	–
Age (years)		ns	ns	ns
Consumption of fresh seafood	Less than once/month	1.00		1.00
	1-3 times/month	ns	ns	**1.35 (1.06–1.71)***
	At least once/week	**1.29 (1.00–1.65)***	ns	ns
Consumption of canned seafood	Less than once/month	1.00		1.00
	1-3 times/month	**1.25 (1.04–1.51)***	ns	**1.30 (1.02–1.65)***
	At least once/week	**1.36 (1.12–1.66)***	ns	**1.45 (1.12–1.86)***
No. of amalgam fillings	0–3	1.00	1.00	1.00
	4–9	**1.89 (1.60–2.24)****	**2.13 (1.64–2.76)****	**1.81 (1.47–2.24)****
	> 9	**3.08 (2.33–4.06)****	**2.99 (1.94–4.16)****	**3.18 (2.25–4.49)****
Hg concentration in soil (twofold change)		**1.09 (1.03–1.15)***	ns	**1.10 (1.04–1.18)***
Hg in hair (ng/g)	*Model R*[b]	*0.22 (p < 0.001)*	*0.20 (p < 0.001)*	*0.25 (p < 0.001)*
		N = 845	*n = 448*	*n = 397*
Sex		ns	ns	ns
Consumption of fresh seafood	Less than once/month	1.00	1.00	1.00
	1-3 times/month	**1.44 (1.26–1.65)****	**1.23 (1.03–1.46)***	**1.71 (1.38–2.12)****
	At least once/week	**2.32 (1.93–2.80)****	**1.91 (1.53–2.39)****	**3.00 (2.17–4.14)****
Consumption of canned seafood	Less than once/month	1.00	1.00	1.00
	1-3 times/month	**1.42 (1.24–1.63)****	**1.44 (1.22–1.71)****	**1.41 (1.13–1.75)***
	At least once/week	**1.83 (1.58–2.11)****	**1.69 (1.39–2.05)****	**1.95 (1.56–2.44)****
Hg concentration in soil (twofold change)		**1.19 (1.12–1.27)****	**1.24 (1.13–1.36)****	**1.18 (1.08–1.29)****
As in blood (μg/L)	*Model R*[b]	*0.13 (p < 0.001)*	*0.12 (p < 0.001)*	*0.14 (p < 0.001)*
		N = 948	*n = 460*	*n = 473*
Sex[a]	Men	1.00	–	–
	Women	1.13 (0.99–1.30)#	–	–
BMI (10% change)		**0.998 (0.997–1.000)***	**0.998 (0.996–1.000)***	ns
Consumption of fresh seafood	Less than once/month	1.00	1.00	1.00
	1-3 times/month	**1.27 (1.13–1.41)****	**1.24 (1.04–1.47)***	**1.29 (1.12–1.49)***
	At least once/week	**1.83 (1.57–2.14)****	**1.92 (1.54–2.41)****	**1.73 (1.39–2.15)****
Consumption of canned seafood	Less than once/month	1.00	1.00	1.00
	1-3 times/month	ns	1.16 (0.98–1.38)#	ns
	At least once/week	**1.15 (1.02–1.30)***	ns	**1.20 (1.02–1.40)***
Consumption of frozen seafood	Less than once/month	1.00		1.00
	1-3 times/month	1.11 (0.99–1.22)#	ns	1.14 (0.99–1.32)#
	At least once/week	**1.17 (1.00–1.37)***	ns	**1.42 (1.15–1.74)***
As concentration in water (twofold change)		**1.06 (1.02–1.12)***	ns	**1.07 (1.00–1.14)***
As in urine (μg/L SG)	*Model R*[b]	*0.06 (p < 0.001)*	*0.06 (p = 0.001)*	*0.05 (p = 0.001)*
		N = 728	*n = 350*	*n = 365*
Sex[a]	Men	1.00	–	–
	Women	**0.74 (0.63–0.86)****	–	–
BMI (10% change)		**0.997 (0.996–0.999)***	0.998 (0.995–1.000)#	0.997 (0.994–1.000)#
Consumption of fresh seafood	Less than once/month	1.00	1.00	1.00
	1-3 times/month	**1.22 (1.03–1.44)***	ns	1.26 (0.98–1.63)#
	At least once/week	**1.52 (1.20–1.93)***	**1.63 (1.21–2.20)***	ns
Consumption of canned seafood	Less than once/month	1.00		
	1-3 times/month	ns	ns	ns
	At least once/week	1.17 (0.97–1.42)#	ns	1.26 (0.96–1.66)#
Consumption of frozen seafood	Less than once/month	1.00	1.00	1.00
	1-3 times/month	ns	ns	ns
	At least once/week	**1.47 (1.15–1.87)***	1.34 (0.95–1.89)#	**1.54 (1.07–2.21)***
As concentration in water (twofold change)		ns	ns	ns
Mn in blood (μg/L)	*Model R*[b]	*0.42 (p < 0.001)*	*0.17 (p < 0.001)*	*0.01 (p = 0.180)*
		N = 711	*n = 337*	*n = 361*
Type of study area	Rural	**1.06 (1.00–1.11)***	**1.09 (1.00–1.18)***	
	Urban	ns	**1.09 (1.01–1.18)***	ns
	Contaminated	1.00	1.00	ns
Sex[a]	Men	1.00	–	–
	Women	**1.64 (1.54–1.75)****	–	–
BMI (10% change)		**0.985 (0.972–0.999)***	0.982 (0.963–1.001)#	ns
Type of water supply	Private	1.00	1.00	
	Public	**1.12 (1.01–1.23)***	**1.17 (1.01–1.34)***	ns
	Botted	**1.19 (1.05–1.35)***	**1.27 (1.05–1.53)***	ns
Consumption of game	Less than once/month	1.00	1.00	
	Once/month or more	**1.06 (1.01–1.11)***	**1.10 (1.00–1.21)***	ns

(*continued on next page*)

Table 4 (*continued*)

		Estimate of change (95% CI), multiplicative factor		
		Total population	Women, lactating	Men
Consumption of poultry	Once/week or less	1.00	1.00	
	More than once/week	**0.94 (0.90–0.98)***	**0.90 (0.85–0.96)***	ns
Consumption of tea or coffee	Once/week or less	1.00		
	2-6 times/week	0.94 (0.89–1.00)#	ns	ns
	At least once/day	ns	ns	ns
Intake of dietary supplements[b]	No	1.00	1.00	1.00
	Yes	**0.95 (0.90–1.00)***	**0.87 (0.81–0.93)****	**1.08 (1.00–1.16)***
Se in blood (μg/L)	*Model R*[b]	*0.30 (p < 0.001)*	*0.15 (p < 0.001)*	*0.11 (p < 0.001)*
		N = 943	*n = 455*	*n = 473*
Type of study area	Rural	1.00	1.00	1.00
	Urban	**1.08 (1.05–1.12)****	**1.12 (1.07–1.17)****	**1.05 (1.00–1.10)***
	Contaminated	**1.08 (1.05–1.12)****	**1.15 (1.10–1.20)****	ns
Sex[a]	Men	1.00	–	–
	Women	**0.82 (0.79–0.85)****	–	–
Consumption of fresh seafood	Less than once/month	1.00	1.00	1.00
	1-3 times/month	**1.04 (1.01–1.07)***	**1.04 (1.00–1.08)***	**1.04 (1.00–1.09)***
	At least once/week	**1.08 (1.04–1.12)****	**1.07 (1.01–1.12)***	**1.10 (1.03–1.16)***
Consumption of canned seafood	Less than once/month	1.00	1.00	1.00
	1-3 times/month	ns	ns	ns
	At least once/week	**1.07 (1.04–1.10)****	**1.04 (1.00–1.09)***	**1.08 (1.04–1.13)****
Consumption of nuts	Less than once/week	1.00	1.00	1.00
	Weekly	**1.04 (1.01–1.06)***	1.03 (0.99–1.06)#	**1.04 (1.00–1.08)***
Intake of dietary supplements[b]	No	1.00		1.00
	Yes	**1.03 (1.00–1.05)***	ns	**1.04 (1.00–1.08)***
Cu in blood (μg/L)	*Model R*[b]	*0.50 (p < 0.001)*	*0.16 (p < 0.001)*	*0.13 (p < 0.001)*
		N = 968	*n = 469*	*n = 484*
Type of area	Urban	1.00	1.00	1.00
	Rural	**1.02 (1.00–1.04)***	**1.07 (1.04–1.11)****	1.03 (1.00–1.06)#
	Contaminated	**1.03 (1.01–1.05)***	**1.03 (1.01–1.06)***	**1.03 (1.00–1.05)***
Sex[a]	Men	1.00	–	–
	Women	**1.24 (1.21–1.27)****	–	–
Alcohol consumption	< once/month	1.00	1.00	
	≥once/month	0.98 (0.96–1.00)#	0.97 (0.95–1.00)#	ns
BMI, 10% change		**1.015 (1.010–1.021)****	**1.014 (1.007–1.022)***	**1.021 (1.013–1.029)****
Zn in blood (μg/L)	*Model R*[b]	*0.07 (p < 0.001)*	*0.06 (p < 0.001)*	*0.07 (p < 0.001)*
		N = 963	*n = 467*	*n = 481*
Type of area	Rural	1.00		1.00
	Urban	**1.03 (0.99–1.05)***	ns	**1.06 (1.02–1.10)***
	Contaminated	**1.02 (1.02–1.05)***	ns	**1.05 (1.02–1.09)***
Sex[a]	Men	1.00	–	–
	Women	**1.11 (1.08–1.14)****	–	–
Fruit consumption	Once/week or less			1.00
	Couple of times/week	ns	ns	ns
	Daily	ns	ns	**0.96 (0.93–1.00)***
Consumption of fresh seafood	Less than once/month	1.00	1.00	
	1-3 times/month	**1.02 (1.00–1.05)***	1.03 (1.00–1.06)#	ns
	At least once/week	**1.04 (1.01–1.07)***	**1.05 (1.01–1.09)***	ns
Intake of dietary supplements[b]	No	1.00	1.00	
	Yes	**0.97 (0.95–1.00)***	**0.96 (0.94–0.99)***	ns

Notes: Margin of statistical significance: #p < 0.1, *p < 0.05, **p ≤ 0.001, ns – not significant. Variables with significance of p < 0.05 in at least one group are bolded.

[a] The sex-related difference may (also) be on account of the lactating status of women.

[b] Supplements containing minerals were taken into account.

where elevated levels of Cd in locally grown vegetables were found (Bešter et al., 2013), which might be a consequence of the local recommendation about consuming purchased rather than home-grown vegetables (Bešter et al., 2013).

Game consumption was shown as an additional potential source of Cd in the blood of the female study group (Table 4). Although Cd concentration is known to be much higher in game kidney and liver than in game meat, several studies across Europe reported a considerable percentage of game meat exceeded the maximum level 0.050 mg/kg wet weight set by Commission Regulation (EC) 1881/2006 or other (Doganoc and Gačnik, 1995; Mitranescu et al., 2011). Furthermore, as observed by Danieli et al. (2012) in their study involving a hunter population and its household members from central Italy, more frequent game consumption could contribute to higher Cd exposure levels.

Both, blood and urinary Cd concentration increased with age, as observed from the statistical model for the whole population (Table 4), which is in agreement with the literature data (ATSDR, 2012) and has been confirmed in various national or regional HBM studies (Castaño et al., 2012; Den Hond et al., 2015; Hoet et al., 2013; Mortensen et al., 2011; Nisse et al., 2017).

3.4. Lead

3.4.1. Basic exposure levels

In line with decreasing Pb exposure levels in industrialised countries starting from the 1990s due to the initiation of unleaded gasoline usage, our study population showed low mean Pb levels in all matrices (Table 2). The observed exposure in males (GM = 19.3 µg/L of blood, Table 2) was lower than that observed in 2001 in a population of male military recruits residing in nine health regions across the country (overall mean = 37.4 µg/L of blood, n = 463, age = 18–27 years) (Eržen and Zaletel Kragelj, 2004). Moreover, the majority of our study population was within the concentration range generally believed not to present elevated health risk (Tables S2 and S24). Only two men from the Pb-smelter area exceeded the population-based reference value of 90 µg/L in the blood (Schulz et al., 2011). In the lack of international consensus for population groups of increased susceptibility (pregnancy, *postpartum*), we referred to the guidelines regarding the screening and management of pregnant and lactating women in the US (Committe on Obstetric Practice, 2012): six women in our study group exceeded the blood Pb level of 50 µg/L (three from the contaminated areas of RA, KR, and ZA, and three from the urban area of LJ), which presents 1% of the study population, similarly to the US population.

3.4.2. Geographic variability within the country

Despite the generally low levels, our study population showed a distinctive geographical pattern of Pb exposure, with peak levels in the Pb-smelter area of RA (GM = 27.2 µg/L, Table S2). As the participants in our study lived in the wider area of RA, we divided them into two subgroups: one living in the vicinity of the Pb-smelter area with higher environmental contamination (Upper RA, n = 22, GM = 39.9 µg/L) and the other living in Lower RA with lower environmental contamination (n = 57, GM = 23.5 µg/L) (Table S2, $p < 0.001$). These results confirm those from the local monitoring of the Pb-smelter community, specifically mothers and preschool and school children, which has been ongoing since 1976. Following the installation of an emission control system in 1978, the Pb levels in blood have decreased slowly from the median levels of 400–500 µg/L in the three study groups (n = 93) to 41–284 µg/L (95% of all values) in 1990 (n = 131), as opposed to the range of 25–123 µg/L in the control area in 1984 (n = 84) (Prpić-Majić et al., 1992, 1984). These findings indicated that Pb-contaminated soil from past exposure was the major source of increased Pb exposure in a population (Prpić-Majić et al., 1992). In 2001–2002, elevated Pb levels in blood (> 100 µg/L) could still be found in over one-third of the studied children from RA area (age = 3 years, n = 47) (Eržen and Janet, 2005), and in year 2017 in one-fifth of the children (age = 3 years, n = 90) (unpublished data). Male military recruits living in nine health regions (Eržen and Zaletel Kragelj, 2004) also showed the highest Pb levels in blood in the RA area (mean = 60.7 µg/L).

3.4.3. Predictors of exposure

Adjusting for potential covariates and confounders revealed that living in Upper RA accounted for about two times higher Pb levels in blood and urine (crt- or SG-normalised) (models not presented). However, as a more predictive variable of Pb contamination, instead of the study area, Pb concentration in moss (Table S40) was used in the statistical models (Table 4). The Pb concentration in the moss species *Hypnum cupressiforme* indicates Pb atmospheric deposition (ICP Vegetation Coordination Centre, 2010). Owing to the extreme persistence of Pb in soil, this and other studies (Prpić-Majić et al., 1992; Zahran et al., 2013) showed that current airborne Pb is most probably from legacy sources in industrialised areas. The following are additional significant determinants of Pb levels in blood that were identified from the statistical models: age (19% higher in participants > 35 years than in those ≤ 25 years), type of water supply (24% higher in private than in public), alcohol consumption (18% higher in those consuming it ≥ once/month than in those < once/month), smoking (15% higher in smokers than in non-smokers) and game consumption (11% higher in consumers than in non-consumers) (Table 4). Estimation of human exposure to Pb mostly relies on levels in blood (Bergdahl and Skerfving, 2008), representing a combination of recent (2–3 months) and past Pb exposure, whereas urinary Pb is considered as an indicator of recent exposure, which is more variable within individuals and does not have a linear correlation with blood Pb (Skerfving and Bergdahl, 2015). Despite that, we identified a similar pattern of exposure using urinary Pb levels, except for the game consumption (Table 4).

The identified predictors were in line with generally known sources of exposure, revealed after the initiation of unleaded gasoline use (Jakubowski, 2012), except in RA, where lifestyle was not found as a significant determinant of Pb exposure. In this area, Pb concentration in moss or soil was the only significant covariate associated with the overall Pb levels in blood and urine (Table S38). Among the male residents of RA, private water supply was additionally found to contribute to 4.5 times higher urinary Pb levels as opposed to bottled water use (Table S38). Increasing Pb levels in blood with increasing levels in tap water have been reported elsewhere due to Pb leaching from the water pipe system (Moore et al., 1977; Pocock et al., 1983). Various studies involving the general adult population whose mean Pb levels ranged between 30 and 90 µg/L of blood confirmed smoking and wine consumption as relevant determinants of Pb exposure (Alimonti et al., 2005; Forte et al., 2011; Hense et al., 1992; Weyermann and Brenner, 1997). While smoking contributes to Pb exposure through the Pb content in tobacco plants, it is commonly argued that alcohol consumption contributes through crystal glassware containing Pb. However, other factors, such as different metabolism rates of drinkers, are possible (Grandjean et al., 1981; Graziano and Blum, 1991). To our best knowledge, the present study is the first to demonstrate smoking and alcohol as significant determinants of Pb exposure at lower levels than reported previously, while increasing Pb levels with age are known (Lee and Kim, 2014).

3.5. Mercury

3.5.1. Geographic variability within the country

The highest Hg levels in the study population's blood and hair were found in the coastal part of Slovenia (KP) (Tables S3 and S37), corresponding to the coastal area residents' lifestyle, which includes the Mediterranean diet. Inhabitants of Mediterranean countries are known to have higher Hg levels in their blood and/or hair than residents of other European countries (Castaño et al., 2015; Višnjevec et al., 2014) because of higher consumption of fish and other seafood, which are the dominant sources of organic Hg exposure (NRC, 2000; UNEP/WHO, 2008). The questionnaire data confirmed that the highest percentage (37%) of participants consuming seafood at least once per week lived in KP, while the percentages in the other study areas ranged between 4% and 19%. The urinary Hg levels (volume expressed, crt- or SG-normalised) were also among the highest in KP (Tables S15–S17) but did not differ statistically from most of the other areas. The Hg levels in breast milk differed minimally among the study areas (Table S32).

The second highest Hg levels in blood and hair were observed in GO and did not differ significantly from KP. Regardless of the normalisation method, the urinary Hg levels of the GO residents were among the highest as well (Tables S15–S17). In the GO area, 11 male participants lived in the former Hg mine area (town of Idrija), and their median creatinine (0.96 µg/g crt, range = 0.3–2.4 µg/g crt) or SG normalised levels in urine (0.90 µg/L SG, range = 0.33–2.35 µg/L SG), but not in blood (2.1 µg/L, range = 1.1–15.0 µg/L) or in hair (466 ng/g, range = 310–5740 ng/g), were somewhat higher than in the overall GO area (0.56 µg/g crt, 0.60 µg/L SG, 2.02 µg/L and 429 ng/g, respectively) (Tables S3, S16, S17, S37), indicating elevated inorganic Hg exposure in the town of Idrija. In this area, increased exposure due to inhalation of Hg vapour in ambient air is still evident despite the mine's closure in the

1990s, with other potential sources of Hg identified (in this site) as ingestion of drinking water and locally grown food or fish from Idrijca River (Horvat et al., 2004; Kobal et al., 2017; Miklavčič et al., 2013b; Žižek et al., 2007). The blood and urine Hg levels of the inhabitants of the former Hg mine area were previously shown to be higher than in a control rural area but did not differ from the ones found in the urban area of LJ (Kobal et al., 2017), similar to our study's findings.

3.5.2. Predictors of exposure

The geographical trend of the highest levels in the surroundings of the Idrija Hg mine (Gosar et al., 2016; Kocman et al., 2011) was confirmed in the statistical models by the positive correlations between Hg concentrations in the soil and in blood, urine and hair. Moreover, Hg concentrations in tap water showed correlations with Hg levels in blood (Table 4). In general, the Hg levels in hair and blood clearly showed exposure that was predominantly associated to seafood consumption with peak exposure in the coastal area. The participants who consumed seafood at least once per week had on average 89% higher Hg levels in their blood and 2.3 times higher Hg levels in their hair than those eating seafood less than once per month, whereas having more than 9 amalgam fillings versus a maximum of 3 contributed to 35% higher Hg levels in blood (Table 4). In contrast, urinary Hg levels depended mainly on the presence of dental amalgams (3 times higher levels in participants with > 9 amalgams than in those with ≤3), with no distinct geographical pattern identified. Seafood consumption contributed to approx. 30% increase in urinary Hg levels (Table 4), confirming that a certain proportion of urinary Hg originates from fish consumption, as first shown by Sherman et al. (2013), who used a stable isotope technique to prove it as a result of demethylation processes in the body (Sherman et al., 2013). Both fresh and canned, but not frozen seafood consumption was significantly associated with Hg levels in blood, hair and urine (Table 4).

3.5.3. Basic exposure levels

Overall, as observed recently in the European-scale pilot HBM study (Den Hond et al., 2015), the Hg levels in hair found in Slovenia were within the European average. In the present study, the percentages of the female study population exceeding the US Environmental Protection Agency (EPA)-based threshold value of 1000 ng/g of hair for developmental neurotoxicity risk (US EPA, 2001) were 22% in the coastal area of KP and 6% in the other study areas. Referring to the updated biological limit of 0.58 μg/g hair, which is based on the outcomes of the highly exposed Faroese population, but is supported by the recent studies of developmental neurotoxicity at low exposure levels (Bellanger et al., 2013), 18% of women in our study were above this cut-off level, 59% in the coastal area (KP). In the absence of a consensus value protecting child development, the data for the susceptible population group (pregnancy and *postpartum*) at low levels of exposure, similarly as in case of Pb, are critically lacking. Among men, four exceeded the HBM II value of 15 μg/L of blood (Schulz et al., 2011); two of them were frequent seafood consumers (residents of the coastal area), while for the other two, no known source of exposure was identified (neither seafood, amalgam nor study area). Ten participants exceeded the HBM I value of 5 μg/g crt in urine (Schulz et al., 2011); among them, no one was from the Hg mine area (Table S13).

Regarding the evaluation of Hg levels in breast milk, scarce data in the literature is available concerning the general population of women. In comparison to other studies, the Hg levels in our study population were very low and geographically independent (Table S32). The previously reported level for mature milk of the women from LJ, using radiochemical neutron activation analysis, was 1 ± 0.2 μg/L (Kosta et al., 1983), while the findings of a recent cohort study are similar to those for our study population (median = 0.2 ng/g, range = LOD–2.9 ng/g, n = 284) (Miklavčič et al., 2013a). In contaminated areas of South Africa and Asia, 10 or 20 times higher concentrations than those in our study were reported in breast milk as a consequence of Hg vapour inhalation or consumption of contaminated fish (Böse-O'Reilly et al., 2008).

3.6. Arsenic

3.6.1. Basic exposure levels

In our study, 161 participants (20%) exceeded the population-based reference value of 15 μg/L of urine, established for people who did not consume seafood 48 h prior to the sampling (Schulz et al., 2011). Within this subgroup, speciation analysis was performed to check for the presence of inorganic As species (n = 138, total As GM = 39.8 μg/L, range = 16–1030 μg/L). The concentrations ranged as follows: AsIII = < LOD–6.1 μg/L (GM = 0.6 μg/L), AsV = < LOD–2.1 (GM = 0.1 μg/L), DMA = < LOD–54.7 μg/L (GM = 3.9 μg/L) and MMA = < LOD–6.6 μg/L (GM = 0.2 μg/L). Their respective percentages were 0.1–80% (GM = 9.6%), 0.1–37% (GM = 1%), 21–100% (GM = 71%) and 0.2–55% (GM = 4%). The respective numbers of samples below LOD were 33, 124, 5, and 84. It is known that the major form of dietary As that predominates in seafood is organic compound AsB (Horvat et al., 2012; Hughes, 2006), whose elevated concentrations can be found in urine at least three days after the consumption of such food (Navas-Acien et al., 2011). Therefore, AsB was determined in samples exceeding 100 μg total As/L of urine (n = 12). The percentages ranged from 65% to 97%, while the levels of inorganic As and MMA were negligible, and the proportion of DMA was low (3–35%). These results excluded any significant environmental exposure to inorganic As and confirmed the exposure mainly to dietary organic As. Some types of seafood can also be rich in DMA (especially mussels, shrimps and some fish) or arsenosugars and also inorganic As (e.g., algae and animals feeding on them) (Cullen and Reimer, 1989; Taylor et al., 2017). Therefore, in some cases, urinary DMA that was found in our samples (together with MMA) may also come from seafood consumption, although both are among the commonly used biomarkers of inorganic As exposure (Aylward et al., 2014; Šlejkovec et al., 2008).

3.6.2. Geographic variability within the country

Similar to the case of Hg, As levels in blood and urine (regardless of the normalisation method) were the highest in the coastal area of KP (Table S4, S18–S20). This finding already indicates that the primary source of total As in our study population was seafood, as previously reported for the Slovene population of pregnant women (Miklavčič et al., 2013a). Among other study areas, excluding the coastal area of KP, total As levels in blood and urine were more or less similar. However, including the potentially As-contaminated areas of Slovenia (ZA, GO, RA, CE and KR) as a confounder in the statistical models, 18% higher As levels in blood were observed in female residents ($p = 0.061$), and 19% higher SG-normalised urinary levels ($p = 0.018$) were found in male and female residents of these areas (models not presented). These results confirm slightly elevated exposure in the listed As-contaminated areas but still below the level regarded as posing a health risk.

3.6.3. Predictors of exposure

Consistent with the speciation analysis, the regression analysis showed that the main predictor of total As exposure was seafood, confirming the predominant exposure to dietary organic As. Consumption of fresh seafood at least once per week increased As levels in blood, on average, by 83% and in urine by about 50% in comparison to intake of less than once per month (Table 4). Consumption of canned and frozen seafood contributed to As levels in blood to a lower extent than fresh seafood, while in urine, the contribution from frozen seafood was also considerable, particularly in men, increasing the urinary As levels by 54% (Table 4). Due to its fast clearance from blood, As levels in blood are normally not used as indicators of chronic exposure to low levels of As (Horvat et al., 2012). However, our study showed similar patterns in blood and urine, probably indicating constant dietary habits

of our study participants.

The As levels in blood also showed a significant association with As concentrations in tap water (Table 4). Drinking water, depending on the geographic region, is the most important medium for inorganic As exposure (Hughes, 2006; Nordstrom, 2002). In Slovenia, As levels in drinking water are generally low (below 10 µg/L); however, certain mineral water springs may contain elevated levels, up to 60 µg inorganic As/L (Van Elteren et al., 2002). Total As concentration in tap water amounting to 0.50 µg/L was observed in ZA, while in the other study areas, the mean concentration was 0.14 µg/L ($p < 0.001$) (Table S42), consistent with the concentration in the vicinity of Znojile mine in Sava Valley, contaminated due to past mining activities (Perharič et al., 2017). Of the 17 samples from ZA that were included in the speciation analysis, none showed elevated inorganic As species, with the levels of AsV, AsIII and MMA below the LOD, and DMA ranging from < LOD to 9.3 µg/L (median = 3.8 µg/L). Likewise, in their study, Perharič et al. (2017) reported no excessive urinary As concentrations, indicating that the levels observed in this particular region did not pose elevated health risks.

3.7. Manganese

3.7.1. Basic levels

As an essential element, Mn has a normal range of 4–15 µg/L of blood (Barceloux, 1999; Burtis et al., 2012). However, Mn levels are known to increase during pregnancy, which is due to changes in absorption, metabolism or tissue mobilisation related to increased oestrogen and progesterone concentrations to satisfy its higher demand during pregnancy (Gunier et al., 2014). The increase in mean blood Mn was demonstrated in the Quebec population of pregnant women (1st trimester: 8.5 µg/L; 3rd trimester 15.6 µg/L) (Takser et al., 2004) and agricultural community of California (26th week of gestation: 14.6 µg/L; delivery: 20.7 µg/L) (Gunier et al., 2014). Consistent with this, 70% of women in our study exceeded the upper reference value of 15 µg/L, while among the men, 10% exceeded the upper level. Among all the participants, no one's level was below the lower reference level of 5 µg/L (Burtis et al., 2012) (Table S5). Similar levels to those of our study (GM_{women} = 17.2 µg/L, Table 3) were reported for maternal blood at 6 weeks *postpartum* in Northern Norway (GM = 17.3 µg/L, n = 211) (Hansen et al., 2011). On the other hand, the US population of non-pregnant women showed markedly lower blood levels than ours (GM ~ 10 µg/L), while men only slightly lower (GM ~ 8.8 µg/L) (CDC, 2018b).

3.7.2. Predictors of Mn status

In accordance with the primary source of Mn intake in the general population through food, adjusting for potential covariates and confounders, our study showed higher Mn levels in the blood of women consuming bottled water (27%) or water from the public supply (17%) than in those using water from private supplies, and somewhat higher in participants consuming game meat (Table 4). An inverse association was observed with poultry consumption in women, likewise for the intake of dietary supplements containing minerals (Table 4). The observed inverse associations may result from co-linearity with some other dietary or lifestyle-related variables that were not covered by our study protocol or in case of dietary supplements from decreased Mn absorbance in the presence of other elements (e.g. iron - Fe) (Kim, 2018).

Along with the dietary determinants, the statistical models (Table 4) indicated slightly higher levels in rural areas, as well as in women in urban areas, compared with contaminated areas. Although the levels in blood did not reflect the levels in soil (Table S43), similar results were observed in pregnant women from Quebec – those who lived in urban and/or agricultural areas had significantly higher Mn levels in their blood compared with the residents of small villages; and higher levels were associated with pesticide spraying less than 1 km from their houses (Takser et al., 2004). The association between Mn exposure and agricultural activities (i.e., the use of Mn-containing fungicides) is further supported by the Mn levels in the blood or hair of pregnant women from the Californian agricultural community (Gunier et al., 2014) and the Costa Rican community of pregnant women (Mora et al., 2014).

Due to its essentiality for maternal health and foetal development, particularly during late pregnancy and neurotoxic potential at excessive levels (Horning et al., 2015), Mn uptake and efflux is highly regulated. Dietary deficiency of Fe can lead to excess absorption of Mn, and it has been shown that interaction between Fe with Mn is the major factor determining sex-specific differences in blood Mn levels (Lee and Kim, 2014). Our study did not monitor Fe levels in blood, however this should be done in future to assure comparability among different study groups and proper interpretation of dose-response relationships.

3.8. Selenium

3.8.1. Basic levels

The majority of our study population had Se levels in their blood within the reference range relevant for European population (Wilhelm et al., 2004): 24% of men and 9% of women exceeded their respective upper reference levels of 130 and 120 µg/L, and only a few participants had levels below the lower reference values of 79 and 60 µg/L, respectively (Table S6). Compared with previous observations in smaller groups of healthy Slovene adults, our study showed somewhat higher Se levels in blood (GM = 105 µg/L). A group of Slovene residents (n = 43) had a mean level of 87 ng/g (SD = 13 ng/g) (Mazej et al., 2003) and 30–62-year-old men living in central and southwestern Slovenia had a mean level of 89.3 µg/L of blood (range = 62–138 µg/L) (Kobal et al., 2004). These findings suggest an increasing time trend for the Slovenian population, which was reported for the Czech adult population monitored between 1996 and 2011 (n = 2414), with a median level of 73.2 µg/L of whole blood in 1996 and 91.5 µg/L in 2001 (Batáriová et al., 2005). The observed Se level in a female study group (95.0 µg/L, Table 3) was lower than those recently determined in the blood of adult non-pregnant women (aged 30–51 years) from central Slovenia (GM = 105 µg/L, range = 68.7–142 µg/L, n = 170) (not published), which is believed to be primarily due to the lactation status of participating women. Selenium levels in serum or plasma are known to decrease with increasing gestational period, due to increased Se requirement to maintain anti-oxidative activity (Izquierdo Álvarez et al., 2007; Kobal et al., 2017). In line with the known higher Se status in the US population (Combs et al., 2011), the most recent Canadian (2009–2011) and US surveys (2011–2016) reported considerably higher levels in the whole blood of their healthy adult population compared with the levels in our study population, GMs of both populations being around 190 µg/L, and in urine of the Canadian population 44 µg/g crt (CDC, 2018b; Health Canada, 2013).

Based on the observed Se levels in breast milk (Tables 3 and S34), with 19% of women below the lower reference value of 10 µg/L, and eight women above the upper value of 25 µg/L (Iyengar, 1998), Slovenia is ranked among the countries with relatively low Se levels (GM = 12.6 µg/L). Similar levels were observed in a group of mothers from a neighbouring region (Northeast Italy, n = 62, 12 ± 3 µg/L) (Mazej et al., 2004). In the past, mature milk of Slovene mothers (Kosta et al., 1983) showed even a lower range (8 ± 2 µg/L, 7–12 µg/L, n = 6).

3.8.2. Predictors of Se status

Based on the statistical models, sex/physiological status was the most distinctive predictor of Se blood levels, men having about 20% higher levels than lactating women. Among the dietary predictors, seafood consumption contributed, on average, up to 10% higher Se levels in blood, while smaller contributions from nuts and intake of dietary supplements were revealed, with the latter only found in men

(Table 4). Fish, a protein-rich food, was previously reported as containing among the highest Se levels on the Slovenian market (Smrkolj et al., 2005). In addition to sex and dietary items, rural areas showed lower Se levels in blood than urban or contaminated areas (Table 4). Unfortunately, Se concentration in soil was not available systematically across the country's regions; therefore, we were unable to check for the correlation between Se levels in blood and soil, which could be present if Se supplementation was used in agriculture (Carvalho et al., 2003).

3.8.3. Geographic variability within the country

Among the specific study areas, LJ was among those with the highest Se levels in blood (GM = 116 µg/L) and in milk (GM = 15.2 µg/L) (Tables S6 and S34). Similar Se levels in breast milk were observed in a recent birth cohort study, which collected breast milk samples 6–8 weeks after delivery (the same as in the present study) and recruited women from LJ and its surroundings (median = 17 ng/g, range = 1.7–69 ng/g, n = 287) (Miklavčič et al., 2013a).

3.9. Copper

3.9.1. Basic levels

Inadequate Cu levels in blood of our study population were more frequent than excessive levels; 31% of men and women had levels below their respective lower reference levels of 800 and 1000 µg/L (Iyengar, 1998). The respective percentages of men and women exceeding the upper reference levels of 1100 and 1400 µg/L were 3% and 4% (Table S7). In contrast to blood, excessive Cu levels in breast milk were more likely than inadequate; 55% of the women had Cu levels above the upper reference value of 400 µg/L and only a few (n = 11) below the lower value of 200 µg/L (Iyengar, 1998) (Table S35). The previously reported levels in the mature milk of women living in LJ were comparable to the mean level in our study (range = 20–500 µg/L, mean = 300 µg/L, n = 7) (Kosta et al., 1983). The sex difference observed in our study population (Table 3) was consistent with the Canadian survey (2009–2011) showing higher blood levels in women (990 µg/L) than men (810 µg/L), similarly also in urine (10 µg/g crt in women; 7.9 µg/g crt in men) (Health Canada, 2013), and also with the US survey with higher serum Cu in women than in men (CDC, 2018b). The Cu levels in serum were demonstrated to increase during the course of pregnancy due to increased physiological need (Izquierdo Álvarez et al., 2007), which might explain the somewhat higher level in the whole blood of Slovenian lactating women compared with Canadian adult non-lactating women (Health Canada, 2013). When comparing specific study areas, no distinct geographical pattern was identified in any of the matrices studied (Table S7, S24–26, S35).

3.9.2. Predictors of Cu status

The most distinctive predictor of Cu in our study population was sex, followed by the type of study area, age and alcohol consumption (Table 4). The levels in rural and contaminated areas prevailed over urban areas. Rural areas may be significant sources of Cu intake due to the potential Cu-containing soil treatments applied in order to prevent Cu deficiency in soils and in livestock or to stimulate the growth of pigs and broiler chickens or due to the use of Cu sulphate as fungicide in agriculture (www.copper.org). A significant influence from the contaminated sites due to industrial activities was somewhat lower than the effect observed in rural areas.

An inverse association between Cu levels in blood and alcohol consumption probably indicates altered Cu metabolism due to alcohol intake (Keen et al., 1998). However, the observed association may also result from co-linearity with some other dietary or lifestyle-related variables that were not covered by our study protocol (Table 4).

3.10. Zinc

3.10.1. Basic levels

According to the reference levels established for blood and breast milk (Iyengar, 1998), the Zn levels in the blood of 37% of the women and 29% of the men were above the upper reference value of 7000 µg/L, while 19% of the women and 26% of the men had levels below the lower value of 6000 µg/L (Table S8). Among women, 71% had Zn levels in their breast milk above 2000 µg/L and 10% had levels below 1000 µg/L (Table S36). The previously reported levels in the mature milk of women living in LJ were comparable to those found in our study (range = 300–3100 µg/L, mean = 1400 µg/L, n = 7) (Kosta et al., 1983). The Canadian survey (2009–2011) showed similar levels in adult men (GM = 6400 µg/L of whole blood and 270 µg/g crt in urine), but the levels in women were much lower than our study's findings (5800 µg/L and 230 µg/g crt, respectively) (Health Canada, 2013), which could be due to the lactating status of our female study group. Supporting this assumption, a case study on 45 pregnant women showed a decreasing trend in Zn levels in plasma during pregnancy, but the levels in whole blood and red cells showed a significant rise (Qvist et al., 1986).

3.10.2. Predictors of Zn status

The statistical model for the total population confirmed 11% higher levels in women than in men, with additional predictors being seafood consumption (positive association), dietary supplement intake (inverse association), and fruit intake (inverse association) (Table 4). Seafood is among the dietary sources of Zn, which was confirmed in the female study group, contributing to an average 5% higher level of Zn in the blood of those consuming seafood at least once per week compared with those who did so less than once per month. Although animal proteins are known major sources of dietary Zn (Lönnerdal, 2000), meat and eggs were not found to be significant sources of Zn in our study population; neither was freshwater fish (not presented). Vegetables and fruits are poor sources of Zn and contain high levels of phytate, which might inhibit Zn absorption from the intestines (Kelsay et al., 1979). This could explain a 4% lower level of Zn in the blood of men consuming fruit daily than those doing so weekly or less (Table 4). Although we would expect increased Zn levels in blood due to dietary supplement intake, the inverse association indicates the possibility of decreased Zn absorption in the presence of other minerals in the supplements (Guyette et al., 1983).

Besides the dietary predictors, higher Zn levels in blood were observed in contaminated and urban areas than in rural areas (Table 4). These results show that Zn status is obviously not compromised due to the elevated presence of metals in industrially active sites. Moreover, none of the elements (Cu, Cd or Hg) that could inhibit Zn absorption was found to have an inverse relationship with Zn (not presented). Adjusted SG-normalised levels showed significantly lower urinary Zn levels of the participants in contaminated areas than those in rural or urban areas (model not presented). This, together with elevated Zn levels in blood and moderate levels of toxic metals, could indicate increased uptake of Zn in a cellular anti-oxidative defence (as part of Cu-Zn superoxide dismutase and other antioxidative enzymes) in contaminated areas.

3.10.3. Geographic variability within the country

Despite relatively uniform levels among the study participants (Tables S8, S27-29, S36), the tendency towards higher levels in the coastal part (KP) of the country supported seafood as an important source of dietary Zn and did not reflect Zn concentrations in soil, which were two to five times higher in KR and Upper RA than in other study areas (Table S46).

Table 5
Proposed reference values (RV_{95}) for selected elements in the Slovenian population of men and lactating *primiparous* women of childbearing age (18–49 years).

Biomarker	Population group	N	P95 [95% CI]	RV_{95}
Cd in blood	Lactating women, non-smoking	452	0.86 [0.78–0.95]	0.9 µg/L
	Non-smoking men	492	0.68 [0.57–0.80]	0.7 µg/L
Cd in urine	Lactating women, non-smoking	410	0.75 [0.65–0.87]	0.8 µg/g crt
		416	0.67 [0.57–0.79]	0.7 µg/L
		399	0.56 [0.52–0.61]	0.6 µg/L SG
	Men, non-smoking	453	0.41 [0.36–0.47]	0.4 µg/g crt
		459	0.61 [0.54–0.70]	0.6 µg/L
		439	0.56 [0.50–0.63]	0.6 µg/L SG
Cd in milk	Lactating women, non-smoking	405	0.19 [0.16–0.22]	0.2 µg/L
Pb in blood	Lactating women, Pb-smelter area excluded	506	33.1 [30.5–36.0]	35 µg/L
	Men, Pb-smelter area excluded	499	42.4 [37.9–47.4]	45 µg/L
Pb in urine	Lactating women, Pb-smelter area excluded	380	1.52 [1.30–1.80]	1.5 µg/g crt
		380	1.12 [0.97–1.31]	1.1 µg/L
		377	1.10 [0.97–1.24]	1.1 µg/L SG
	Men, Pb-smelter area excluded	353	1.30 [1.15–1.47]	1.3 µg/g crt
		353	2.13 [1.86–2.44]	2.1 µg/L
		349	1.77 [1.58–1.97]	1.8 µg/L SG
Pb in milk	Lactating women, Pb-smelter area excluded	324	0.98 [0.77–1.25]	1.0 µg/L
Hg in blood	Lactating women consuming fish ≤ 3 times/month	431	3.65 [3.17–4.20]	4.0 µg/L
	Men consuming fish ≤ 3 times/month	479	4.78 [4.10–5.58]	5.0 µg/L
Hg in urine	Lactating women without amalgam dental fillings	84	2.54 [1.51–4.26]	3.0 µg/g crt
		87	3.73 [1.35–10.3]	4.0 µg/L
		80	2.06 [1.19–3.57]	2.0 µg/L SG
	Men without amalgam dental fillings	73	1.22 [0.81–1.84]	1.2 µg/g crt
		73	2.42 [1.24–4.73]	2.4 µg/L
		65	2.09 [1.15–3.81]	2.1 µg/L SG
Hg in hair	Lactating women consuming fish ≤ 3 times/month	404	841 [742–953]	900 ng/g
	Men consuming fish ≤ 3 times/month	388	1253 [1041–1510]	1200 ng/g
Hg in milk	Lactating women without amalgam dental fillings	84	0.53 [0.38–0.75]	0.5 µg/L
As in blood	Lactating women consuming fish ≤ 3 times/month	432	4.26 [3.25–5.56]	4.0 µg/L
	Men consuming fish ≤ 3 times/month	479	3.09 [2.41–3.95]	3.0 µg/L
As in urine	Lactating women consuming fish ≤ 3 times/month	324	41.6 [31.1–55.7]	40 µg/g crt
		324	33.2 [24.7–44.6]	30 µg/L
		321	33.3 [24.6–45.1]	30 µg/L SG
	Men consuming fish ≤ 3 times/month	353	47.5 [29.6–76.5]	50 µg/g crt
		353	65.7 [45.5–95.0]	70 µg/L
		348	84.2 [43.4–163]	80 µg/L SG
As in milk	Lactating women consuming fish ≤ 3 times/month	380	0.74 [0.58–0.94]	0.7 µg/L

3.11. Sex-related differences?

Among the potentially toxic elements, blood samples showed higher Cd levels in women than in men ($p < 0.001$) (Table 2), the difference that is typically observed in adult healthy populations (CDC, 2013; Černá et al., 2012; Health Canada, 2013), mainly due to low ferritin concentrations in women. Cadmium absorption is additionally enhanced during late pregnancy on account of upregulated Fe absorption (Lee and Kim, 2014). On the other hand, men had higher Pb and Hg levels in their blood than women ($p < 0.001$) (Table 2), consistent with the findings reported in the literature (Becker et al., 2002; Bjermo et al., 2013; CDC, 2018b; Černá et al., 2012; Health Canada, 2013) and with higher haematocrit levels in men and in case of Pb decreasing effect of oestrogen on blood Pb levels in women (Lee and Kim, 2014; Vahter et al., 2007). However, after adjustments for possible predictors and confounders (including haematocrit level), the Pb levels in blood showed 10% higher levels in women than in men (Tabe 4), speculatively due to increased release of Pb from bone compartment particularly during *postpartum* period (Manton et al., 2003). In case of the Hg, the difference was lost, which could be explained by the haematocrit level included as a confounder into the model (Table 4).

Sex-related difference in the levels of toxic metals in urine, expressed per volume or creatinine, was consistent with other HBM studies (higher in women than men) (Casta□o et al., 2012; CDC, 2013; Puklová et al., 2010), but not in cases expressed per SG. Urinary Cd did not differ between men and women (Table 2), while Pb, Hg and As showed higher urinary levels in men than women (Table 2). The differences in SG-normalised levels were confirmed in the models (Table 4). It should be noted that crt-normalised levels present the risk of underestimating the exposure due to creatinine over-compensation, particularly for Cd and As (Balakrishnan et al., 2018; Drobná et al., 2016; Hoet et al., 2016; Skröder et al., 2018), therefore SG normalisation is suggested as a more reliable alternative.

Speciation analysis of the samples > 15 µg/L of urine (n = 138) showed no sex difference in the sum of As species (AsV, AsIII, MMA and DMA) ($p = 0.548$). Although on the margin of statistical significance, the percentage of AsIII was lower in women than in men ($p = 0.061$), while the percentage of DMA was higher in women ($p = 0.165$). The difference was observed also in a study including central European population (median total As = 8.0 µg/L of urine, n = 415) (Lindberg et al., 2007), supported by more efficient methylation in females due to the sex hormone influence on women of childbearing age. The oestrogen influence is even more pronounced in the pregnancy or lactation phase as was demonstrated in the recent work by Stajnko et al. (2019). The US survey (NHANES, 2003–2004, n = 2568) with GM for total As of 8.24 µg/g crt also showed slightly higher levels of DMA in women compared to men, but the reverse was observed when non-normalised levels were used (Caldwell et al., 2009).

Among the essential elements, women in our study population had significantly higher Mn, Cu and Zn levels in their blood than the men, which was confirmed by the models (Tables 3 and 4). This is consistent with the abovementioned normal increase in blood levels during the course of pregnancy (Gunier et al., 2014; Izquierdo Álvarez et al., 2007; Qvist et al., 1986). The sex-difference in Cu blood levels is typical, regardless of the pregnancy, because the levels of ceruloplasmin, a Cu-containing plasma protein, are elevated by oestrogen (Fitzgerald et al.,

Table 6
Proposed reference intervals ($RI_{2.5–97.5}$) for selected essential elements in the Slovenian population of men and lactating *primiparous* women of childbearing age (18–49 years).

Biomarker	Population group	N	P2.5 [95% CI]	P97.5 [95% CI]	$RI_{2.5-97.5}$
Mn in blood	Lactating women	409	9.39 [8.63–10.2]	30.3 [28.3–32.5]	9.0–30 µg/L
	Men	402	7.13 [6.78–7.49]	17.6 [16.6–18.7]	7.0–18 µg/L
Se in blood	Lactating women	536	66.6 [64.1–69.1]	138 [131–144]	70–140 µg/L
	Men	548	81.5 [79.0–84.1]	179 [165–195]	80–180 µg/L
Se in urine	Lactating women	410	7.03 [6.58–7.52]	26.2 [24.7–27.8]	7.0–26 µg/g crt
		410	1.83 [1.41–2.38]	37.3 [32.4–42.9]	2.0–40 µg/L
		407	5.03 [4.46–5.68]	22.4 [20.9–24.1]	5.0–22 µg/L SG
	Men	402	7.69 [7.22–8.20]	28.1 [26.1–30.1]	8.0–28 µg/g crt
		402	4.87 [3.93–6.04]	68.4 [59.1–79.2]	5.0–70 µg/L
		397	8.72 [7.58–10.1]	44.3 [40.6–48.4]	9.0–45 µg/L SG
Se in milk	Lactating women	470	7.58 [7.16–8.02]	23.3 [20.6–26.5]	8.0–23 µg/L
Cu in blood	Lactating women	536	809 [784–835]	1432 [1380–1496]	800–1400 µg/L
	Men	548	668 [651–687]	1108 [1057–1161]	700–1100 µg/L
Cu in urine	Lactating women	410	1.41 [1.12–1.79]	36.7 [26.9–50.0]	1.5–40 µg/g crt
		410	1.50 [1.39–1.61]	30.0 [23.8–37.9]	1.5–30 µg/L
		407	1.16 [0.93–1.45]	30.2 [22.1–41.3]	1.0–30 µg/L SG
	Men	402	0.71 [0.30–1.67]	19.5 [15.9–23.8]	1.0–20 µg/g crt
		402	1.50 [1.32–1.70]	28.3 [24.5–32.7]	1.5–30 µg/L
		397	1.14 [0.74–1.77]	22.7 [20.4–25.3]	1.0–23 µg/L SG
Cu in milk	Lactating women	470	196 [183–211]	629 [591–668]	200–600 µg/L
Zn in blood	Lactating women	536	4869 [4628–5123]	9025 [8717–9344]	5000–9000 µg/L
	Men	548	4875 [4694–5062]	8383 [8181–8589]	5000–8400 µg/L
Zn in urine	Lactating women	410	98.9 [75.7–129]	1179 [1029–1350]	100–1200 µg/g crt
		410	52.0 [41.5–65.2]	1034 [898–1190]	50–1100 µg/L
		407	85.1 [67.8–107]	909 [807–1024]	85–900 µg/L SG
	Men	402	38.8 [13.0–116]	618 [564–677]	40–600 µg/g crt
		402	50.3 [35.0–72.3]	1335 [1116–1596]	50–1300 µg/L
		397	54.3 [25.1–118]	983 [883–1096]	50–1000 µg/L SG
Zn in milk	Lactating women	470	579 [461–728]	4799 [4438–5189]	600–5000 µg/L

2008). In contrast, physiological status of women resulted in their lower Se levels in blood (Tables 3 and 4), which normally does not differ according to the sex (Batáriová et al., 2005; Health Canada, 2013).

3.12. Reference values

To derive the RV_{95}, the population groups were selected by considering the following exclusion criteria: smoking (Cd), living in a Pb-smelter area (Pb), consuming fish more than thrice a month (Hg and As in blood and hair) and presence of dental amalgam fillings (Hg in urine). The selected population groups for each biomarker are defined in Table 5. For essential elements (Table 6), no exclusion criteria were considered. For all elements, the partition criteria used to characterise subsets of the reference population was sex.

The proposed levels for toxic elements are mainly comparable to the reference values reported for various populations internationally, as recently reviewed by Saravanabhavan et al. (2017). Among the elements, Hg in blood showed markedly higher reference values in Slovenian than in German, Czech and Canadian populations, and urinary Hg levels were higher in Slovenian than in German, Canadian and Belgian populations. However, it should be noted, that the percentage of our study population without amalgam fillings was very small and was therefore not representative for the Slovenian general population. Similar results were observed for urinary As, but it should be noted that our study lacked information on seafood consumption prior to the sampling; therefore, recent seafood consumption could not be excluded.

The reference intervals proposed for essential elements should be taken as informative because concentrations in whole blood and urine are not used as the most appropriate indicators of essential element status. When comparing the blood and urine RIs with the Canadian reference values (Saravanabhavan et al., 2017), similarities were observed for Zn and Cu, while Se levels were expectedly considerably lower in the Slovenian than the Canadian population. The reference levels for the selected elements are compared internationally in Table 7.

4. Conclusions

The presented study assessed, for the first time, exposure to selected elements in a Slovenian population of childbearing age (18–49 years), selected from lactating *primiparous* women and men. The complete dataset of potentially toxic and essential elements, accompanied by environmental and life-style data, and even distribution of male and female participants across diverse geographical areas of Slovenia enabled us to estimate the exposure levels, compare them geographically and identify the main predictors (lifestyle, particularly diet, and environmental) of exposure. Additionally, the first reference values for the selected Slovenian population have been established, providing important data for the sensitive period of pregnancy/lactation, which is critically lacking in the literature.

Limitations emerging from our study include a lack of parameters which interact crucially with trace elements, particularly in childbearing age, as discussed above (e.g. Fe status), small sample size per study area, and differences in spatial resolutions and the coverage of various datasets for HBM and environmental data, respectively. Despite that, the findings of the study provide the basis to establish recommendations regarding food intake for pregnant or lactating women (e.g. seafood and vegetable intake) and could help focus future biomonitoring efforts for specific metals, especially Pb and Hg, in the selected geographic areas.

Declaration of competing interests

The authors report no competing interests.

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Table 7
International comparison of reference values (RV_{95}) for the selected trace elements in general adult population.

Element/Survey	Study period	Population group (age in years)	N	Exclusion criteria	RV_{95}	Reference
Cd in blood						
Czech Republic	2005–2009	18–58	896	smoking	1.0 µg/L	Černá et al. (2012)
Germany, GerES III	1997–1999	18–69	3061	smoking	1.0 µg/L	Schulz et al. (2011)
Italy, PROBE	2008–2010	18–65	831	smoking	1.08 µg/L	Alimonti et al. (2011)
Canada	2012–2013	20–79	2507		0.83 µg/L	Saravanabhavan et al. (2017)
Cd in urine						
Czech Republic	2005–2009	18–58	896	smoking	1.3 µg/L	Černá et al. (2012)
Germany, GerES III	1997–1999	18–69	3128	smoking	0.8 µg/L	Schulz et al. (2011)
Belgium	2010–2011	> 18	620	smoking	0.7 µg/L[a]	Hoet et al. (2013)
Spain, BIOAMBIENT.ES	2009	18–65	691	smoking	0.74 µg/L	
Canada	2009–2011	20–79	1196		1.3 µg/L	Saravanabhavan et al. (2017)
Pb in blood						
Czech Republic	2001–2003	18–58	1188		75 µg/L	Černá et al. (2012)
Germany, GerES III	1997–1999	18–69	2303		70 µg/L (F)	Schulz et al. (2011)
			2342		90 µg/L (M)	
Italy, PROBE	2008–2010	18–65	1423		51.7 µg/L	Alimonti et al. (2011)
Spain, BIOAMBIENT.ES	2009	18–65	1880		56.8 µg/L	
Canada	2012–2013	20–79	3142		33 µg/L	Saravanabhavan et al. (2017)
Pb in urine						
Belgium	2010–2011	> 18	1001		2.81 µg/L	Hoet et al. (2013)
Canada	2009–2011	20–79	3210		1.9 µg/L	Saravanabhavan et al. (2017)
Total Hg in blood						
Czech Republic	2005–2009	18–58	1221	Seafood[b]	2.6 µg/L	Černá et al. (2012)
Germany, GerES III	1997–1999	18–69	2310	Seafood[c]	2.0 µg/L	Schulz et al. (2011)
Italy, PROBE	2008–2010	18–65	1423		5.16 µg/L	
Canada	2012–2013	20–79	1229		2.3 µg/L **	Saravanabhavan et al. (2017)
Total Hg in urine						
Czech Republic	2005–2009	18–58	1227		3 µg/L	Černá et al. (2012)
Germany, GerES III	1997–1999	18–69	1560	amalgams	1.0 µg/L	Schulz et al. (2011)
Canada	2012–2013	20–79	241		NA (F)	Saravanabhavan et al. (2017)
			217		0.73 µg/L (M)	
Total As in blood						
Italy, PROBE	2008–2010	18–65	1423		5.32 µg/L	
Canada	2007–2009	20–79	996		2.0 µg/L	Saravanabhavan et al. (2017)
Total As in urine						
Germany, GerES III	1997–1999	18–69	3924	Seafood[d]	15 µg/L	Schulz et al. (2011)
Belgium	2010–2011	> 18	1001	Seafood[e]	48.8 µg/L	Hoet et al. (2013)
Canada	2009–2011	3–79	2480		27 µg/L	Saravanabhavan et al. (2017)
Mn in blood						
Italy, PROBE	2008–2010	18–65	470		14.8 µg/L (F)	
			953		14.1 µg/L (M)	
Canada	2009–2011	20–79	1937		16 µg/L (F)	Saravanabhavan et al. (2017)
			1676		14 µg/L (M)	
Cu in blood, Canada	2009–2011	3–79	3124		1300 µg/L (F)	Saravanabhavan et al. (2017)
			2940		1000 µg/L (M)	
Se in blood, Canada	2009–2011	20–79	3598		240 µg/L	Saravanabhavan et al. (2017)
Zn in blood, Canada	2009–2011	20–79	947		6700 µg/L (F)	Saravanabhavan et al. (2017)
			821		7900 µg/L (M)	

[a] P95; **provisional due to high coefficient of variation.
[b] Average fish consumption ≥ 1 time per week.
[c] Average fish consumption > 3 times per month.
[d] Fish consumption 48 h prior to sample collection.
[e] Fish consumption within 96 h of sample collection.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2019.02.008.

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Supplementary data

1 Chemical analysis

1.1 Cadmium, lead, arsenic, manganese, selenium, copper and zinc in blood

An aliquot of 0.3 mL of blood sample was diluted ten times with an alkaline (Merck, suprapur) solution containing Triton X-100 (Sigma Aldrich, sigmaultra) and ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA, Fisher Scientific, analytical reagent grade) (Barany et al., 1997). An aliquot of internal standard solution containing Ga, Gd, Y and Sc was added. For calibration the standard addition procedure was performed. Measurements of prepared solutions were made by Inductively Coupled Plasma Mass Spectrometer (ICP-MS, 7500ce, Agilent, Tokyo, Japan) equipped with an Octapole Reaction System (ORS) and an ASX-510 Autosampler (Cetac). Instrumental conditions were as follows: Babington nebulizer, Scott-type spray chamber, spray chamber temperature 5 °C, plasma gas flow rate 15 L/min, carrier gas flow rate 0.8 L/min, make-up gas flow rate 0.1 L/min, sample solution uptake flow rate 1 mL/min, RF power 1500 W, reaction cell gas helium 4 mL/min in ORS to avoid interference, isotopes monitored 55Mn, 63Cu, 66Zn, 75As, 78Se, 111Cd, 114Cd, 206-208Pb. Tuning of the instrument was made daily. To ensure quality, blank samples, control samples and reference materials were run together with the samples on a daily basis. Analytical precision was 5 % for Pb, Mn, Cu, Zn and Se, 10 % for As and 20 % for Cd. The limits of detection (LOD), calculated as three times the standard deviations of the blank sample, were 0.2, 0.4, 0.1, 0,4, 8, 7, 30 ng/mL for Cd, Pb, As, Mn, Se, Cu and Zn, respectively. A reference material Seronorm® (Trace Elements in Whole Blood L-1) was used to check the accuracy of the results (Jagodic et al., 2017).

1.2 Cadmium, lead, arsenic, selenium, copper and zinc in breast milk samples

For multi-elemental analysis of milk samples, two procedures of sample preparation were used. Procedures are described in detail in Potočnik et al., 2016. Briefly: (a) Microwave digestion for the determination of Se, Cu; Zn: About 1 mL of milk sample (0.15 g of lyophilized sample) was weighed into a quartz tubes. 1 mL 65 % HNO3, (Merck, Germany, suprapur) and 1 mL 30 % H2O2, (Merck, Germany, suprapur) were added and samples were subjected to closed vessel microwave digestion (Microwave system ETHOS 1, MILESTONE SN 130471) at a max. power of 1500 W: ramp to 130°C 10 min, ramp to 200 °C 10 min, hold 20 min and cooling 20 min. Then the samples were equilibrated to room temperature. Solution was quantitatively transferred to a polyethylene graduated tube and filled to 10 mL with Mili-Q water. External calibration was made ICP Multi Element Standard solution XVI CertiPUR (Merck).

(b) Dilution for determination of As, Pb, Cd: An aliquot of 1 mL of milk sample was diluted five times with alkaline (Merck, suprapur) solution containing Triton X-100 (Sigma Aldrich, sigmaultra) and ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA, Fisher Scientific, analytical reagent grade). An aliquot of the internal standard solution containing Ga, Gd, Y and Sc was added. For calibration the standard addition procedure was utilised. Measurements of prepared solutions by Inductively Coupled Plasma Mass Spectrometer (ICP-MS) were described in Section 2.4.1. The only difference is the use of the nebulizer in measurements of digested milk samples, were Micro Mist nebulizer replaced Babington.

To ensure quality, blank samples, control samples and reference materials were run together with the samples on a daily basis. Analytical precision for Cd, Pb and As was 15%, for Se 10%, for Cu and Zn 5%, and for Hg 6%. LODs for Cd, Pb, As, Se, Cu and Zn, calculated as described above were 0.1, 0.2, 0.05, 2, 6 and 35 ng/mL milk sample, respectively. The accuracy of the results was checked by

analysing the certified reference and control material Whole milk powder NIST 8435, Non-fat Milk Powder NIST 1549 (both National Institute of Standard and Technology) and Skim Milk Powder BCR 150 (EC-JRC-IRMM), Whole milk powder NIST 1549a and FAPAS 7190/7172. For As there are no suitable reference materials, therefore, comparison of results of a laboratory control milk sample was made by an independent method, radiochemical neutron activation analysis. Quality control procedures for milk samples are described in detail in (Potočnik et al., 2016).

1.3 Cadmium, lead, arsenic, selenium, copper and zinc in urine samples

An aliquot of 1 ml of urine sample was diluted five times with 1 % nitric acid (Merck, suprapur), and internal standard solution containing Ga, Gd, Y and Sc was added (Stajnko et al, 2017). For calibration the standard addition procedure was performed. Measurement of prepared solutions by ICP-MS is described in Section 2.4.1. To ensure quality, blank samples, control samples and reference materials were run together with the samples on a daily basis. Analytical precision was 5 % for Cu, Zn, Se and As and 7 % for Cd and Pb. LODs were 0.03, 0.3, 0.1, 2, 3 and 3 ng/mL for Cd, Pb, As, Se, Cu and Zn, respectively. Reference material Clinchek® (Urine Control, Level 1) was used to check the accuracy of the results.

1.4 Mercury

The total Hg in biological samples was determined by cold vapour atomic absorption and fluorescence spectrometry (CVAAS and CVAFS) using three different analytical procedures, depending on the matrix type and the sampling period. All measurements were made under strict quality control procedures. Blank samples, control samples and reference materials were tested together with the samples on a daily basis. The accuracy of the total Hg measurements was assured using reference materials IAEA-086 (human hair), Seronorm® (trace elements in whole blood L-1), ClinCheck® (urine control, Level 1) and Skim Milk Powder BCR 150 (EC-JRC-IRMM). Both analytical and quality control procedures used are described in detail elsewhere (Akagi, 1997; Horvat et al., 1991; Miklavčič et al., 2013, 2011). The estimated measurement uncertainty rates were 10% for blood, 8% for urine and breast milk and 4% for hair (95% confidence interval). The LODs of the procedures were <0.1 ng/mL of blood and urine, <0.05 in breast milk and <1 ng/g of hair.

S1. Levels of **cadmium in blood** [µg/L] of Slovenian study population of men and lactating women living in 12 study areas. Different letters indicate that the study groups (according to the study area or sampling period) differed statistically significantly. RV=reference value (1 µg/L, population-based) (Schulz et al., 2011).

Population group	N	N < LOD	N > RV	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [a,b]	79	8	1	**0.29** [0.25-0.34]	0.30	<0.15-2.78	<0.15	<0.15	0.56	0.80
Men/Women	37/42	8/0	1/0	**0.22** [0.17-0.27]/ **0.38** [0.33-0.44]	0.23/0.40	<0.15-2.78/0.15-0.97	<0.15/0.17	<0.15/0.23	0.36/0.67	0.50/0.80
POSOČJE and IDRIJA (GO) [a,b]	96	20	3	**0.26** [0.22-0.30]	0.29	<0.15-1.60	<0.15	<0.15	0.62	0.69
Men/Women	48/48	19/1	3/0	**0.19** [0.15-0.25]/ **0.35** [0.31-0.39]	0.22/0.33	<0.15-1.60/<0.15-0.80	<0.15/0.18	<0.15/0.20	0.49/0.65	1.20/0.68
KOPER (KP) [a,b]	102	11	3	**0.30** [0.27-0.33]	0.30	<0.15-1.50	<0.15	<0.15	0.63	0.86
Men/Women	51/51	9/2	2/1	**0.24** [0.20-0.29]/ **0.36** [0.32-0.42]	0.24/0.37	<0.15-1.50/<0.15-1.08	<0.15/0.19	<0.15/0.20	0.54/0.68	0.92/0.86
JESENICE (KR) [a,b]	83	7	2	**0.32** [0.27-0.37]	0.32	<0.15-1.44	<0.15	0.16	0.71	0.89
Men/Women	43/40	7/0	1/1	**0.24** [0.19-0.29]/ **0.44** [0.38-0.51]	0.23/0.42	<0.15-1.44/0.16-1.36	<0.15/0.21	<0.15/0.25	0.53/0.76	0.67/0.94
MARIBOR (MB) [a]	98	6	7	**0.38** [0.33-0.44]	0.35	<0.15-4.80	<0.15	0.17	0.84	1.75
Men/Women	42/56	5/1	5/2	**0.39** [0.29-0.51]/ **0.38** [0.33-0.43]	0.31/0.37	<0.15-4.80/<0.15-1.15	<0.15/0.15	<0.15/0.22	1.75/0.67	2.74/0.97
POMURJE (MS) [a,b]	90	11	6	**0.30** [0.26-0.35]	0.29	<0.15-3.08	<0.15	<0.15	0.80	1.23
Men/Women	40/50	7/4	4/2	**0.23** [0.18-0.30]/ **0.35** [0.30-0.42]	0.25/0.33	<0.15-1.36/<0.15-3.08	<0.15/<0.15	<0.15/0.18	1.09/0.64	1.24/0.96
MEŽICA VALLEY (RA) [b]	79	11	2	**0.27** [0.24-0.31]	0.29	<0.15-1.22	<0.15	<0.15	0.50	0.86
Men/Women	49/30	6/5	2/0	**0.27** [0.23-0.32]/ **0.27** [0.22-0.34]	0.28/0.31	<0.15-1.22/<0.15-0.97	<0.15/<0.15	<0.15/<0.15	0.47/0.55	0.86/0.68
SAVINJSKO-POSAVSKA (SP) [a,b]	80	10	5	**0.32** [0.28-0.38]	0.32	<0.15-1.63	<0.15	<0.15	0.72	1.08
Men/Women	41/39	10/0	4/1	**0.25** [0.19-0.31]/ **0.43** [0.38-0.49]	0.23/0.45	<0.15-1.63/0.18-1.32	<0.15/0.20	<0.15/0.23	0.93/0.70	1.12/0.74
ZASAVJE (ZA) [a,b]	104	20	5	**0.30** [0.25-0.35]	0.34	<0.15-1.78	<0.15	<0.15	0.76	0.98
Men/Women	52/52	19/1	4/1	**0.21** [0.16-0.27]/ **0.43** [0.38-0.49]	0.22/0.41	<0.15-1.78/<0.15-1.04	<0.15/0.24	<0.15/0.26	0.59/0.83	1.13/0.94
LJUBLJANA (LJ) [b]	100	36	3	**0.20** [0.16-0.24]	0.20	<0.15-2.64	<0.15	<0.15	0.65	0.84
Men/Women	50/50	21/15	3/0	**0.17** [0.12-0.23]/ **0.23** [0.18-0.29]	0.19/0.26	<0.15-2.64/<0.15-0.87	<0.15/<0.15	<0.15/<0.15	0.75/0.61	1.24/0.68
BELA KRAJINA (BK) [a,b]	101	33	9	**0.22** [0.17-0.28]	0.26	<0.15-2.27	<0.15	<0.15	0.96	1.32
Men/Women	54/47	39/4	6/3	**0.16** [0.11-0.23]/ **0.33** [0.25-0.43]	0.13/0.33	<0.15-2.27/<0.15-1.64	<0.15/<0.15	<0.15/0.16	1.03/0.77	1.62/1.13
KOČEVJE and CERKNICA (KO) [a,b]	71	27	9	**0.21** [0.16-0.29]	0.22	<0.15-2.93	<0.15	<0.15	1.15	1.32
Men/Women	41/30	20/7	7/2	**0.17** [0.11-0.27]/ **0.28** [0.20-0.41]	0.16/0.34	<0.15-2.39/<0.15-2.93	<0.15/<0.15	<0.15/<0.15	1.17/0.75	1.25/1.32
Sampling period 2008-2010 [a]	272	96	21	**0.21** [0.18-0.24]	0.22	<0.15-2.93	<0.15	<0.15	0.82	1.24
Sampling period 2011-2014 [b]	811	104	34	**0.30** [0.29-0.32]	0.30	<0.15-4.80	<0.15	<0.15	0.68	0.97

S2. Levels of **lead in blood** [µg/L] of Slovenian study population of men and lactating women living in 12 study areas. Different letters indicate that the study groups (according to the study area or sampling period) differed statistically significantly. RV=reference value (men 90 µg/L; women 70 µg/L (population-based) (Schulz et al., 2011).

Population group	N	N < LOD	N > RV	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [b,c]	79	0	0	**16.8** [15.3-18.4]	16.7	6.78-47.0	9.16	10.4	28.9	34.3
Men/Women	37/42	0/0	0/0	**17.0** [14.5-19.9]/ **16.6** [14.9-18.5]	17.9/16.1	6.78-47.0/9.40-35.6	8.05/10.6	9.16/10.8	29.1/27.4	33.2/34.3
POSOČJE and IDRIJA (GO) [b,c]	96	0	0	**15.7** [14.3-17.1]	15.0	6.05-52.9	7.56	9.19	27.6	33.9
Men/Women	48/48	0/0	0/0	**16.8** [14.6-19.4]/ **14.6** [13.0-16.2]	16.6/13.8	6.64-52.9/6.05-27.6	7.98/7.56	9.13/9.19	33.9/25.1	38.5/26.0
KOPER (KP) [b,c]	102	0	0	**17.4** [16.1-18.8]	16.9	7.30-85.0	10.2	11.4	26.2	36.6
Men/Women	51/51	0/0	0/0	**18.6** [16.6-20.9]/ **16.3** [14.8-18.1]	17.9/16.6	9.25-85.0/7.30-40.1	10.7/10.0	11.7/10.3	32.5/23.6	38.5/36.6
JESENICE (KR) [b,c]	83	0	0	**17.2** [15.5-19.1]	17.0	5.99-69.5	9.26	9.92	30.6	42.3
Men/Women	43/40	0/0	0/0	**16.1** [14.0-18.4]/ **18.5** [15.7-21.8]	15.3/17.7	7.65-47.5/5.99-69.5	9.75/7.97	9.86/10.3	27.0/39.0	41.5/43.5
MARIBOR (MB) [b,c]	98	0	0	**18.1** [16.5-19.8]	17.6	7.84-52.5	9.32	10.1	34.9	42.6
Men/Women	42/56	0/0	0/0	**18.9** [16.2-22.0]/ **17.5** [15.6-19.6]	19.0/16.8	7.84-52.5/8.50-45.4	9.32/9.85	9.89/10.5	36.6/30.9	42.8/38.4
POMURJE (MS) [c]	90	0	0	**15.4** [14.1-16.9]	15.5	4.25-47.8	7.60	9.59	26.5	31.2
Men/Women	40/50	0/0	0/0	**18.5** [16.3-21.1]/ **13.3** [11.8-14.9]	18.9/13.6	5.25-47.8/4.25-32.3	10.3/5.51	11.3/7.74	28.3/21.1	33.0/24.3
MEŽICA VALLEY (RA) [a]	79	0	2	**27.2** [24.0-30.8]	26.8	7.83-116	12.9	14.4	64.8	74.6
Men/Women	49/30	0/0	2/0	**31.0** [26.1-36.7]/ **22.0** [18.9-25.5]	29.1/24.8	7.83-116/8.90-50.2	14.4/10.9	15.9/13.2	73.1/32.4	84.7/34.2
SAVINJSKO-POSAVSKA (SP) [b,c]	81	0	0	**18.8** [17.0-20.7]	18.8	6.96-48.2	8.37	10.8	32.3	42.4
Men/Women	41/40	0/0	0/0	**20.6** [17.7-24.0]/ **17.0** [15.1-19.2]	20.8/18.0	6.96-48.2/7.28-32.3	8.68/8.05	12.2/10.5	42.4/25.9	44.7/31.4
ZASAVJE (ZA) [b,c]	104	0	0	**17.3** [16.1-18.7]	16.2	6.97-57.8	10.1	10.5	28.8	36.5
Men/Women	52/52	0/0	0/0	**17.9** [15.9-20.2]/ **16.8** [15.2-18.6]	16.2/16.1	8.77-48.5/6.97-57.8	9.63/10.3	10.1/11.7	32.9/26.6	42.0/30.1
LJUBLJANA (LJ) [b,c]	100	0	1	**18.0** [16.4-19.6]	17.5	6.53-71.9	8.72	11.0	31.7	42.6
Men/Women	50/50	0/0	0/1	**17.8** [16.0-19.8]/ **18.1** [15.6-21.0]	17.2/17.7	7.31-45.5/6.53-71.9	10.6/7.43	11.7/9.23	31.1/31.7	34.7/53.7
BELA KRAJINA (BK) [b]	101	0	0	**19.3** [17.6-21.1]	19.7	3.86-50.8	9.31	12.1	33.2	37.1
Men/Women	54/47	0/0	0/0	**21.2** [18.5-24.2]/ **17.3** [15.5-19.3]	22.9/16.7	3.86-50.8/6.48-47.3	8.43/9.31	12.1/11.9	36.2/29.4	40.1/33.2
KOČEVJE and CERKNICA (KO) [b,c]	71	0	0	**18.0** [16.0-20.2]	16.8	6.53-67.8	8.94	10.0	33.8	46.1
Men/Women	41/30	0/0	0/0	**19.6** [16.4-23.5]/ **15.9** [14.0-18.1]	20.2/15.8	6.53-67.8/9.22-35.5	6.95/9.31	10.0/10.4	45.8/24.6	52.8/33.1
Sampling period 2008-2010 [a]	272	0	1	**18.4** [17.4-19.5]	17.7	3.86-71.9	8.82	11.1	33.2	40.8
Sampling period 2011-2014 [a]	812	0	2	**17.8** [17.3-18.4]	17.3	4.25-116	9.16	10.2	32.3	42.0
Lower Mežica valley [a]	57	0	0	**23.5** [20.6-26.7]	24.0	7.83-74.6	10.9	13.5	50.2	70.8
Men/Women	31/26	0/0	0/0	**26.0** [21.3-31.8]/ **20.8** [17.7-24.4]	24.0/24.0	7.83-74.6/8.90-50.2	13.3/10.9	14.7/12.9	55.7/30.2	73.1/32.0
Upper Mežica valley [b]	22	0	2	**39.9** [31.8-50.0]	33.5	18.8-116	20.7	23.9	84.7	103
Men/Women	18/4	0/0	2/0	**41.9** [31.8-55.3]/ **31.8** [28.5-35.4]	41.3/31.9	18.8-116/29.3-34.2	18.8/29.3	20.7/29.3	103/34.2	116/34.2

S3. Levels of **mercury in blood** [µg/L] of Slovenian study population of men and lactating women living in 12 study areas. Different letters indicate that the study groups (according to the study area or sampling period) differed statistically significantly. RV=reference value (5 µg/L, HBM I) (Schulz et al., 2011).

Population group	N	N < LOD	N > RV	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [c]	79	0	2	**0.98** [0.78-1.21]	1.04	0.09-5.23	0.14	0.24	3.51	4.00
Men/Women	37/42	0/0	0/2	**0.98** [0.70-1.36]/ **0.98** [0.72-1.32]	1.41/0.96	0.12-4.09/0.09-5.23	0.12/0.15	0.19/0.34	2.87/3.61	4.00/3.85
POSOČJE and IDRIJA (GO) [a,b]	95	0	6	**1.77** [1.53-2.04]	1.78	0.25-15.0	0.50	0.78	4.00	5.25
Men/Women	48/47	0/0	4/2	**1.95** [1.58-2.42]/ **1.59** [1.31-1.93]	2.02/1.61	0.25-15.0/0.32-5.38	0.50/0.58	0.94/0.71	4.62/3.91	5.51/4.68
KOPER (KP) [a]	102	0	15	**2.25** [1.91-2.65]	2.31	0.21-31.0	0.60	0.72	5.82	7.43
Men/Women	51/51	0/0	8/7	**2.36** [1.82-3.06]/ **2.14** [1.74-2.63]	2.31/2.31	0.21-31.0/0.34-7.43	0.60/0.61	0.71/0.91	6.75/5.46	10.3/5.83
JESENICE (KR) [c]	83	0	4	**1.03** [0.84-1.27]	1.11	0.09-15.4	0.21	0.31	3.10	4.11
Men/Women	43/40	0/0	3/1	**1.04** [0.78-1.37]/ **1.02** [0.74-1.41]	1.08/1.28	0.17-15.4/0.09-10.2	0.28/0.14	0.38/0.30	2.92/3.54	5.13/4.05
MARIBOR (MB) [c]	98	2	4	**0.92** [0.73-1.16]	1.06	<0.08-6.40	0.12	0.26	2.88	3.85
Men/Women	42/56	1/1	3/1	**0.94** [0.63-1.41]/ **0.91** [0.68-1.21]	1.20/1.04	<0.08-6.40/<0.08-5.97	0.21/0.12	0.26/0.30	3.58/2.87	5.48/3.09
POMURJE (MS) [c]	90	0	3	**1.08** [0.91-1.27]	1.09	0.18-5.51	0.31	0.40	3.08	4.74
Men/Women	40/50	0/0	1/2	**1.19** [0.93-1.51]/ **1.00** [0.79-1.26]	1.18/0.97	0.18-5.38/0.24-5.51	0.36/0.25	0.47/0.34	2.93/3.12	4.37/4.74
MEŽICA VALLEY (RA) [c]	79	0	2	**1.03** [0.89-1.20]	1.00	0.11-8.13	0.36	0.44	2.27	3.55
Men/Women	49/30	0/0	2/0	**1.14** [0.93-1.41]/ **0.87** [0.71-1.07]	1.07/0.85	0.11-8.13/0.31-2.27	0.43/0.36	0.54/0.39	2.58/2.05	4.48/2.17
SAVINJSKO-POSAVSKA (SP) [c]	81	0	1	**0.97** [0.83-1.13]	0.92	0.31-7.39	0.39	0.43	2.61	3.33
Men/Women	41/40	0/0	1/0	**1.06** [0.84-1.33]/ **0.88** [0.71-1.09]	0.99/0.83	0.37-7.39/0.31-4.78	0.42/0.32	0.46/0.41	2.66/2.31	3.49/2.66
ZASAVJE (ZA) [b]	104	0	2	**1.24** [1.10-1.39]	1.23	0.32-7.38	0.47	0.54	2.60	3.04
Men/Women	52/52	0/0	2/0	**1.36** [1.14-1.62]/ **1.13** [0.97-1.32]	1.30/1.13	0.32-7.38/0.39-3.78	0.42/0.47	0.67/0.49	2.73/2.04	4.89/3.04
LJUBLJANA (LJ) [b]	100	0	3	**1.33** [1.14-1.56]	1.45	0.11-6.12	0.29	0.59	3.48	4.19
Men/Women	50/50	0/0	1/2	**1.38** [1.08-1.77]/ **1.28** [1.04-1.57]	1.54/1.43	0.11-5.85/0.12-6.12	0.23/0.32	0.55/0.63	3.95/2.82	4.33/3.53
BELA KRAJINA (BK) [c]	101	0	1	**0.75** [0.65-0.87]	0.78	0.15-5.08	0.22	0.26	1.79	2.54
Men/Women	54/47	0/0	0/1	**0.69** [0.56-0.86]/ **0.83** [0.69-1.01]	0.64/0.84	0.15-3.94/0.22-5.08	0.19/0.22	0.24/0.35	1.84/1.53	2.54/2.61
KOČEVJE and CERKNICA (KO) [b]	71	0	4	**1.33** [1.10-1.60]	1.21	0.17-15.9	0.39	0.58	3.86	5.04
Men/Women	41/30	0/0	4/0	**1.61** [1.23-2.10]/ **1.02** [0.82-1.28]	1.46/1.07	0.32-15.9/0.17-2.80	0.52/0.39	0.68/0.48	4.99/2.22	6.42/2.64
Sampling period 2008-2010 [a]	272	0	8	**1.08** [0.98-1.19]	1.06	0.11-15.9	0.24	0.36	3.05	4.06
Sampling period 2011-2014 [b]	812	2	39	**1.21** [1.14-1.29]	1.26	<0.08-31.0	0.31	0.43	3.55	4.96
Idrija (men)	11	0	2	**2.7** [1.66-4.30]	2.1	1.1–15.0	1.12	1.57	5.25	15.0

S4. Levels of **arsenic in blood** [µg/L] of Slovenian study population of men and lactating women living in 12 study areas. Different letters indicate that the study groups (according to the study area or sampling period) differed statistically significantly. RV=reference value (20 µg/L, population-based) (Iyengar, 1998).

Population group	N	N < LOD	N > RV	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [b,c]	79	0	0	**0.89** [0.75-1.07]	0.76	0.32-13.0	0.37	0.40	2.55	5.78
Men/Women	37/42	0/0	0/0	**0.75** [0.59-0.96]/ **1.04** [0.80-1.34]	0.64/0.89	0.34-13.0/0.32-11.5	0.37/0.37	0.40/0.42	2.11/2.88	3.44/5.78
POSOČJE and IDRIJA (GO) [b,c]	96	0	0	**0.93** [0.81-1.07]	0.85	0.32-12.3	0.40	0.44	2.90	3.37
Men/Women	48/48	0/0	0/0	**0.92** [0.75-1.11]/ **0.95** [0.77-1.17]	0.85/0.87	0.39-12.3/0.32-3.67	0.44/0.37	0.47/0.40	2.43/3.04	3.58/3.36
KOPER (KP) [a]	102	0	1	**1.81** [1.52-2.16]	1.70	0.44-28.9	0.55	0.64	6.00	8.29
Men/Women	51/51	0/0	0/1	**1.43** [1.15-1.79]/ **2.29** [1.76-2.98]	1.31/2.20	0.44-6.34/0.55-28.9	0.51/0.60	0.57/0.76	4.69/8.29	5.72/10.5
JESENICE (KR) [b,c]	83	0	1	**0.90** [0.77-1.05]	0.81	0.27-22.4	0.36	0.42	1.95	3.40
Men/Women	43/40	0/0	1/0	**0.92** [0.71-1.19]/ **0.88** [0.74-1.06]	0.79/0.82	0.27-22.4/0.32-6.88	0.37/0.35	0.41/0.52	3.09/1.76	3.73/1.90
MARIBOR (MB) [b,c]	98	0	0	**0.91** [0.79-1.04]	0.77	0.32-12.8	0.42	0.47	2.26	3.17
Men/Women	42/56	0/0	0/0	**0.79** [0.66-0.95]/ **1.00** [0.82-1.23]	0.72/0.91	0.36-10.1/0.32-12.8	0.42/0.44	0.47/0.50	1.44/2.33	1.79/5.75
POMURJE (MS) [b,c]	90	0	0	**0.73** [0.65-0.82]	0.66	0.24-5.71	0.36	0.41	1.74	2.17
Men/Women	40/50	0/0	0/0	**0.72** [0.64-0.82]/ **0.73** [0.60-0.88]	0.68/0.63	0.41-2.39/0.24-5.71	0.42/0.35	0.46/0.37	1.24/1.92	1.69/2.44
MEŽICA VALLEY (RA) [b,c]	79	0	0	**0.80** [0.68-0.94]	0.67	0.23-10.7	0.32	0.37	1.95	3.72
Men/Women	49/30	0/0	0/0	**0.75** [0.62-0.91]/ **0.87** [0.64-1.18]	0.62/0.75	0.23-4.99/0.29-10.7	0.36/0.29	0.37/0.34	1.81/2.16	2.23/5.25
SAVINJSKO-POSAVSKA (SP) [b,c]	81	0	0	**0.82** [0.71-0.94]	0.75	0.25-4.60	0.38	0.41	1.81	2.30
Men/Women	41/40	0/0	0/0	**0.87** [0.73-1.05]/ **0.76** [0.62-0.94]	0.83/0.69	0.40-4.60/0.25-4.04	0.44/0.33	0.45/0.38	1.80/1.84	2.29/2.76
ZASAVJE (ZA) [b]	104	0	0	**0.94** [0.82-1.08]	0.93	0.19-7.42	0.35	0.37	2.33	2.93
Men/Women	52/52	0/0	0/0	**0.79** [0.66-0.93]/ **1.12** [0.90-1.40]	0.78/1.16	0.19-6.53/0.26-7.42	0.35/0.35	0.37/0.40	1.51/2.80	2.12/5.16
LJUBLJANA (LJ) [b,c]	100	0	0	**0.92** [0.78-1.07]	0.79	0.20-18.7	0.31	0.38	2.17	4.10
Men/Women	50/50	0/0	0/0	**0.93** [0.77-1.12]/ **0.91** [0.70-1.18]	0.91/0.76	0.30-18.7/0.20-16.8	0.38/0.30	0.46/0.33	1.90/3.50	2.08/4.56
BELA KRAJINA (BK) [c]	101	0	0	**0.66** [0.59-0.75]	0.60	0.22-6.38	0.32	0.37	1.53	2.74
Men/Women	54/47	0/0	0/0	**0.70** [0.59-0.84]/ **0.62** [0.53-0.73]	0.62/0.57	0.32-6.38/0.22-3.13	0.33/0.30	0.39/0.32	2.05/1.46	3.10/1.53
KOČEVJE and CERKNICA (KO) [b,c]	71	1	0	**0.66** [0.54-0.81]	0.60	<0.08-6.47	0.26	0.28	2.27	3.51
Men/Women	41/30	1/0	0/0	**0.59** [0.45-0.76]/ **0.79** [0.57-1.08]	0.52/0.71	<0.08-3.53/0.25-6.47	0.26/0.27	0.27/0.28	1.92/2.95	2.41/3.52
Sampling period 2008-2010 [a]	272	1	0	**0.75** [0.68-0.82]	0.67	<0.08-18.7	0.29	0.33	2.05	3.12
Sampling period 2011-2014 [b]	812	0	2	**0.95** [0.90-1.00]	0.81	0.19-28.9	0.37	0.42	2.74	4.28

S5. Levels of **manganese in blood** [µg/L] of Slovenian study population of men and lactating women living in 9 study areas. There was no statistical difference between the study areas (indicated by [a]). LRV=lower reference value (4 µg/L); URV=upper reference value (15 µg/L) (Barceloux, 1999).

Population group	N	N < LOD	N<LRV	N>URV	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [a]	79	0	0	29	**13.1** [12.2-14.1]	13.3	6.47-27.6	7.71	8.43	19.7	20.5
Men/Women	37/42	0/0	0/0	4/25	**10.7** [9.83-11.6]/ **15.7** [14.6-17.0]	10.3/15.6	6.47-17.1/9.96-27.6	7.54/10.7	7.71/10.9	15.8/20.2	16.6/24.0
POSOČJE and IDRIJA (GO) [a]	96	0	0	36	**13.2** [12.3-14.3]	13.0	6.33-33.4	6.90	8.37	21.3	24.0
Men/Women	48/48	0/0	0/0	4/32	**10.6** [9.88-11.4]/ **16.5** [15.0-18.2]	10.8/17.4	6.33-18.4/6.55-33.4	6.60/9.28	7.16/10.2	14.4/24.0	15.7/27.1
KOPER (KP) [a]	102	0	0	41	**14.4** [13.3-15.6]	13.1	7.13-33.7	8.10	8.88	26.2	28.6
Men/Women	51/51	0/0	0/0	3/38	**10.7** [10.0-11.5]/ **19.3** [17.6-21.2]	10.8/19.5	7.13-29.8/9.15-33.7	7.39/11.4	8.10/12.4	13.2/28.4	15.4/29.7
JESENICE (KR) [a]	82	0	0	38	**14.3** [13.3-15.4]	14.2	7.91-30.8	8.91	9.35	23.0	26.7
Men/Women	42/40	0/0	0/0	9/29	**11.9** [11.1-12.9]/ **17.3** [15.7-19.2]	11.8/17.3	7.91-20.2/9.29-30.8	8.31/10.2	8.91/11.4	15.9/26.7	16.4/27.5
MARIBOR (MB) [a]	98	0	0	44	**14.1** [13.1-15.2]	14.0	6.51-40.5	7.97	9.10	24.2	26.8
Men/Women	42/56	0/0	0/0	3/41	**10.7** [9.87-11.6]/ **17.4** [16.1-18.8]	10.3/17.2	6.51-26.8/10.1-40.5	7.23/10.6	7.97/12.4	14.1/26.2	17.3/28.1
POMURJE (MS) [a]	90	0	0	37	**13.9** [13.0-14.8]	13.5	6.99-30.5	8.37	9.35	21.0	22.7
Men/Women	40/50	0/0	0/0	2/35	**10.9** [10.3-11.6]/ **16.9** [15.8-18.1]	11.3/16.7	6.99-15.9/9.87-30.5	8.03/11.9	8.32/12.2	13.7/22.3	14.5/25.3
MEŽICA VALLEY (RA) [a]	79	0	0	23	**12.8** [12.0-13.6]	12.3	5.83-26.3	8.02	9.32	17.7	20.7
Men/Women	49/30	0/0	0/0	7/16	**11.9** [11.1-12.7]/ **14.3** [12.8-15.9]	11.9/15.5	6.96-26.3/5.83-22.7	8.02/9.03	9.24/10.1	16.0/19.7	17.3/21.9
SAVINJSKO-POSAVSKA (SP) [a]	81	0	0	38	**15.0** [13.8-16.3]	14.1	7.20-35.2	8.94	9.81	24.1	27.2
Men/Women	41/40	0/0	0/0	4/34	**11.5** [10.7-12.4]/ **19.7** [18.0-21.6]	11.0/19.8	7.20-24.0/10.6-35.2	8.60/12.0	8.94/13.1	14.5/29.5	17.8/33.6
ZASAVJE (ZA) [a]	104	0	0	38	**13.5** [12.7-14.5]	13.4	5.69-29.0	8.26	8.82	21.5	23.4
Men/Women	52/52	0/0	0/0	5/33	**10.9** [10.2-11.7]/ **16.8** [15.5-18.2]	10.9/17.1	5.69-17.6/8.45-29.0	7.38/10.2	8.26/11.1	14.7/23.4	16.4/26.7

S6. Levels of **selenium in blood** [µg/L] of Slovenian study population of men and lactating women living in 12 study areas. Different letters indicate that the study groups (according to the study area or sampling period) differed statistically significantly. LRV=lower reference value (females 60 µg/L; males 79 µg/L); URV=upper reference value (females 120 µg/L; males 130 µg/L) (Wilhelm et al., 2004).

Population group	N	N < LOD	N<LRV	N>URV	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [b]	79	0	0	10	**105** [101-108]	104	71.1-163	81.5	87.9	127	146
Men/Women	37/42	0/0	0/0	5/5	**110** [104-115]/ **101** [95.7-106]	106/100	86-163/71.1-146	89.1/76.2	92.8/85.0	138/124	158/126
POSOČJE and IDRIJA (GO) [a,b,c]	96	0	0	16	**106** [102-110]	106	62.5-177	75.7	84.9	138	152
Men/Women	48/48	0/0	0/0	14/2	**119** [114-125]/ **94.5** [90.3-99.0]	117/96.7	90.8-177/62.5-137	91.9/68.2	98.1/75.7	152/113	161/115
KOPER (KP) [b,c]	102	0	0	12	**103** [99.3-106]	103	73.0-152	80.5	84.0	131	144
Men/Women	51/51	0/0	0/0	10/2	**114** [109-119]/ **92.7** [89.3-96.3]	113/90.1	81.3-151/73.0-152	86.1/77.4	91.5/80.5	143/108	146/117
JESENICE (KR) [b,c]	83	0	0	14	**105** [101-110]	106	61.0-153	80.6	84.1	136	140
Men/Women	43/40	0/0	0/0	7/7	**111** [105-117]/ **99.9** [94.5-106]	112/99.0	80.4-153/61.0-146	81.3/79.0	89.4/82.9	139/124	147/135
MARIBOR (MB) [b,c]	98	0	0	11	**106** [103-110]	106	63.6-175	83.2	87.5	129	150
Men/Women	42/56	0/0	0/0	7/5	**117** [112-123]/ **98.9** [95.0-103]	113/97.3	88.1-175/63.6-167	97.6/75.1	99.1/84.1	146/117	155/126
POMURJE (MS) [d]	90	0	4	3	**87.2** [83.3-91.3]	86.8	53.9-179	62.7	67.9	117	123
Men/Women	40/50	0/0	1/3	2/1	**103** [97.6-109]/ **76.3** [73.0-79.8]	101/75.9	76.6-179/53.9-120	81.5/59.9	86.8/63.3	123/90.7	137/105
MEŽICA VALLEY (RA) [b,c]	79	0	0	8	**103** [99.5-107]	104	68.9-176	77.3	83.2	123	142
Men/Women	49/30	0/0	0/0	7/1	**112** [107-116]/ **91.2** [86.7-96.0]	109/89.9	90.3-176/68.9-120	92.3/73.2	95.1/76.7	140/109	142/110
SAVINJSKO-POSAVSKA (SP) [c]	81	0	0	9	**98.3** [93.9-103]	97.0	66.6-178	72.1	77.3	132	140
Men/Women	41/40	0/0	0/0	9/0	**112** [106-119]/ **85.7** [82.2-89.4]	109/84.7	80.6-178/66.6-117	88.4/70.7	91.0/71.9	140/104	152/108
ZASAVJE (ZA) [a,b]	104	0	2	34	**113** [108-118]	114	66.1-197	75.2	83.5	150	164
Men/Women	52/52	0/0	2/0	21/13	**123** [115-132]/ **104** [98.0-109]	125/103	66.1-197/71.7-150	81.5/74.4	92.6/76.4	164/136	180/146
LJUBLJANA (LJ) [a]	100	0	0	34	**116** [111-122]	116	72.8-217	78.9	81.8	162	177
Men/Women	50/50	0/0	0/0	24/10	**131** [124-139]/ **102** [96.6-108]	129/104	95.3-217/72.8-176	99.9/75.8	102/78.9	177/127	190/136
BELA KRAJINA (BK) [c]	101	0	1	13	**101** [96.7-105]	99.2	60.5-196	74.1	77.3	130	138
Men/Women	54/47	0/0	1/0	9/4	**109** [103-115]/ **92.5** [87.5-97.7]	107/93.8	72.7-196/60.5-138	81.5/70.7	85.7/74.1	137/120	172/125
KOČEVJE and CERKNICA (KO) [a,b]	71	0	1	16	**114** [108-120]	113	60.3-226	81.0	89.3	163	184
Men/Women	41/30	0/0	1/0	15/1	**124** [114-134]/ **101** [96.2-107]	117/103	60.3-226/71.9-131	91.4/79.1	95.4/81.5	182/118	184/119
Sampling period 2008-2010 [a]	272	0	2	63	**110** [107-113]	108	60.3-226	77.2	81.4	153	175
Sampling period 2011-2014 [b]	812	0	6	118	**103** [102-104]	103	53.9-197	73.2	79.9	136	147

S7. Levels of **copper in blood** [µg/L] of Slovenian study population of men and lactating women living in 12 study areas. Different letters indicate that the study groups (according to the study area or sampling period) differed statistically significantly. LRV=lower reference value (female 1000 µg/L; male 800 µg/L); URV=upper reference value (female 1400 µg/L; male 1100 µg/L) (Iyengar, 1998).

Population group	N	N < LOD	N<LRV	N>URV	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [a,b]	79	0	21	0	**966** [934-998]	961	641-1341	762	807	1165	1234
Men/Women	37/42	0/0	7/14	0/0	**864** [836-893]/ **1065** [1028-1103]	863/1064	641-1041/810-1341	741/865	762/947	964/1213	1031/1243
POSOČJE and IDRIJA (GO) [a,b]	96	0	22	7	**987** [947-1028]	973	707-1820	739	769	1259	1414
Men/Women	48/48	0/0	13/9	1/6	**857** [832-883]/ **1136** [1079-1196]	864/1111	707-1107/725-1820	732/908	756/946	981/1414	1022/1565
KOPER (KP) [b,c]	102	0	32	1	**933** [902-965]	937	669-1387	734	750	1173	1230
Men/Women	51/51	0/0	23/9	1/0	**820** [796-846]/ **1061** [1025-1098]	806/1058	669-1261/669-1387	721/857	737/944	936/1227	988/1274
JESENICE (KR) [a,b]	83	0	18	2	**961** [931-992]	961	709-1386	762	797	1154	1195
Men/Women	43/40	0/0	8/10	2/0	**879** [849-910]/ **1058** [1020-1097]	876/1064	709-1143/830-1386	740/858	762/901	1033/1218	1075/1298
MARIBOR (MB) [a,b]	98	0	32	2	**963** [931-996]	954	657-1390	724	782	1204	1337
Men/Women	42/56	0/0	12/20	2/1	**863** [833-894]/ **1046** [1002-1091]	851/1054	714-1204/657-1390	738/724	756/904	980/1328	1081/1358
POMURJE (MS) [a,b]	90	0	19	4	**1001** [963-1041]	1003	649-1632	753	787	1278	1373
Men/Women	40/50	0/0	11/8	0/4	**855** [828-884]/ **1135** [1093-1179]	868/1127	649-1046/875-1632	737/928	749/969	978/1368	1038/1463
MEŽICA VALLEY (RA) [a,b,c]	79	0	20	3	**947** [911-985]	923	688-2004	746	789	1219	1252
Men/Women	49/30	0/0	9/11	1/2	**876** [848-905]/ **1076** [1004-1153]	885/1084	688-1404/762-2004	740/821	759/851	975/1251	1015/1465
SAVINJSKO-POSAVSKA (SP) [a,b]	81	0	14	3	**994** [953-1036]	943	737-1747	792	794	1253	1357
Men/Women	41/40	0/0	10/4	0/3	**849** [830-869]/ **1167** [1120-1216]	842/1159	737-1033/910-1747	758/965	792/1008	922/1370	943/1455
ZASAVJE (ZA) [a]	104	0	15	6	**1009** [979-1040]	1026	735-1405	794	824	1215	1280
Men/Women	52/52	0/0	7/8	4/2	**917** [884-950]/ **1110** [1076-1146]	888/1112	735-1272/816-1405	762/923	794/968	1086/1280	1211/1331
LJUBLJANA (LJ) [c]	100	0	55	1	**882** [857-908]	877	608-1478	698	731	1037	1126
Men/Women	50/50	0/0	23/33	0/1	**801** [778-824]/ **972** [940-1006]	807/956	608-965/770-1478	665/816	698/837	895/1126	945/1163
BELA KRAJINA (BK) [c]	101	0	55	2	**870** [838-904]	848	532-1404	649	706	1140	1191
Men/Women	54/47	0/0	33/22	1/1	**768** [741-797]/ **1005** [965-1046]	763/1007	532-1195/738-1404	604/804	649/878	872/1191	983/1240
KOČEVJE and CERKNICA (KO) [b,c]	71	0	31	2	**914** [881-948]	926	577-1273	712	767	1116	1227
Men/Women	41/30	0/0	15/16	2/0	**842** [806-879]/ **1023** [987-1061]	827/997	577-1244/853-1273	708/917	722/924	946/1193	1060/1261
Sampling period 2008-2010 [a]	272	0	142	5	**885** [867-902]	884	532-1478	686	718	1110	1182
Sampling period 2011-2014 [b]	812	0	193	29	**974** [962-986]	963	641-2004	753	789	1221	1298

S8. Levels of **zinc in blood** [µg/L] of Slovenian study population of men and lactating women living in 12 study areas. Different letters indicate that the study groups (according to the study area or sampling period) differed statistically significantly. LRV=lower reference value (6000 µg/L); URV=upper reference value (7000 µg/L) (Iyengar, 1998).

Population group	N	N<LRV	N>URV	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [b,c]	79	17	21	**6610** [6424-6803]	6629	4614-10157	5180	5650	7605	8244
Men/Women	37/42	9/8	9/12	**6491** [6234-6760]/**6717** [6444-7001]	6491/6640	4614-8506/5058-10157	5180/5499	5650/5748	7555/8012	7783/8244
POSOČJE and IDRIJA (GO) [c]	96	35	23	**6219** [6017-6428]	6296	4342-8356	4681	4850	7563	7950
Men/Women	48/48	26/9	10/13	**5938** [5652-6237]/**6514** [6249-6790]	5794/6520	4470-8356/4342-8298	4669/4738	4820/5616	7472/7835	7491/8124
KOPER (KP) [a,b]	102	11	43	**6869** [6703-7038]	6903	4875-9198	5459	5996	8027	8163
Men/Women	51/51	7/4	20/23	**6762** [6531-7001]/**6977** [6738-7224]	6909/6897	4875-8760/5435-9198	5128/5488	5879/6107	7645/8077	8121/8614
JESENICE (KR) [b,c]	83	13	28	**6614** [6461-6771]	6638	4430-8376	5495	5777	7403	7748
Men/Women	43/40	5/8	15/13	**6672** [6489-6860]/**6552** [6299-6815]	6637/6698	5335-8001/4430-8376	5777/5292	5958/5601	7335/7543	7553/7756
MARIBOR (MB) [b,c]	98	27	31	**6514** [6292-6744]	6479	3010-10301	4594	5573	7868	8572
Men/Women	42/56	15/12	8/24	**6324** [6028-6634]/**6661** [6341-6998]	6279/6634	4421-10301/3010-9886	5021/4568	5460/5782	7608/7956	7916/8643
POMURJE (MS) [c]	90	31	13	**6249** [6093-6409]	6292	4105-8011	5199	5449	7222	7535
Men/Women	40/50	19/12	2/11	**6030** [5841-6226]/**6429** [6197-6669]	6053/6629	4515-7433/4105-8011	5172/5274	5394/5455	6814/7516	7177/7771
MEŽICA VALLEY (RA) [b,c]	79	22	25	**6457** [6275-6645]	6530	4780-8038	5099	5333	7572	7728
Men/Women	49/30	7/15	20/5	**6707** [6494-6926]/**6070** [5784-6370]	6836/6149	4923-8038/4780-7445	5342/4915	5797/5115	7702/7147	7773/7283
SAVINJSKO-POSAVSKA (SP) [b,c]	81	26	18	**6444** [6229-6666]	6381	4550-9743	5184	5285	7810	8581
Men/Women	41/40	19/7	4/14	**6088** [5848-6338]/**6831** [6496-7182]	6131/6819	5007-9743/4550-9733	5184/5124	5263/5862	6803/8466	7316/8984
ZASAVJE (ZA) [a]	104	11	64	**7218** [6997-7446]	7308	3400-11733	5640	5980	8603	9012
Men/Women	52/52	7/4	28/37	**7009** [6682-7352]/**7433** [7144-7735]	7076/7520	3400-10243/5637-11733	5548/5972	5701/6114	8285/8774	9012/9375
LJUBLJANA (LJ) [b]	100	19	39	**6653** [6478-6833]	6717	4026-9136	5189	5555	7798	8251
Men/Women	50/50	9/10	18/18	**6692** [6475-6916]/**6614** [6335-6907]	6654/6764	4929-8706/4026-9136	5500/5150	5896/5430	7750/7847	8276/8227
BELA KRAJINA (BK) [b,c]	101	21	30	**6583** [6423-6746]	6615	4891-9108	5351	5568	7806	8053
Men/Women	54/47	14/7	12/18	**6412** [6205-6625]/**6785** [6543-7035]	6380/6791	4891-8534/5102-9108	5118/5464	5500/5781	7461/8053	7907/8454
KOČEVJE and CERKNICA (KO) [a,b]	71	11	27	**6815** [6581-7057]	6835	4911-10544	5479	5841	8173	8779
Men/Women	41/30	7/4	15/13	**6736** [6447-7037]/**6925** [6524-7351]	6835/6848	4911-9579/4987-10544	5479/5851	5596/5889	8155/8664	8383/10195
Sampling period 2008-2010 [a]	272	51	94	**6670** [6564-6779]	6670	4026-10544	5346	5596	7907	8383
Sampling period 2011-2014 [a]	812	193	268	**6584** [6517-6652]	6619	3010-11733	5099	5495	7783	8245
Lower Mežica Valley [a]	79	18	17	**6388** [6167-6618]	6530	4780-8038	4923	5244	7402	7702
Men/Women	37/42	6/12	12/5	**6644** [6365-6936]/ **6096** [5767-6444]	6797/6357	4923-8038/4780-7445	5342/4915	5797/5099	7690/7154	7898/7283
Upper Mežica Valley [a]	96	4	8	**6640** [6320-6976]	6630	5341-7773	5633	5742	7680	7728
Men/Women	48/48	1/3	8/0	**6815** [6471-7178]/ **5905** [5452-6394]	6946/5848	5341-7773/5633-6312	5341/5633	6011/5633	7728/6312	7773/6312

S9. Levels of **cadmium in urine** [µg/L] of Slovenian study population of men and lactating women living in 12 study areas. Different letters indicate that the study groups (according to the study area or sampling period) differed statistically significantly. LOD=0.03 µg Cd/L. RV=reference value (1 µg/L, health-based HBM I) (Schulz et al., 2011).

Population group	N	N < LOD	N > RV	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [a,b]	79	1	4	**0.20** [0.16-0.24]	0.19	<LOD-1.68	0.05	0.07	0.59	1.12
Men/Women	37/42	0/1	1/3	**0.18** [0.14-0.22]/ **0.22** [0.16-0.29]	0.19/0.21	0.05-1.16/<LOD-1.68	0.05/0.06	0.07/0.10	0.38/0.70	0.71/1.12
POSOČJE and IDRIJA (GO) [a,b]	98	0	2	**0.19** [0.16-0.22]	0.19	0.04-3.83	0.05	0.06	0.50	0.68
Men/Women	49/49	0/0	0/2	**0.24** [0.20-0.28]/ **0.15** [0.12-0.19]	0.22/0.13	0.05-0.88/0.04-3.83	0.08/0.05	0.10/0.05	0.57/0.35	0.68/0.50
KOPER (KP) [a,b]	101	0	2	**0.22** [0.19-0.25]	0.22	0.04-1.17	0.07	0.08	0.50	0.66
Men/Women	50/51	0/0	1/1	**0.25** [0.21-0.30]/ **0.18** [0.15-0.23]	0.25/0.18	0.07-1.01/0.04-1.17	0.10/0.04	0.11/0.07	0.54/0.49	0.68/0.66
JESENICE (KR) [a]	82	2	0	**0.15** [0.13-0.19]	0.15	<LOD-0.90	0.03	0.05	0.48	0.77
Men/Women	42/40	0/2	0/0	**0.18** [0.14-0.23]/ **0.13** [0.10-0.17]	0.17/0.13	0.03-0.90/<LOD-0.80	0.05/<LOD	0.07/0.04	0.60/0.37	0.84/0.66
MARIBOR (MB) [a,b]	99	1	1	**0.17** [0.15-0.20]	0.18	<LOD-1.95	0.05	0.07	0.46	0.67
Men/Women	42/57	0/1	0/1	**0.16** [0.13-0.20]/ **0.18** [0.14-0.23]	0.17/0.20	0.04-0.74/<LOD-1.95	0.06/0.05	0.07/0.07	0.42/0.52	0.53/0.75
POMURJE (MS) [a,b]	89	6	1	**0.18** [0.15-0.22]	0.24	<LOD-1.11	<LOD	0.05	0.50	0.54
Men/Women	40/49	1/5	1/0	**0.24** [0.19-0.31]/ **0.14** [0.10-0.19]	0.29/0.14	<LOD-1.11/<LOD-0.81	0.07/<LOD	0.09/<LOD	0.49/0.51	0.53/0.72
MEŽICA VALLEY (RA) [a,b]	79	2	0	**0.19** [0.16-0.23]	0.22	<LOD-0.88	0.04	0.08	0.54	0.73
Men/Women	49/30	0/2	0/0	**0.23** [0.19-0.28]/ **0.14** [0.10-0.21]	0.24/0.16	0.07-0.88/<LOD-0.81	0.08/<LOD	0.10/0.03	0.54/0.52	0.62/0.73
SAVINJSKO-POSAVSKA (SP) [b]	81	0	4	**0.25** [0.21-0.29]	0.24	0.04-2.00	0.08	0.10	0.68	0.99
Men/Women	41/40	0/0	1/3	**0.23** [0.18-0.29]/ **0.27** [0.20-0.35]	0.21/0.25	0.06-1.75/0.04-2.00	0.10/0.08	0.10/0.10	0.56/0.88	0.78/1.26
ZASAVJE (ZA) [a,b]	104	2	1	**0.18** [0.15-0.21]	0.19	<LOD-3.65	0.04	0.06	0.45	0.67
Men/Women	52/52	1/1	0/1	**0.18** [0.14-0.23]/ **0.18** [0.14-0.23]	0.20/0.19	<LOD-0.99/<LOD-3.65	0.04/0.05	0.06/0.06	0.45/0.45	0.88/0.67
LJUBLJANA (LJ) [a]	61	0	0	**0.15** [0.13-0.17]	0.13	<LOD-0.38	<LOD	0.10	0.30	0.31
Men/Women	25/36	0/0	0/0	**0.17** [0.14-0.21]/ **0.14** [0.12-0.16]	0.17/0.12	<LOD-0.35/<LOD-0.38	<LOD/<LOD	<LOD/<LOD	0.33/0.23	0.35/0.31
BELA KRAJINA (BK) [a,b]	84	0	0	**0.21** [0.19-0.23]	0.23	<LOD-0.49	0.10	0.10	0.36	0.43
Men/Women	45/39	0/0	0/0	**0.20** [0.18-0.23]/ **0.21** [0.18-0.25]	0.22/0.24	<LOD-0.49/<LOD-0.46	0.10/0.10	0.10/0.10	0.30/0.43	0.38/0.46
KOČEVJE and CERKNICA (KO) [a,b]	43	0	0	**0.19** [0.17-0.22]	0.20	<LOD-0.37	0.10	0.10	0.33	0.35
Men/Women	34/10	0/0	0/0	**0.21** [0.18-0.24]/ **0.16** [0.12-0.20]	0.23/0.15	<LOD-0.49/<LOD-0.46	0.10/0.10	0.11/0.10	0.35/0.26	0.35/0.29
Sampling period 2008-2010 [α]	189	0	0	**0.18** [0.17-0.20]	0.19	<LOD-0.49	0.10	0.10	0.33	0.37
Sampling period 2011-2014 [α]	812	14	15	**0.19** [0.18-0.20]	0.20	<LOD-3.83	0.05	0.07	0.52	0.73

S10. Levels of **cadmium in urine** [µg/g creatinine] of Slovenian study population of men and lactating women living in 12 study areas. Different letters indicate that the study groups (according to the study area or sampling period) differed statistically significantly.

Population group	N	N < LOD	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [a,b]	79	1	**0.21** [0.18-0.24]	0.20	<LOD-0.88	0.10	0.10	0.48	0.74
Men/Women	37/42	0/1	**0.13** [0.12-0.16]/ **0.30** [0.25-0.36]	0.14-0.30	<LOD-0.30/0.09-0.88	0.07/0.11	0.08/0.14	0.21/0.68	0.25/0.81
POSOČJE and IDRIJA (GO) [a,b]	98	0	**0.20** [0.18-0.22]	0.19	0.08-1.44	0.11	0.12	0.33	0.50
Men/Women	49/49	0/0	**0.18** [0.16-0.20]/ **0.23** [0.20-0.27]	0.16/0.23	0.08-0.50/0.09-1.44	0.11/0.11	0.11/0.14	0.32/0.48	0.38/0.53
KOPER (KP) [a,b]	101	0	**0.22** [0.19-0.24]	0.21	0.08-0.61	0.09	0.11	0.43	0.49
Men/Women	50/51	0/0	**0.17** [0.15-0.19]/ **0.27** [0.24-0.31]	0.17/0.31	0.08-0.47/0.09-0.61	0.09/0.10	0.10/0.17	0.32/0.49	0.41/0.55
JESENICE (KR) [a,b]	82	2	**0.18** [0.15-0.21]	0.16	<LOD-0.77	0.07	0.08	0.47	0.54
Men/Women	42/40	0/2	**0.14** [0.12-0.17]/ **0.23** [0.19-0.28]	0.14/0.24	0.03-0.53/0.07-0.77	0.06/0.08	0.07/0.10	0.34/0.60	0.46/0.69
MARIBOR (MB) [b]	99	1	**0.17** [0.15-0.19]	0.18	<LOD-0.82	0.05	0.06	0.42	0.54
Men/Women	42/57	0/1	**0.11** [0.09-0.14]/ **0.22** [0.20-0.26]	0.12/0.20	0.04-0.54/0.08-0.82	0.04/0.10	0.05/0.12	0.25/0.51	0.33/0.59
POMURJE (MS) [a,b]	89	6	**0.21** [0.18-0.23]	0.19	<LOD-0.94	<LOD	0.11	0.47	0.55
Men/Women	40/49	1/5	**0.17** [0.15-0.19]/ **0.25** [0.21-0.29]	0.16/0.21	0.07-0.41/0.07-0.94	0.08/0.09	0.09/0.11	0.26/0.56	0.38/0.67
MEŽICA VALLEY (RA) [a,b]	79	2	**0.21** [0.19-0.24]	0.20	<LOD-1.19	0.10	0.12	0.46	0.54
Men/Women	49/30	0/2	**0.19** [0.16-0.21]/ **0.26** [0.21-0.32]	0.20/0.22	0.06-0.53/0.12-1.19	0.09/0.13	0.11/0.13	0.32/0.57	0.41/0.66
SAVINJSKO-POSAVSKA (SP) [a]	81	0	**0.24** [0.20-0.28]	0.22	<LOD -1.23	0.08	0.12	0.66	0.81
Men/Women	41/40	0/0	**0.19** [0.16-0.23]/ **0.31** [0.24-0.40]	0.17/0.30	0.08-1.14/<LOD-1.23	0.08/0.12	0.11/0.15	0.52/0.79	0.54/1.06
ZASAVJE (ZA) [a,b]	104	2	**0.19** [0.16-0.21]	0.18	0.05-2.79	0.06	0.08	0.37	0.44
Men/Women	52/52	1/1	**0.14** [0.12-0.16]/ **0.25** [0.21-0.29]	0.14/0.25	0.05-0.44/0.07-2.79	0.06/0.10	0.06/0.13	0.30/0.43	0.37/0.49
LJUBLJANA (LJ) [a,b]	57	0	**0.21** [0.17-0.26]	0.21	0.05-1.32	0.06	0.08	0.60	0.88
Men/Women	22/35	0/0	**0.14** [0.10-0.20]/ **0.27** [0.21-0.34]	0.12/0.26	0.05-0.74/0.0 -1.32	0.05/0.10	0.06/0.12	0.38/0.71	0.44/0.97
BELA KRAJINA (BK) [a,b]	76	0	**0.19** [0.16-0.23]	0.17	0.04-1.40	0.05	0.07	0.67	0.98
Men/Women	42/34	0/0	**0.14** [0.11-0.17]/ **0.28** [0.21-0.38]	0.14/0.24	0.05-0.67/0.04-1.40	0.05/0.11	0.06/0.12	0.38/0.98	0.45/1.23
KOČEVJE and CERKNICA (KO) [a,b]	43	0	**0.22** [0.16-0.29]	0.22	0.04-1.73	0.05	0.06	0.70	1.04
Men/Women	34/10	0/0	**0.17** [0.13-0.23]/ **0.47** [0.27-0.81]	0.17/0.41	0.04-1.04/0.21-1.73	0.04/0.21	0.06/0.22	0.51/1.50	0.63/1.73
Sampling period 2008-2010 [α]	178	0	**0.20** [0.18-0.23]	0.19	<LOD-1.73	0.05	0.07	0.67	0.97
Sampling period 2011-2014 [α]	811	14	**0.20** [0.19-0.21]	0.19	<LOD-2.79	0.08	0.09	0.46	0.54

S11. Levels of **cadmium in urine, SG normalized** [µg/L SG] of Slovenian study population of men and lactating women living in 12 study areas. Different letters indicate that the study groups (according to the study area or sampling period) differed statistically significantly.

Population group	N	N < LOD	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [a,b]	77	1	**0.21** [0.19-0.24]	0.20	<LOD-0.95	0.09	0.11	0.43	0.69
Men/Women	36/41	0/1	**0.20** [0.17-0.23]/ **0.23** [0.19-0.29]	0.19/0.23	0.09-0.50/<LOD-0.95	0.11/0.07	0.11/0.10	0.39/0.56	0.43/0.75
POSOČJE and IDRIJA (GO) [a,b]	96	0	**0.21** [0.19-0.24]	0.19	0.6-1.92	0.12	0.12	0.43	0.62
Men/Women	48/48	0/0	**0.24** [0.21-0.27]/ **0.19** [0.16-0.23]	0.23/0.16	0.12-0.73/<LOD-1.92	0.12/0.10	0.13/0.12	0.43/0.46	0.56/0.83
KOPER (KP) [a,b]	100	0	**0.24** [0.22-0.26]	0.22	0.06-0.85	0.12	0.16	0.44	0.50
Men/Women	49/51	0/0	**0.26** [0.23-0.30]/ **0.22** [0.20-0.26]	0.23/0.21	0.12-0.79/0.06-0.85	0.16/0.11	0.17/0.13	0.44/0.41	0.51/0.50
JESENICE (KR) [a,b]	81	2	**0.19** [0.16-0.22]	0.17	<LOD-0.80	0.06	0.08	0.56	0.60
Men/Women	41/40	0/2	**0.19** [0.15-0.24]/ **0.19** [0.15-0.24]	0.17/0.20	0.03-0.66/<LOD-0.80	0.07/0.06	0.09/0.07	0.60/0.53	0.60/0.59
MARIBOR (MB) [b]	98	1	**0.18** [0.16-0.21]	0.18	<LOD-1.10	0.06	0.07	0.39	0.55
Men/Women	42/56	0/1	**0.16** [0.13-0.20]/ **0.20** [0.17-0.23]	0.15/0.19	0.06-0.56/<LOD-1.10	0.06/0.06	0.07/0.11	0.36/0.45	0.45/0.61
POMURJE (MS) [a,b]	89	6	**0.22** [0.19-0.24]	0.24	<LOD-0.73	0.07	0.09	0.47	0.50
Men/Women	40/49	1/5	**0.25** [0.22-0.29]/ **0.19** [0.16-0.23]	0.24/0.22	<LOD-0.73/<LOD-0.64	0.14/0.07	0.17/0.07	0.44/0.47	0.50/0.48
MEŽICA VALLEY (RA) [a,b]	78	2	**0.24** [0.21-0.27]	0.24	<LOD-1.05	0.09	0.11	0.52	0.56
Men/Women	48/30	0/2	**0.27** [0.23-0.31]/ **0.20** [0.15-0.26]	0.30/0.17	0.07-0.56/<LOD-1.05	0.11/0.07	0.12/0.09	0.52/0.53	0.56/0.56
SAVINJSKO-POSAVSKA (SP) [a]	81	0	**0.26** [0.23-0.30]	0.25	0.08-1.42	0.11	0.13	0.54	0.85
Men/Women	41/40	0/0	**0.26** [0.21-0.30]/ **0.26** [0.21-0.33]	0.25/0.25	0.08-1.11/0.08-1.42	0.12/0.09	0.14/0.12	0.54/0.69	0.71/0.96
ZASAVJE (ZA) [a,b]	103	2	**0.19** [0.17-0.22]	0.19	<LOD-3.65	0.07	0.09	0.42	0.51
Men/Women	52/51	1/1	**0.19** [0.16-0.23]/ **0.19** [0.16-0.23]	0.19/0.17	<LOD-0.76/<LOD-3.65	0.07/0.08	0.08/0.10	0.47/0.36	0.55/0.42
LJUBLJANA (LJ) [a,b]	47	0	**0.19** [0.16-0.23]	0.18	0.06-0.65	0.09	0.10	0.39	0.50
Men/Women	21/26	0/0	**0.22** [0.17-0.27]/ **0.18** [0.14-0.22]	0.24/0.16	0.10-0.65/0.06-0.65	0.10/0.08	0.11/0.09	0.36/0.39	0.46/0.50
BELA KRAJINA (BK) [a,b]	61	0	**0.22** [0.18-0.26]	0.20	0.06-0.92	0.08	0.10	0.54	0.65
Men/Women	31/30	0/0	**0.21** [0.16-0.26]/ **0.23** [0.18-0.29]	0.20/0.20	0.06-0.92/0.07-0.75	0.07/0.10	0.10/0.11	0.36/0.62	0.57/0.69
KOČEVJE and CERKNICA (KO) [a,b]	40	0	**0.22** [0.18-0.27]	0.22	0.08-0.81	0.08	0.09	0.56	0.68
Men/Women	30/10	0/0	**0.21** [0.16-0.27]/ **0.27** [0.18-0.40]	0.22/0.23	0.08-0.81/0.14-0.65	0.08/0.14	0.09/0.15	0.55/0.60	0.70/0.65
Sampling period 2008-2010 [α]	148	0	**0.21** [0.19-0.23]	0.20	<LOD-0.92	0.08	0.10	0.54	0.65
Sampling period 2011-2014 [α]	803	14	**0.21** [0.21-0.22]	0.20	<LOD-3.65	0.07	0.10	0.47	0.56

S12. Levels of **lead in urine** [µg/L] of Slovenian study population of men and lactating women living in 9 study areas. Different letters indicate that the study groups (according to the study area) differed statistically significantly. LOD=0.30 µg Pb/L.

Population group	N	N < LOD	GM [CI 95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [a]	79	27	**0.40** [0.33-0.48]	0.40	<LOD-3.48	<LOD	<LOD	1.14	1.81
Men/Women	37/42	10/17	**0.51** [0.37-0.69]/ **0.33** [0.26-0.41]	0.50/0.37	<LOD-3.48/<LOD-1.21	<LOD/<LOD	<LOD/<LOD	1.81/0.89	2.16/1.08
POSOČJE and IDRIJA (GO) [a,b]	98	31	**0.46** [0.39-0.56]	0.51	<LOD-4.13	<LOD	<LOD	1.66	1.81
Men/Women	49/49	5/26	**0.73** [0.59-0.91]/ **0.30** [0.23-0.38]	0.71/<LOD	<LOD-3.70/<LOD-4.13	<LOD/<LOD	<LOD/<LOD	1.79/1.02	1.85/1.44
KOPER (KP) [a,b]	101	28	**0.51** [0.43-0.61]	0.57	<LOD-3.53	<LOD	<LOD	1.43	1.79
Men/Women	50/51	4/24	**0.84** [0.68-1.04]/ **0.32** [0.25-0.40]	0.94/0.33	<LOD-3.53/<LOD-1.79	<LOD/<LOD	0.35/<LOD	1.97/0.95	3.01/1.17
JESENICE (KR) [a]	82	27	**0.42** [0.35-0.50]	0.45	<LOD-3.22	<LOD	<LOD	1.07	1.40
Men/Women	42/40	10/17	**0.49** [0.38-0.63]/ **0.32** [0.25-0.40]	0.55/0.38	<LOD-3.22/<LOD-1.65	<LOD/<LOD	<LOD/<LOD	1.31/0.38	1.41/1.02
MARIBOR (MB) [a,b]	99	30	**0.45** [0.38-0.53]	0.48	<LOD-4.41	<LOD	<LOD	1.42	1.86
Men/Women	42/57	6/24	**0.60** [0.47-0.76]/ **0.36** [0.28-0.46]	0.61/0.33	<LOD-3.24/<LOD-4.41	<LOD/<LOD	<LOD/<LOD	1.51/1.10	1.86/1.86
POMURJE (MS) [a,b]	89	22	**0.51** [0.42-0.60]	0.58	<LOD-2.31	<LOD	<LOD	1.42	1.56
Men/Women	40/49	3/19	**0.83** [0.67-1.03]/ **0.34** [0.27-0.41]	0.94/0.38	<LOD-2.31/<LOD-1.16	<LOD/<LOD	0.41/<LOD	1.67/0.97	2.13/1.03
MEŽICA VALLEY (RA) [b]	79	15	**0.67** [0.54-0.83]	0.72	<LOD-3.83	<LOD	<LOD	2.52	3.41
Men/Women	49/30	5/10	**0.85** [0.65-1.10]/ **0.46** [0.32-0.65]	0.94/0.50	<LOD-3.83/<LOD-3.74	<LOD/<LOD	<LOD/<LOD	2.62/1.41	3.41/2.36
SAVINJSKO-POSAVSKA (SP) [a,b]	81	24	**0.47** [0.39-0.58]	0.51	<LOD-4.40	<LOD	<LOD	1.48	1.89
Men/Women	41/40	6/18	**0.71** [0.54-0.93]/ **0.31** [0.24-0.40]	0.77/0.32	<LOD-4.40/<LOD-1.50	<LOD/<LOD	<LOD/<LOD	1.89/1.03	2.02/1.28
ZASAVJE (ZA) [a]	103	43	**0.37** [0.31-0.44]	0.40	<LOD-2.74	<LOD	<LOD	1.07	1.48
Men/Women	52/51	16/27	**0.48** [0.37-0.62]/ **0.29** [0.23-0.36]	0.59/<LOD	<LOD-2.74/<LOD-1.98	<LOD/<LOD	<LOD/<LOD	1.31/0.83	2.00/1.05
Lower Mežica valley [a]	57	14	**0.54** [0.42-0.70]	0.54	<LOD-3.74	<LOD	>LOD	2.04	3.07
Men/Women	31/26	4/10	**0.73** [0.52-1.01]/ **0.29** [0.23-0.36]	0.84/0.42	<LOD-3.41/<LOD-3.74	<LOD/<LOD	<LOD/<LOD	2.52/1.05	3.07/1.27
Upper Mežica valley [b]	22	1	**1.16** [0.80-1.67]	1.35	<LOD-3.83	0.35	0.41	2.56	3.53
Men/Women	18/4	1/0	**1.10** [0.70-1.72]/ **1.45** [0.80-2.62]	1.35/1.37	<LOD-3.83/1.01-2.36	<LOD/1.01	0.35/1.01	3.53/2.36	3.83/2.36

S13. Levels of **lead in urine** [µg/g creatinine] of Slovenian study population of men and lactating women living in 9 study areas. Different letters indicate that the study groups (according to the study area) differed statistically significantly. LOD=0.30 µg Pb/L.

Population group	N	N < LOD	GM [CI 95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [a,c]	79	27	**0.42** [0.35-0.50]	0.41	<LOD-1.93	<LOD	<LOD	1.17	1.49
Men/Women	37/42	10/17	**0.39** [0.29-0.51]/ **0.45** [0.36-0.56]	0.38/0.45	<LOD-1.52/<LOD-1.93	<LOD/<LOD	<LOD/<LOD	1.17/1.15	1.52/1.37
POSOČJE and IDRIJA (GO) [a,c]	98	31	**0.50** [0.44-0.56]	0.47	<LOD-2.35	<LOD	<LOD	1.18	1.51
Men/Women	49/49	5/26	**0.54** [0.46-0.64]/ **0.46** [0.38-0.55]	0.50/<LOD	<LOD-2.27/<LOD-2.35	<LOD/<LOD	<LOD/<LOD	1.22/1.18	1.51/1.27
KOPER (KP) [a,c]	101	28	**0.51** [0.45-0.58]	0.52	<LOD-2.54	<LOD	<LOD	0.99	1.27
Men/Women	50/51	4/24	**0.55** [0.48-0.64]/ **0.47** [0.39-0.58]	0.52/0.54	<LOD-2.35/<LOD-2.54	<LOD/<LOD	0.24/<LOD	0.99/0.99	1.20/1.37
JESENICE (KR) [a,c]	82	27	**0.49** [0.41-0.57]	0.47	<LOD-2.88	<LOD	<LOD	1.33	1.85
Men/Women	42/40	10/17	**0.38** [0.31-0.47]/ **0.63** [0.49-0.80]	0.38/0.56	<LOD-1.47/<LOD-2.88	<LOD/<LOD	<LOD/<LOD	0.85/2.03	1.08/2.81
MARIBOR (MB) [a,c]	99	30	**0.43** [0.37-0.50]	0.45	<LOD-3.44	<LOD	<LOD	1.07	1.62
Men/Women	42/57	6/24	**0.42** [0.33-0.53]/ **0.44** [0.36-0.55]	0.42/0.46	<LOD-2.19/<LOD-3.44	<LOD/<LOD	<LOD/<LOD	0.92/1.07	1.29/1.81
POMURJE (MS) [a,b]	89	22	**0.57** [0.51-0.64]	0.58	<LOD-2.68	<LOD	<LOD	1.27	1.33
Men/Women	40/49	3/19	**0.56** [0.49-0.65]/ **0.58** [0.49-0.70]	0.57/0.62	<LOD-1.37/<LOD-2.68	<LOD/<LOD	0.30/<LOD	1.03/1.32	1.30/1.52
MEŽICA VALLEY (RA) [b]	79	15	**0.74** [0.62-0.88]	0.74	<LOD-5.31	<LOD	<LOD	2.29	2.71
Men/Women	49/30	5/10	**0.69** [0.54-0.87]/ **0.82** [0.63-1.07]	0.70/0.91	<LOD-5.31/<LOD-2.64	<LOD/<LOD	<LOD/<LOD	2.56/1.58	2.80/2.30
SAVINJSKO-POSAVSKA (SP) [a]	81	24	**0.46** [0.39-0.55]	0.55	<LOD-2.16	<LOD	<LOD	1.13	1.29
Men/Women	41/40	6/18	**0.58** [0.46-0.74]/ **0.36** [0.28-0.47]	0.62/0.40	<LOD-2.16/<LOD-1.19	<LOD/<LOD	<LOD/<LOD	1.29/0.98	1.41/1.12
ZASAVJE (ZA) [c]	103	43	**0.38** [0.34-0.44]	0.40	<LOD-2.78	<LOD	<LOD	0.82	1.03
Men/Women	52/51	16/27	**0.37** [0.30-0.45]/ **0.40** [0.33-0.48]	0.39/0.40	<LOD-2.78/<LOD-1.32	<LOD/<LOD	<LOD/<LOD	0.79/1.29	1.00/1.29
Lower Mežica valley [a]	56	14	**0.71** [0.59-1.09]	0.71	<LOD-3.41	<LOD	0.24	1.51	3.19
Men/Women	30/26	4/10	**0.83** [0.63-1.09]/ **0.60** [0.46-0.78]	0.77/0.65	<LOD-3.41/<LOD-1.87	<LOD/<LOD	0.37/0.24	2.67/1.24	3.24/1.31
Upper Mežica valley [b]	22	1	**1.24** [0.87-1.76]	1.24	<LOD-5.21	0.36	0.57	3.05	3.82
Men/Women	18/4	1/0	**1.32** [0.86-2.02]/ **0.94** [0.56-1.58]	1.31/1.08	<LOD-5.21/<LOD-1.14	<LOD/0.57	0.36/0.57	3.82/1.14	5.21/1.14

S14. Levels of **lead in urine, SG normalized** [µg/L SG] of Slovenian study population of men and lactating women living in 9 study areas. Different letters indicate that the study groups (according to the study area) differed statistically significantly.

Population group	N	N < LOD	GM [CI 95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [a]	77	27	**0.44** [0.38-0.52]	0.47	<LOD-1.62	<LOD	<LOD	1.16	1.46
Men/Women	36/41	10/17	**0.57** [0.45-0.73]/ **0.35** [0.29-0.43]	0.59/0.39	<LOD-1.62/<LOD-1.62	<LOD/<LOD	<LOD/<LOD	1.42/0.66	1.52/0.76
POSOČJE and IDRIJA (GO) [a,c]	96	31	**0.52** [0.45-0.60]	0.49	<LOD-6.11	<LOD	<LOD	1.21	1.89
Men/Women	48/48	5/26	**0.71** [0.60-0.84]/ **0.38** [0.31-0.47]	0.71/<LOD	<LOD-3.05/<LOD-6.11	<LOD/<LOD	<LOD/<LOD	1.40/1.06	1.89/1.24
KOPER (KP) [a]	100	28	**0.58** [0.50-0.66]	0.65	<LOD-3.37	<LOD	<LOD	1.24	1.70
Men/Women	49/51	4/24	**0.87** [0.76-1.01]/ **0.39** [0.33-0.46]	0.85/0.40	<LOD-3.37/<LOD-1.05	<LOD/<LOD	0.47/<LOD	1.93/0.80	2.07/1.02
JESENICE (KR) [a,c]	81	26	**0.53** [0.45-0.62]	0.56	<LOD-1.95	<LOD	<LOD	1.19	1.48
Men/Women	41/40	9/17	**0.54** [0.43-0.69]/ **0.52** [0.42-0.64]	0.58/0.51	<LOD-1.85/<LOD-1.95	<LOD/<LOD	<LOD/<LOD	1.19/1.28	1.57/1.45
MARIBOR (MB) [a,c]	98	29	**0.48** [0.41-0.55]	0.49	<LOD-3.18	<LOD	<LOD	1.42	1.68
Men/Women	42/56	6/23	**0.60** [0.48-0.75]/ **0.40** [0.32-0.49]	0.61/0.39	<LOD-2.25/<LOD-3.18	<LOD/<LOD	<LOD/<LOD	1.47/1.02	1.65/1.68
POMURJE (MS) [a,b]	89	22	**0.60** [0.53-0.69]	0.65	<LOD-3.14	<LOD	<LOD	1.24	1.58
Men/Women	40/49	3/19	**0.85** [0.74-0.98]/ **0.45** [0.38-0.54]	0.85/0.49	<LOD-3.14/<LOD-2.37	<LOD/<LOD	0.49/<LOD	1.56/0.89	1.69/1.01
MEŽICA VALLEY (RA) [b]	78	15	**0.83** [0.70-0.99]	0.86	<LOD-5.21	<LOD	<LOD	2.51	2.24
Men/Women	48/30	5/10	**0.99** [0.78-1.25]/ **0.64** [0.51-0.80]	1.08/0.69	<LOD-5.21/<LOD-1.87	<LOD/<LOD	<LOD/<LOD	3.19/1.19	3.41/1.31
SAVINJSKO-POSAVSKA (SP) [a,c]	81	24	**0.49** [0.41-0.60]	0.60	<LOD-3.35	<LOD	<LOD	1.36	1.52
Men/Women	41/40	6/18	**0.78** [0.62-0.99]/ **0.31** [0.24-0.39]	0.92/0.30	<LOD-3.35/<LOD-1.02	<LOD/<LOD	<LOD/<LOD	1.52/0.79	2.03/0.85
ZASAVJE (ZA) [c]	103	43	**0.40** [0.35-0.46]	0.42	<LOD-3.06	<LOD	<LOD	0.96	1.26
Men/Women	52/51	16/27	**0.51** [0.42-0.63]/ **0.31** [0.26-0.37]	0.62/<LOD	<LOD-3.06/<LOD-1.51	<LOD/<LOD	<LOD/<LOD	1.26/0.66	1.28/0.77
Lower Mežica valley [a]	57	14	**0.67** [0.55-0.80]	0.67	<LOD-3.53	<LOD	0.28	1.56	2.56
Men/Women	31/26	4/10	**0.60** [0.46-0.78]/ **0.75** [0.57-1.00]	0.51/0.87	<LOD-3.53/<LOD-2.30	<LOD/<LOD	0.29/0.24	1.42/1.56	2.80/1.59
Upper Mežica valley [b]	22	1	**0.96** [0.65-1.44]	0.90	<LOD-5.31	0.29	0.54	2.64	2.71
Men/Women	18/4	1/0	**0.88** [0.55-1.41]/ **1.47** [0.69-3.11]	0.80/1.44	<LOD-5.31/<LOD-2.64	<LOD/0.84	0.29/0.84	2.71/2.64	5.31/2.64

S15. Levels of **mercury in urine** [µg/L] of Slovenian study population of men and lactating women living in 12 study areas. Different letters indicate that the study groups (according to the study area or sampling period) differed statistically significantly. LOD=0.01 µg/L. RV=reference value (7 µg/L, health-based, HBM I) (Schulz et al., 2011).

Population group	N	N < LOD	N > RV	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [a,b,c]	76	0	1	**0.40** [0.30-0.52]	0.31	0.02-7.62	0.08	0.11	2.18	3.31
Men/Women	35/41	0/0	0/1	**0.33** [0.23-0.47]/ **0.46** [0.31-0.69]	0.28/0.36	0.02-7.62/0.02-3.79	0.11/0.05	0.12/0.11	1.11/2.18	3.02/3.31
POSOČJE and IDRIJA (GO) [a,b]	96	0	0	**0.54** [0.43-0.68]	0.63	<LOD-5.44	0.06	0.13	2.06	3.30
Men/Women	49/47	0/0	0/0	**0.78** [0.62-0.98]/ **0.37** [0.25-0.55]	0.84/0.46	0.16-4.68/<LOD-5.44	0.19/0.06	0.21/0.06	2.06/2.40	3.18/3.30
KOPER (KP) [a]	101	0	0	**0.70** [0.57-0.84]	0.69	0.05-6.69	0.17	0.22	2.39	3.17
Men/Women	50/51	0/0	0/0	**0.97** [0.73-1.28]/ **0.50** [0.40-0.64]	1.17/0.50	0.05-6.69/0.07-3.62	0.17/0.14	0.30/0.18	3.08/1.46	4.78/2.00
JESENICE (KR) [b,c]	82	1	0	**0.30** [0.23-0.40]	0.34	<LOD-4.17	0.05	0.08	1.59	2.67
Men/Women	42/40	0/1	0/0	**0.44** [0.30-0.63]/ **0.21** [0.14-0.30]	0.46/0.23	0.05-4.17/<LOD-1.57	0.07/0.03	0.10/0.06	2.67/0.77	3.13/1.02
MARIBOR (MB) [c]	99	6	0	**0.26** [0.19-0.37]	0.36	<LOD-4.97	<LOD	0.02	2.25	3.69
Men/Women	41/58	0/6	0/0	**0.42** [0.29-0.59]/ **0.19** [0.11-0.32]	0.41/0.20	0.03-4.27/<LOD-4.97	0.07/<LOD	0.08/<LOD	1.27/2.25	3.69/3.61
POMURJE (MS) [b,c]	89	2	1	**0.34** [0.25-0.47]	0.32	<LOD-12.3	0.02	0.07	2.96	4.23
Men/Women	40/49	1/1	0/1	**0.58** [0.34-1.00]/ **0.22** [0.15-0.31]	0.53/0.24	<LOD-12.3/<LOD-2.60	0.05/0.02	0.09/0.04	4.28/0.81	5.78/2.22
MEŽICA VALLEY (RA) [a,b,c]	79	3	0	**0.40** [0.29-0.55]	0.47	<LOD-4.77	0.02	0.08	2.05	2.64
Men/Women	49/30	1/2	0/0	**0.48** [0.33-0.70]/ **0.30** [0.17-0.53]	0.56/0.34	<LOD-3.40/<LOD-4.77	0.05/0.34	0.09/0.05	2.18/1.50	2.48/2.99
SAVINJSKO-POSAVSKA (SP) [a,b,c]	70	0	0	**0.50** [0.39-0.63]	0.53	0.04-4.41	0.08	0.13	1.99	3.30
Men/Women	32/38	0/0	0/0	**0.63** [0.44-0.90]/ **0.41** [0.29-0.57]	0.60/0.38	0.07-3.72/0.04-4.41	0.11/0.08	0.19/0.11	1.96/2.01	3.41/3.30
ZASAVJE (ZA) [a,b]	104	0	0	**0.61** [0.53-0.70]	0.53	0.21-5.76	0.27	0.29	1.81	2.29
Men/Women	52/52	0/0	0/0	**0.71** [0.57-0.88]/ **0.52** [0.44-0.62]	0.60/0.44	0.21-5.76/0.24-3.64	0.27/0.27	0.30/0.27	2.20/1.13	3.40/1.45
LJUBLJANA (LJ) [a,b]	93	0	0	**0.59** [0.45-0.77]	0.51	0.02-6.40	0.05	0.13	3.64	4.63
Men/Women	45/48	0/0	0/0	**0.79** [0.55-1.13]/ **0.44** [0.30-0.66]	0.71/0.36	0.03-5.69/0.02-6.40	0.13/0.04	0.22/0.06	3.80/3.60	5.09/3.97
BELA KRAJINA (BK) [a,b,c]	97	0	1	**0.40** [0.32-0.49]	0.34	0.04-7.05	0.08	0.09	1.72	2.84
Men/Women	51/46	0/0	1/0	**0.35** [0.26-0.48]/ **0.45** [0.33-0.61]	0.32/0.39	0.04-5.09/0.08-7.05	0.07/0.10	0.08/0.13	1.41/2.00	2.50/3.18
KOČEVJE and CERKNICA (KO) [a,b,c]	69	0	0	**0.45** [0.33-0.61]	0.49	<LOD-5.80	0.06	0.11	2.02	4.22
Men/Women	41/28	0/0	0/0	**0.46** [0.29-0.73]/ **0.43** [0.29-0.62]	0.51/0.47	<LOD-5.80/0.09-1.82	0.05/0.10	0.11/0.11	2.67/1.52	4.31/1.64
Sampling period 2008-2010 [a]	259	0	1	**0.47** [0.40-0.55]	0.44	<LOD-7.05	0.07	0.11	2.67	3.97
Sampling period 2011-2014 [a]	796	12	2	**0.43** [0.40-0.47]	0.45	<LOD-12.3	0.05	0.10	2.18	3.30
Idrija (men)	11	0	0	**0.85** [0.50-1.43]	0.99	0.19-2.09	0.19	0.27	1.91	2.09

S16. Levels of **mercury in urine** [µg/g creatinine] of Slovenian study population of men and lactating women living in 12 study areas. Different letters indicate that the study groups (according to the study area or sampling period) differed statistically significantly. LOD=0.01 µg/L. RV=reference value (5 µg/g crea., health-based, HBM I) (Schulz et al., 2011).

Population group	N	N < LOD	N > RV	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [a,b,c]	76	0	0	**0.42** [0.33-0.53]	0.40	0.05-3.99	0.08	0.11	1.57	1.90
Men/Women	35/41	0/0	0/0	**0.26** [0.19-0.35]/ **0.64** [0.48-0.85]	0.23/0.66	0.05-3.99/0.07-3.02	0.05/0.11	0.08/0.17	1.01/1.77	1.34/1.90
POSOČJE and IDRIJA (GO) [a,b]	96	0	0	**0.57** [0.48-0.68]	0.56	0.06-3.92	0.15	0.17	1.68	2.55
Men/Women	49/47	0/0	0/0	**0.58** [0.46-0.72]/ **0.56** [0.43-0.74]	0.56/0.56	0.09-3.92/<LOD-2.81	0.16/0.11	0.23/0.16	1.68/2.33	1.96/2.65
KOPER (KP) [a]	101	0	0	**0.70** [0.59-0.81]	0.80	0.09-3.09	0.15	0.21	1.82	2.04
Men/Women	50/51	0/0	0/0	**0.64** [0.50-0.82]/ **0.75** [0.61-0.92]	0.75/0.83	0.09-3.09/0.10-2.31	0.12/0.15	0.18/0.29	1.92/1.75	2.59/1.95
JESENICE (KR) [b,c]	82	1	0	**0.35** [0.28-0.45]	0.38	<LOD-2.91	0.05	0.08	1.17	1.56
Men/Women	42/40	0/1	0/0	**0.34** [0.24-0.49]/ **0.37** [0.27-0.50]	0.34/0.39	0.03-2.91/<LOD-1.81	0.03/0.06	0.06/0.08	1.40/1.13	1.62/1.31
MARIBOR (MB) [c,e]	99	6	0	**0.26** [0.19-0.35]	0.31	<LOD-4.52	0.02	0.02	1.41	2.21
Men/Women	41/58	0/6	0/0	**0.29** [0.21-0.41]/ **0.24** [0.15-0.37]	0.31/0.30	0.02-2.73/<LOD-4.52	0.04/<LOD	0.07/<LOD	0.84/1.43	2.19/2.23
POMURJE (MS) [b,d,e]	89	2	1	**0.39** [0.29-0.51]	0.47	<LOD-5.52	0.03	0.09	1.59	2.50
Men/Women	40/49	1/1	1/0	**0.39** [0.25-0.63]/ **0.38** [0.27-0.54]	0.38/0.48	<LOD-5.52/<LOD-2.84	0.04/0.02	0.07/0.11	2.00/1.42	3.11/1.59
MEŽICA VALLEY (MS) [a,b,c]	79	3	0	**0.44** [0.34-0.58]	0.49	<LOD-4.16	0.06	0.09	1.83	2.76
Men/Women	49/30	1/2	0/0	**0.39** [0.29-0.52]/ **0.54** [0.31-0.94]	0.45/0.65	<LOD-2.76/<LOD-4.16	0.07/0.01	0.09/0.10	1.27/2.73	1.44/3.15
SAVINJSKO-POSAVSKA (SP) [a,b]	70	0	2	**0.49** [0.39-0.62]	0.48	0.03-5.34	0.09	0.13	1.61	1.95
Men/Women	32/38	0/0	0/2	**0.53** [0.40-0.70]/ **0.46** [0.31-0.68]	0.56/0.39	0.09-1.47/0.03-5.34	0.12/0.05	0.16/0.10	1.35/1.95	1.46/5.03
ZASAVJE (ZA) [a,d]	104	0	0	**0.63** [0.54-0.73]	0.63	0.10-3.40	0.19	0.22	1.79	2.34
Men/Women	52/52	0/0	0/0	**0.54** [0.43-0.68]/ **0.73** [0.60-0.89]	0.58/0.69	0.10-3.38/0.16-3.40	0.18/0.29	0.20/0.37	1.42/1.79	2.34/2.34
LJUBLJANA (LJ) [a]	86	0	4	**0.72** [0.57-0.92]	0.84	0.03-8.67	0.10	0.17	3.01	3.67
Men/Women	42/44	0/0	0/4	**0.58** [0.42-0.81]/ **0.88** [0.62-1.26]	0.78/0.85	0.03-3.67/0.04-8.67	0.10/0.10	0.16/0.24	1.54/3.38	3.10/5.45
BELA KRAJINA (BK) [b,c]	83	0	2	**0.36** [0.28-0.46]	0.39	0.03-8.49	0.07	0.08	1.47	2.14
Men/Women	44/39	0/0	0/2	**0.24** [0.18-0.34]/ **0.54** [0.39-0.77]	0.28/0.55	0.03-2.17/0.09-8.49	0.03/0.09	0.07/0.11	1.08/2.10	1.47/5.01
KOČEVJE and CERKNICA (KO) [a,b,c]	55	0	1	**0.47** [0.30-0.73]	0.70	<LOD-7.92	0.02	0.06	3.03	4.77
Men/Women	40/15	0/0	1/0	**0.36** [0.21-0.64]/ **0.92** [0.51-1.64]	0.57/0.95	<LOD-7.92/0.14-4.78	0.02/0.14	0.05/0.15	2.98/3.40	4.75/4.78
Sampling period 2008-2010 [a]	224	0	7	**0.50** [0.42-0.59]	0.61	<LOD-8.67	0.06	0.09	2.41	3.45
Sampling period 2011-2014 [b]	796	12	3	**0.46** [0.42-0.49]	0.50	<LOD-5.52	0.06	0.11	1.63	2.19
Idrija (men)	11	0	0	**0.79** [0.50-1.26]	0.96	0.31-2.39	0.31	0.31	1.42	2.39

S17. Levels of **mercury in urine, SG normalized** [µg/L SG] of Slovenian study population of men and lactating women living in 12 study areas. Different letters indicate that the study groups (according to the study area or sampling period) differed statistically significantly.

Population group	N	N < LOD	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [a,b,c]	75	0	**0.45** [0.36-0.55]	0.36	0.05-6.03	0.11	0.14	1.35	2.05
Men/Women	34/41	0/0	**0.39** [0.29-0.54]/ **0.50** [0.37-0.67]	0.29/0.48	0.11-6.03/0.05-2.26	0.13/0.09	0.14/0.16	1.33/1.35	2.30/1.75
POSOČJE and IDRIJA (GO) [a,b,c]	95	0	**0.59** [0.48-0.71]	0.60	0.03-5.05	0.12	0.16	2.08	2.80
Men/Women	48/47	0/0	**0.77** [0.61-0.97]/ **0.44** [0.33-0.60]	0.76/0.44	0.12-5.05/0.03-3.12	0.23/0.09	0.25/0.12	2.21/1.73	2.80/2.68
KOPER (KP) [b]	100	0	**0.78** [0.66-0.92]	0.89	0.07-5.34	0.16	0.25	2.05	2.86
Men/Women	49/51	0/0	**1.00** [0.77-1.28]/ **0.61** [0.49-0.77]	1.14/0.75	0.11-5.34/0.07-2.31	0.23/0.14	0.27/0.17	2.87/1.56	3.75/1.84
JESENICE (KR) [c,d]	81	1	**0.38** [0.30-0.48]	0.34	<LOD-3.30	0.07	0.08	1.54	2.09
Men/Women	41/40	0/1	**0.46** [0.32-0.67]/ **0.31** [0.22-0.42]	0.41/0.31	0.05-3.30/<LOD-1.65	0.07/0.06	0.10/0.08	2.09/1.04	2.20/1.50
MARIBOR (MB) [d]	98	6	**0.28** [0.20-0.38]	0.36	<LOD-5.07	<LOD	0.03	1.86	3.34
Men/Women	41/57	0/6	**0.42** [0.30-0.60]/ **0.21** [0.13-0.33]	0.40/0.28	0.04-5.07/<LOD-4.18	0.05/<LOD	0.10/<LOD	1.86/1.81	3.34/2.58
POMURJE (MS) [c,d]	89	2	**0.41** [0.30-0.54]	0.38	<LOD-10.2	0.06	0.09	2.53	3.05
Men/Women	40/49	1/1	**0.60** [0.37-0.96]/ **0.30** [0.21-0.42]	0.50/0.35	<LOD-10.2/<LOD-2.25	0.07/0.02	0.10/0.08	3.12/1.17	4.13/1.61
MEŽICA VALLEY (RA) [a,b,c]	78	3	**0.49** [0.37-0.65]	0.59	<LOD-3.45	0.05	0.08	1.72	2.08
Men/Women	48/30	1/2	**0.55** [0.41-0.74]/ **0.42** [0.23-0.74]	0.62/0.58	<LOD-3.08/<LOD-3.45	0.07/0.01	0.14/0.06	1.67/2.06	1.85/3.01
SAVINJSKO-POSAVSKA (SP) [a,b,c]	70	0	**0.52** [0.42-0.65]	0.53	0.05-3.30	0.13	0.15	1.58	2.73
Men/Women	32/38	0/0	**0.71** [0.54-0.94]/ **0.40** [0.29-0.56]	0.76/0.34	0.13-2.83/0.05-3.30	0.13/0.07	0.18/0.14	1.41/2.34	2.40/2.90
ZASAVJE (ZA) [a,b]	103	0	**0.66** [0.57-0.76]	0.61	0.14-7.88	0.26	0.28	1.77	3.08
Men/Women	52/51	0/0	**0.76** [0.62-0.93]/ **0.57** [0.47-0.70]	0.72/0.58	0.26-4.05/0.14-7.88	0.28/0.21	0.32/0.27	1.94/1.02	3.40/2.46
LJUBLJANA (LJ) [a,b]	75	0	**0.78** [0.61-1.00]	0.81	0.04-4.32	0.10	0.20	2.83	3.72
Men/Women	39/36	0/0	**0.91** [0.65-1.27]/ **0.66** [0.45-1.00]	0.90/0.67	0.04-4.32/0.04-3.08	0.16/0.08	0.30/0.11	3.72/2.67	4.07/2.89
BELA KRAJINA (BK) [c,d]	67	0	**0.35** [0.28-0.45]	0.31	0.04-3.53	0.07	0.11	1.64	2.00
Men/Women	35/32	0/0	**0.31** [0.21-0.45]/ **0.41** [0.30-0.56]	0.26/0.38	0.04-2.35/0.08-3.53	0.05/0.11	0.07/0.16	1.64/1.31	2.26/2.00
KOČEVJE and CERKNICA (KO) [a,b,c,d]	58	1	**0.48** [0.34-0.66]	0.58	<LOD-5.33	0.05	0.09	3.49	4.09
Men/Women	36/22	1/0	**0.46** [0.29-0.73]/ **0.51** [0.31-0.82]	0.48/0.71	<LOD-4.26/0.07-5.33	0.04/0.9	0.10/0.09	3.62/1.29	4.09/1.39
Sampling period 2008-2010 [a]	200	0	**0.52** [0.44-0.61]	0.54	<LOD-5.33	0.08	0.11	2.34	3.51
Sampling period 2011-2014 [a]	789	12	**0.49** [0.45-0.53]	0.52	<LOD-10.2	0.07	0.13	1.86	2.70

S18. Levels of **arsenic in urine** [µg/L] of Slovenian study population of men and lactating women living in 9 study areas. Different letters indicate that the study groups (according to the study area) differed statistically significantly. RV=reference value (15 µg/L, population-based) (Schulz et al., 2011).

Population group	N	N < LOD	N > RV	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [b]	79	0	17	**5.86** [4.45-7.71]	4.66	0.90-350	1.21	1.45	30.7	95.3
Men/Women	37/42	0/0	9/8	**7.09** [4.78-10.5]/ **4.95** [3.35-7.31]	5.25/3.69	1.45-350/0.90-209	1.45/1.17	2.00/1.30	37.0/26.0	95.3/30.7
POSOČJE and IDRIJA (GO) [b]	98	0	18	**7.40** [5.91-9.26]	7.16	0.87-1026	1.40	1.80	31.4	44.8
Men/Women	49/49	0/0	12/6	**10.3** [7.23-14.5/ **5.33** [4.10-6.94]	8.79/5.56	1.08-1026/0.87-35.1	1.63/1.07	2.82/1.46	44.8/19.5	90.2/20.7
KOPER (KP) [a]	101	0	45	**15.1** [11.8-19.2]	12.5	1.30-556	2.09	3.25	93.1	117
Men/Women	50/51	0/0	26/19	**18.0** [12.6-25.8]/ **12.7** [9.09-17.7]	15.2/10.0	1.30-556/1.34-266	2.57/2.01	3.43/2.86	110/61.1	147/101
JESENICE (KR) [b]	82	0	16	**5.33** [3.99-7.12]	4.09	0.50-135	0.72	1.32	40.9	75.1
Men/Women	42/40	0/0	13/3	**7.51** [4.79-11.8]/ **3.71** [2.63-5.24]	5.14/3.71	0.71-135/0.50-75.1	0.98/0.63	1.52/1.06	44.2/13.2	122/28.4
MARIBOR (MB) [b]	99	0	11	**4.65** [3.93-5.50]	4.70	0.78-52.6	1.06	1.71	16.4	26.8
Men/Women	42/57	0/0	4/7	**4.74** [3.78-5.95]/ **4.58** [3.58-5.85]	5.01/4.23	0.86-26.4/0.78-52.6	1.66/1.03	1.93/1.36	14.0/21.0	16.4/32.8
POMURJE (MS) [b]	89	0	13	**4.73** [3.81-5.87]	4.59	0.60-71.6	1.01	1.29	20.1	27.0
Men/Women	40/49	0/0	9/4	**6.82** [4.96-9.39]/ **3.51** [2.67-4.61]	5.81/3.22	1.18-71.6/0.60-35.5	1.54/0.73	1.88/1.01	25.7/14.4	32.5/15.6
MEŽICA VALLEY (RA) [b]	79	0	12	**5.96** [4.67-7.60]	5.18	0.48-110	1.28	1.83	31.9	61.8
Men/Women	49/30	0/0	10/2	**6.81** [4.89-9.49]/ **4.78** [3.36-6.81]	5.76/4.86	0.48-110/0.60-66.5	1.57/1.06	1.83/1.77	52.5/11.3	61.8/46.5
SAVINJSKO-POSAVSKA (SP) [b]	81	0	13	**5.58** [4.42-7.04]	5.35	0.26-136	1.18	1.95	17.6	28.5
Men/Women	41/40	0/0	8/5	**6.72** [4.81-9.39]/ **4.61** [3.32-6.39]	6.55/4.32	0.64-136/0.26-72.4	1.27/1.07	2.42/1.64	21.2/16.3	32.6/23.1
ZASAVJE (ZA) [b]	103	0	15	**5.95** [5.00-7.08]	5.95	0.85-65.0	1.54	2.00	18.1	28.2
Men/Women	52/51	0/0	8/7	**6.22** [4.86-7.97]/ **5.95** [4.42-7.32]	5.87/5.95	1.16-65.0/0.85-35.7	1.57/1.52	2.00/2.02	17.3/18.1	28.2/31.3

S19. Levels of **arsenic in urine** [µg/g creatinine] of Slovenian study population of men and lactating women living in 9 study areas. Different letters indicate that the study groups (according to the study area) differed statistically significantly.

Population group	N	N < LOD	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [b,c]	79	0	**6.12** [4.74-7.90]	4.76	0.76-499	1.52	2.26	23.1	82.2
Men/Women	37/42	0/0	**5.41** [3.50-8.38]/ **6.81** [5.03-9.22]	4.37/6.92	0.76-499/2.03-191	0.77/2.40	1.52/2.45	23.4/13.1	92.9/64.9
POSOČJE and IDRIJA (CE) [b]	98	0	**7.92** [6.54-9.60]	6.51	1.82-403	2.24	2.50	26.9	52.2
Men/Women	49/49	0/0	**7.58** [5.59-10.3]/ **8.27** [6.48-10.6]	6.04/6.54	2.11-403/1.82-66.1	2.24/2.26	2.47/3.14	29.2/26.9	52.9/39.3
KOPER (KP) [a]	101	0	**15.0** [12.1-18.7]	12.9	2.25-267	3.23	4.01	64.5	152
Men/Women	50/51	0/0	**11.9** [8.75-16.2]/ **18.9** [13.9-25.6]	9.72/13.9	2.25-267/2.60-219	2.63/3.39	3.47/5.84	67.6/64.5	85.9/203
JESENICE (KR) [b,c]	82	0	**6.21** [4.94-7.81]	5.63	0.88-118	1.38	1.82	30.2	54.9
Men/Women	42/40	0/0	**5.83** [3.95-8.61]/ **6.63** [5.18-8.50]	4.01/6.05	0.98-118/0.88-54.9	1.23/1.95	1.59/2.50	34.3/14.2	70.8/31.1
MARIBOR (MB) [b,c]	99	0	**4.49** [3.81-5.30]	4.24	0.85-48.8	1.18	1.83	14.4	22.8
Men/Women	42/57	0/0	**3.30** [2.61-4.18]/ **5.64** [4.54-7.01]	2.68/5.12	0.85-22.2/1.16-48.8	1.15/1.39	1.42/2.17	8.82/20.1	12.9/26.5
POMURJE (MS) [b,c]	89	0	**5.38** [4.62-6.27]	4.73	1.36-39.8	1.78	2.42	13.9	20.8
Men/Women	40/49	0/0	**4.63** [3.69-5.81]/ **6.08** [4.93-7.50]	4.27/5.69	1.36-33.9/1.49-39.8	1.69/2.15	1.82/2.53	11.8/17.3	13.9/24.9
MEŽICA VALLEY (RA) [b,c]	79	0	**6.56** [5.09-8.45]	4.86	1.41-103	1.51	1.65	50.5	65.2
Men/Women	49/30	0/0	**5.55** [3.96-7.77]/ **8.62** [5.87-12.6]	4.80/7.17	1.41-103/2.30-65.9	1.49/2.41	1.60/2.86	50.5/47.5	65.2/57.9
SAVINJSKO-POSAVSKA (SP) [b,c]	81	0	**5.43** [4.40-6.70]	5.52	0.37-84.3	1.49	1.92	25.0	27.2
Men/Women	41/40	0/0	**5.51** [4.16-7.31]/ **5.35** [3.87-7.40]	5.72/5.37	1.26-84.3/0.37-65.3	1.54/1.13	1.92/1.74	13.0/25.0	27.2/27.1
ZASAVJE (ZA) [b,c]	103	0	**6.13** [5.22-7.20]	5.59	1.28-49.0	2.03	2.36	20.3	29.7
Men/Women	52/51	0/0	**4.76** [3.90-5.81]/ **7.93** [6.24-10.1]	4.24/8.05	1.28-32.3/1.97-49.0	1.89/2.34	2.19/2.56	13.6/26.1	20.3/39.1

S20. Levels of **arsenic in urine, SG normalized** [µg/L SG] of Slovenian study population of men and lactating women living in 9 study areas. Different letters indicate that the study groups (according to the study area) differed statistically significantly.

Population group	N	N < LOD	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [a,c]	77	0	**6.59** [5.08-8.54]	5.54	1.46-665	1.72	1.87	28.5	97.4
Men/Women	36/41	0/0	**8.18** [5.35-12.5]/ **5.45** [3.95-7.51]	6.26/4.85	1.58-665/1.46-143	1.67/1.72	2.53/1.87	33.5/16.2	121/36.7
POSOČJE and IDRIJA (GO) [a]	96	0	**8.38** [6.78-10.4]	7.09	1.55-672	2.26	2.82	30.4	56.7
Men/Women	48/48	0/0	**10.3** [7.44-14.2]/ **6.84** [5.19-9.00]	8.26/5.57	2.33-672/1.55-269	2.82/1.99	3.09/2.26	48.8/24.2	71.5/30.4
KOPER (KP) [b]	100	0	**17.0** [13.7-21.1]	14.1	2.06-459	3.41	5.28	91.1	130
Men/Women	49/51	0/0	**18.8** [13.5-26.1]/ **15.5** [11.5-20.8]	16.2/12.4	2.87-459/2.06-257	3.33/4.07	5.24/5.43	108/60.5	129/138
JESENICE (KR) [a,c]	81	0	**6.62** [5.19-8.44]	5.16	0.71-116	1.55	2.13	27.7	90.1
Men/Women	41/40	0/0	**7.95** [5.28-12.0]/ **5.48** [4.23-7.11]	5.07/5.29	0.97-116/0.71-51.4	1.55/1.50	2.32/1.92	90.1/14.1	95.1/23.3
MARIBOR (MB) [c]	98	0	**4.91** [4.20-5.74]	3.99	1.26-68.2	1.68	2.29	16.5	25.1
Men/Women	42/56	0/0	**4.76** [3.80-5.97]/ **5.03** [4.04-6.26]	3.85/4.10	1.26-26.0/1.26-68.2	1.68/1.80	2.47/2.29	13.6/18.4	15.7/27.8
POMURJE (MS) [a,c]	89	0	**5.64** [4.81-6.62]	5.43	1.34-45.3	1.83	2.13	17.7	23.0
Men/Women	40/49	0/0	**6.98** [5.44-8.96]/ **4.74** [3.87-5.81]	6.14/4.20	1.63-45.3/1.34-29.3	2.28/1.74	3.06/1.95	22.5/11.9	29.4/17.7
MEŽICA VALLEY (RA) [a,c]	78	0	**7.36** [5.78-9.36]	6.14	1.58-147	2.06	2.36	37.6	61.3
Men/Women	48/30	0/0	**7.82** [5.64-10.9]/ **6.67** [4.64-9.58]	6.00/6.34	1.58-147/1.90-54.9	2.06/2.09	2.37/2.32	58.6/35.2	83.7/45.1
SAVINJSKO-POSAVSKA (SP) [a,c]	81	0	**5.82** [4.76-7.13]	5.56	0.97-104	1.66	2.14	18.6	28.4
Men/Women	41/40	0/0	**7.41** [5.56-9.87]/ **4.55** [3.46-5.99]	7.59/3.75	1.42-104/0.97-62.8	2.401.39	2.44/1.77	21.7/14.0	36.4/23.5
ZASAVJE (ZA) [a,c]	103	0	**6.42** [5.52-7.46]	6.23	1.47-76.5	2.17	2.51	18.6	22.6
Men/Women	52/51	0/0	**6.67** [5.40-8.22]/ **6.17** [4.94-7.71]	5.96/6.43	1.49-76.5/1.47-33.7	2.52/1.79	3.05/2.19	19.8/17.7	37.5/21.6

S21. Levels of **selenium in urine** [µg/L] of Slovenian study population of men and lactating women living in 9 study areas. Different letters indicate that the study groups (according to the study area) differed statistically significantly.

Population group	N	N < LOD	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [a]	79	0	**14.0** [11.8-16.7]	13.9	2.20-121	3.38	5.49	43.4	48.4
Men/Women	37/42	0/0	**18.1**[14.1-23.0]/ **11.2** [8.81-14.3]	17.1/11.2	5.29-51.6/2.20-51.6	6.38/2.92	7.06/3.99	47.9/26.0	51.3/38.7
POSOČJE and IDRIJA (GO) [a]	98	0	**13.8** [11.7-16.3]	15.7	2.22-79.9	2.98	3.93	37.9	44.1
Men/Women	49/49	0/0	**20.6** [17.1-24.8]/ **9.26** [7.37-11.6]	20.8/10.1	4.87-79.9/2.22-44.1	5.30/2.43	7.24/2.98	42.9/28.0	56.9/37.9
KOPER (KP) [a]	101	0	**16.4** [13.8-19.6]	18.0	1.55-108	2.77	5.61	45.8	55.5
Men/Women	50/51	0/0	**26.8** [22.5-32.0]/ **10.2** [8.01-12.9]	28.8/11.7	7.11-108/1.55-51.9	8.63/2.06	9.78/2.77	56.2/28.6	64.4/33.6
JESENICE (KR) [a]	82	0	**12.3** [10.2-14.7]	12.4	2.19-83.0	3.07	3.85	34.8	50.2
Men/Women	42/40	0/0	**17.7** [13.9-22.6]/ **8.30** [6.63-10.4]	18.1/9.46	3.62-83.0/2.19-27.9	4.61/2.50	6.05/3.05	50.2/20.8	56.4/23.8
MARIBOR (MB) [a]	99	0	**12.2** [10.6-14.1]	12.8	1.47-45.1	2.80	4.04	29.6	34.3
Men/Women	42/57	0/0	**16.2** [14.1-18.6]/ **9.91** [7.96-12.3]	16.8/11.9	6.14-34.5/1.47-45.1	8.71/2.02	9.56/3.04	29.6/28.3	31.7/34.3
POMURJE (MS) [a]	89	0	**11.5** [9.43-14.1]	14.9	1.29-102	2.02	2.65	34.5	41.9
Men/Women	40/49	0/0	**19.6** [15.3-25.0]/ **7.51** [5.81-9.72]	21.3/7.44	2.81-102/1.29-34.5	3.16/1.55	7.51/2.02	43.9/21.2	51.2/24.2
MEŽICA VALLEY (RA) [a]	79	0	**13.1** [10.8-16.1]	13.4	0.50-75.8	2.32	4.26	34.7	46.4
Men/Women	49/30	0/0	**18.9** [15.7-22.7]/ **7.29** [5.15-10.3]	20.1/8.30	4.26-75.8/0.50-33.5	6.59/1.75	8.01/2.11	46.1/21.4	46.5/32.1
SAVINJSKO-POSAVSKA (SP) [a]	81	0	**14.3** [12.4-16.6]	14.4	2.62-50.5	5.00	5.56	33.4	41.8
Men/Women	41/40	0/0	**19.0** [15.8-22.8]/ **10.7** [8.79-13.1]	18.7/11.4	5.00-50.5/2.62-44.2	5.57/3.07	9.09/5.05	41.5/24.4	42.7/26.6
ZASAVJE (ZA) [a]	103	0	**13.8** [12.1-15.9]	13.7	2.88-106	5.07	5.76	32.4	42.2
Men/Women	52/51	0/0	**19.0** [15.6-23.3]/ **10.0** [8.61-11.6]	19.5/10.1	3.35-106/2.88-27.0	6.05/4.66	7.13/5.23	42.2/20.1	106/27.0

S22. Levels of **selenium in urine** [µg/g creatinine] of Slovenian study population of men and lactating women living in 9 study areas. Different letters indicate that the study groups (according to the study area) differed statistically significantly.

Population group	N	N < LOD	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [a,b]	79	0	**14.6** [13.2-16.2]	15.0	1.57-134	8.13	10.1	20.6	22.6
Men/Women	37/42	0/0	**13.8** [11.7-16.1]/ **15.4** [13.6-17.5]	16.0/14.3	1.57-22.6/9.47-134	6.69/10.2	8.13/11.4	21.2/20.4	22.4/23.6
POSOČJE and IDRIJA (GO) [a,b]	98	0	**14.8** [13.9-15.7]	14.8	5.87-31.4	8.32	10.1	22.5	25.9
Men/Women	49/49	0/0	**15.3** [14.0-16.6]/ **14.4** [13.1-15.7]	14.9/14.3	5.87-31.4/6.15-28.9	10.2/8.26	11.1/8.84	23.9/22.3	24.5/26.2
KOPER (KP) [a]	101	0	**16.4** [15.4-17.4]	17.0	3.99-46.7	10.5	10.9	22.3	25.0
Men/Women	50/51	0/0	**17.7** [16.7-18.9]/ **15.1** [13.6-16.8]	18.1/15.3	10.7-30.3/3.99-46.7	12.9/9.38	13.3/10.5	22.4/22.1	24.8/25.4
JESENICE (KR) [a,b]	82	0	**14.3** [13.0-15.7]	14.8	2.13-54.3	8.07	9.42	20.4	24.7
Men/Women	42/40	0/0	**13.8** [11.8-16.1]/ **14.8** [13.3-16.6]	14.2/15.6	2.13-54.3/5.47-40.7	5.24/8.48	9.89/9.40	22.4/20.2	23.2/24.7
MARIBOR (MB) [c]	99	0	**11.8** [11.1-12.6]	11.6	6.70-35.4	7.39	7.97	18.7	22.1
Men/Women	42/57	0/0	**11.3** [10.4-12.3]/ **12.2** [11.1-13.4]	10.7/12.2	7.12-22.9/6.70-35.4	7.86/7.16	8.24/7.95	16.1/21.4	17.6/24.0
POMURJE (MS) [b,c]	89	0	**13.1** [12.1-14.2]	13.1	2.85-48.4	7.42	8.98	20.6	22.3
Men/Women	40/49	0/0	**13.3** [11.7-15.0]/ **13.0** [11.7-14.5]	12.4/13.5	4.32-48.4/2.85-26.2	8.55/7.40	9.24/8.69	20.6/20.3	22.6/22.3
MEŽICA VALLEY (RA) [a,b]	79	0	**14.5** [13.3-15.7]	14.6	3.16-43.4	7.71	10.3	22.1	26.2
Men/Women	49/30	0/0	**15.3** [14.1-16.7]/ **13.1** [11.1-15.6]	14.6/14.3	7.96-43.4/3.16-26.2	10.4/5.08	11.3/7.28	22.5/21.0	27.4/22.3
SAVINJSKO-POSAVSKA (SP) [b,c]	81	0	**13.9** [12.6-15.4]	13.3	1.00-49.9	9.12	9.85	22.4	23.6
Men/Women	41/40	0/0	**15.5** [14.1-17.1]/ **12.5** [10.5-14.8]	15.6/12.5	8.68-30.4/1.00-49.9	9.73/8.31	10.6/9.49	23.3/17.5	23.6/29.4
ZASAVJE (ZA) [a,b]	103	0	**14.3** [13.4-15.1]	13.6	7.97-34.2	8.78	10.0	21.0	24.5
Men/Women	52/51	0/0	**14.6** [13.3-16.0]/ **13.9** [12.9-15.0]	13.5/13.7	7.97-34.2/8.30-25.6	8.36/8.78	10.2/9.72	23.1/20.3	31.8/21.4

S23. Levels of **selenium in urine, SG normalized** [µg/L SG] of Slovenian study population of males and lactating females living in 9 study areas. Different letters indicate that the study groups (according to the study area) differed statistically significantly.

Population group	N	N < LOD	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [a,b]	77	0	**15.5** [14.1-17.0]	14.6	7.16-40.7	7.88	9.50	26.6	34.8
Males/Females	36/41	0/0	**20.3** [18.0-23.0]/ **12.2** [11.2-13.4]	20.6/12.4	10.2-40.7/7.16-23.9	10.6/7.78	12.6/8.35	34.8/16.7	36.4/19.4
POSOČJE and IDRIJA (GO) [a,b]	96	0	**15.5** [13.9-17.3]	15.5	4.82-138	6.14	7.76	28.3	36.5
Males/Females	48/48	0/0	**20.8** [18.4-22.9]/ **11.7** [10.0-13.7]	19.8/11.4	9.25-52.3/4.82-138	11.6/5.54	12.6/6.15	33.920.5	37.3/22.0
KOPER (KP) [b]	100	0	**18.4** [16.6-20.3]	18.8	6.00-63.9	8.01	9.15	33.8	38.6
Males/Females	49/51	0/0	**27.6** [25.5-29.9]/ **12.4** [11.4-13.6]	27.4/12.3	13.7-63.9/6.00-25.0	16.6/7.69	20.1/8.04	39.1/17.8	42.7/20.8
JESENICE (KR) [a,b]	81	0	**15.3** [13.5-17.3]	15.3	3.13-56.3	6.09	7.93	33.4	35.8
Males/Females	41/40	0/0	**19.0** [15.7-23.0]/ **12.3** [10.7-14.1]	20.1/12.9	3.13-56.3/3.38-45.9	6.29/5.24	8.30/7.31	35.7/18.2	52.0/20.5
MARIBOR (MB) [a]	98	0	**12.9** [11.8-14.0]	12.4	4.32-33.9	6.03	7.89	23.4	25.4
Males/Females	42/56	0/0	**16.3** [14.6-18.2]/ **10.8** [9.71-12.0]	16.2/10.3	8.11-33.9/4.32-26.6	9.15/5.48	10.6/6.35	25.1/19.0	29.0/22.1
POMURJE (MS) [a]	89	0	**13.8** [12.3-15.4]	13.0	4.20-64.7	6.01	6.76	28.7	35.8
Males/Females	40/49	0/0	**20.0** [17.3-23.2]/ **10.2** [9.17-11.3]	20.8/11.2	7.01-64.7/4.20-22.5	9.54/5.30	11.3/6.01	36.3/15.3	39.6/16.0
MEŽICA VALLEY (RA) [a,b]	78	0	**16.2** [14.4-18.4]	16.7	2.17-42.3	6.41	7.84	30.8	32.6
Males/Females	48/30	0/0	**21.8** [19.8-25.0]/ **10.2** [8.47-12.2]	22.2/10.7	10.8-42.3/2.17-18.0	12.7/3.47	13.5/5.74	31.7/16.5	33.8/16.8
SAVINJSKO-POSAVSKA (SP) [a,b]	81	0	**14.9** [13.6-16.5]	14.8	6.54-37.2	7.70	8.38	25.7	31.6
Males/Females	41/40	0/0	**20.9** [19.2-22.8]/ **10.6** [9.67-11.6]	19.9/10.6	12.6-37.2/6.54-23.0	13.9/6.81	14.6/7.42	31.6/15.6	34.5/17.4
ZASAVJE (ZA) [a,b]	103	0	**14.9** [13.5-16.5]	14.2	4.75-57.5	7.16	8.33	29.0	37.2
Males/Females	52/51	0/0	**20.4** [18.1-23.0]/ **10.8** [9.86-11.9]	20.4/11.2	5.75-57.5/4.75-31.9	10.3/6.31	13.2/7.19	37.2/15.3	44.3/19.1

S24. Levels of **copper in urine** [µg/L] of Slovenian study population of men and lactating women living in 9 study areas. Different letters indicate that the study groups (according to the study area) differed statistically significantly. LOD=3.0 µg/L.

Population group	N	N < LOD	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [b]	79	28	**4.23** [3.46-5.18]	4.47	<LOD-38.6	<LOD	<LOD	13.8	18.0
Men/Women	37/42	13/15	**4.30** [3.15-5.86]/ **4.17** [3.17-5.50]	4.47/4.47	<LOD-38.6/<LOD-20.4	<LOD/<LOD	<LOD/<LOD	13.8/12.5	18.0/16.0
POSOČJE and IDRIJA (GO) [a,b]	98	24	**6.20** [5.11-7.52]	7.40	<LOD-34.1	<LOD	<LOD	20.5	27.4
Men/Women	49/49	5/19	**8.16** [6.56-10.2]/ **4.70** [3.46-6.40]	9.08/4.51	<LOD-27.4/<LOD-34.1	<LOD/<LOD	<LOD/<LOD	20.5/24.1	23.0/30.5
KOPER (KP) [a]	101	20	**7.00** [5.74-8.55]	8.45	<LOD-67.3	<LOD	<LOD	20.3	32.8
Men/Women	50/51	3/17	**9.18** [7.24-11.7]/ **5.37** [3.94-7.31]	9.42/6.46	<LOD-64.5/<LOD-67.3	<LOD/<LOD	3.52/<LOD	24.6/20.3	57.5/32.8
JESENICE (KR) [c]	82	34	**3.86** [3.16-4.72]	4.25	<LOD-28.7	<LOD	<LOD	13.3	17.2
Men/Women	42/40	15/19	**4.74** [3.46-6.51]/ **3.11** [2.44-3.96]	4.84/3.33	<LOD-28.7/<LOD-11.7	<LOD/<LOD	<LOD/<LOD	17.2/8.29	26.9/9.72
MARIBOR (MB) [b,c]	99	32	**5.12** [4.22-6.22]	5.97	<LOD-37.2	<LOD	<LOD	16.8	21.9
Men/Women	42/57	11/21	**4.63** [3.62-5.93]/ **5.51** [4.13-7.35]	5.19/6.76	<LOD-18.1/<LOD-37.2	<LOD/<LOD	<LOD/<LOD	12.3/21.2	14.1/24.0
POMURJE (MS) [a]	89	19	**6.69** [5.53-8.09]	7.82	<LOD-34.6	<LOD	<LOD	19.4	20.4
Men/Women	40/49	3/16	**8.98** [7.20-11.2]/ **5.26** [3.96-6.98]	10.7/6.85	<LOD-21.4/<LOD-34.6	<LOD/<LOD	4.57/<LOD	18.9/20.2	20.2/24.6
MEŽICA VALLEY (RA) [a,b,c]	79	15	**5.62** [4.69-6.74]	6.67	<LOD-33.8	<LOD	<LOD	12.6	22.5
Men/Women	49/30	6/9	**6.39** [5.18-7.87]/ **4.57** [3.26-6.40]	7.02/4.80	<LOD-33.8/<LOD-31.9	<LOD/<LOD	<LOD/<LOD	17.0/12.1	18.8/27.0
SAVINJSKO-POSAVSKA (SP) [a,b]	81	10	**7.70** [6.44-9.21]	8.64	<LOD-61.3	<LOD	<LOD	19.2	22.5
Men/Women	41/40	5/5	**8.40** [6.57-10.7]/ **7.05** [5.39-9.21]	9.12/7.11	<LOD-23.6/<LOD-61.3	<LOD/<LOD	<LOD/<LOD	18.7/20.5	22.3/26.0
ZASAVJE (ZA) [c]	103	41	**3.72** [3.16-4.39]	4.36	<LOD-39.3	<LOD	<LOD	11.2	15.6
Men/Women	52/51	20/21	**4.14** [3.21-5.35]/ **3.34** [2.70-4.13]	5.23/3.94	<LOD-39.3/<LOD-16.4	<LOD/<LOD	<LOD/<LOD	11.6/9.55	17.0/12.5

S25. Levels of **copper in urine** [µg/g creatinine] of Slovenian study population of men and lactating women living in 9 study areas. Different letters indicate that the study groups (according to the study area) differed statistically significantly.

Population group	N	N < LOD	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [c,d]	79	28	**4.42** [3.74-5.22]	4.81	<LOD-53.2	<LOD	<LOD	9.47	12.1
Men/Women	37/42	13/15	**3.28** [2.53-4.24]/ **5.74** [4.73-6.97]	3.81/5.69	<LOD-8.63/<LOD -53.2	<LOD/<LOD	<LOD/<LOD	7.75/11.1	7.98/12.5
POSOČJE and IDRIJA (GO) [b,e]	98	24	**6.64** [5.83-7.56]	5.86	1.29-35.0	<LOD	<LOD	16.6	19.1
Men/Women	49/49	5/19	**6.04** [5.07-7.19]/ **7.30** [6.00-8.87]	4.93/6.91	<LOD-21.5/<LOD-35.0	<LOD/<LOD	<LOD/<LOD	16.0/17.6	19.0/19.1
KOPER (KP) [a,b]	101	20	**6.97** [6.10-7.97]	6.48	1.58-46.5	<LOD	<LOD	16.2	24.9
Men/Women	50/51	3/17	**6.07** [5.21-7.07]/ **7.99** [6.42-9.93]	5.56/8.46	<LOD-41.1/<LOD-46.5	<LOD/<LOD	3.34/<LOD	11.1/17.2	22.9/28.7
JESENICE (KR) [c,d]	82	34	**4.50** [3.81-5.33]	5.76	0.61-19.1	<LOD	<LOD	9.73	10.7
Men/Women	42/40	15/19	**3.68** [2.83-4.79]/ **5.56** [4.57-6.76]	5.39/5.99	<LOD-10.3/<LOD-19.1	<LOD/<LOD	<LOD/<LOD	8.05/11.2	9.73/12.3
MARIBOR (MB) [c,d,e]	99	32	**4.95** [4.11-5.96]	4.35	0.89-86.2	<LOD	<LOD	19.8	33.0
Men/Women	42/57	11/21	**3.23** [2.68-3.88]/ **6.79** [5.19-8.88]	3.58/5.97	<LOD-9.01/<LOD-86.2	<LOD/<LOD	<LOD/<LOD	6.48/25.5	7.44/44.2
POMURJE (MS) [a,b]	89	19	**7.60** [6.61-8.74]	7.37	1.07-54.8	<LOD	<LOD	18.0	23.8
Men/Women	40/49	3/16	**6.10** [5.06-7.35]/ **9.11** [7.51-11.0]	6.80/8.53	<LOD-30.2/<LOD-54.8	<LOD/<LOD	3.08/<LOD	9.45/22.9	13.6/24.6
MEŽICA VALLEY (RA) [a,b,c]	79	15	**6.19** [5.45-7.04]	6.19	1.49-47.0	<LOD	<LOD	11.7	15.2
Men/Women	49/30	6/9	**5.20** [4.61-5.87]/ **8.24** [6.43-10.6]	5.29/8.03	<LOD-13.9/<LOD-47.0	<LOD/<LOD	<LOD/<LOD	7.87/15.3	9.87/42.6
SAVINJSKO-POSAVSKA (SP) [a,b]	81	10	**7.50** [6.43-8.75]	8.01	0.72-34.9	<LOD	<LOD	15.9	34.9
Men/Women	41/40	5/5	**6.89** [5.72-8.30]/ **8.18** [6.35-10.5]	6.87/9.74	<LOD-27.3/<LOD-34.9	<LOD/<LOD	<LOD/<LOD	13.5/17.9	19.5/24.5
ZASAVJE (ZA) [d]	103	41	**3.83** [3.27-4.49]	4.01	0.35-39.9	<LOD	<LOD	9.03	9.41
Men/Women	52/51	20/21	**3.17** [2.50-4.02]/ **4.65** [3.81-5.69]	3.38/5.10	<LOD-39.9/<LOD-28.7	<LOD/<LOD	<LOD/<LOD	8.29/9.08	9.34/11.3

S26. Levels of **copper in urine, SG normalized** [µg/L SG] of Slovenian study population of men and lactating women living in 9 study areas. Different letters indicate that the study groups (according to the study area) differed statistically significantly.

Population group	N	N < LOD	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [a]	77	28	**4.60** [3.95-5.36]	4.88	<LOD-17.0	<LOD	<LOD	10.1	11.6
Men/Women	36/41	13/15	**4.80** [3.68-6.26]/ **4.43** [3.70-5.29]	5.24/4.88	<LOD-17.0/<LOD-10.4	<LOD/<LOD	<LOD/<LOD	11.6/8.86	14.1/9.05
POSOČJE and IDRIJA (GO) [b,c]	96	24	**6.89** [5.89-8.07]	6.59	<LOD-80.2	<LOD	<LOD	19.3	22.6
Men/Women	48/48	5/19	**8.08** [6.70-9.74]/ **5.88** [4.57-7.56]	7.00/4.96	<LOD-22.7/<LOD-80.2	<LOD/<LOD	<LOD/<LOD	19.3/19.5	21.8/26.4
KOPER (KP) [b]	100	20	**7.81** [6.75-9.02]	8.26	<LOD-51.4	<LOD	<LOD	19.6	25.2
Men/Women	49/51	3/17	**9.38** [7.90-11.1]/ **6.55** [5.22-8.21]	8.89/6.66	<LOD-42.4/<LOD-51.4	<LOD/<LOD	4.07/<LOD	22.0/16.1	37.7/23.7
JESENICE (KR) [a,c]	81	34	**4.85** [4.05-5.80]	5.55	<LOD-20.7	<LOD	<LOD	13.8	15.2
Men/Women	41/40	15/19	**5.11**[3.75-6.95]/ **4.59** [3.79-5.57]	6.20/5.06	<LOD-20.7/<LOD-15.2	<LOD/<LOD	<LOD/<LOD	15.1/8.96	17.0/10.1
MARIBOR (MB) [a,c]	98	32	**5.34** [4.49-6.34]	5.31	<LOD-43.9	<LOD	<LOD	16.1	23.8
Men/Women	42/56	11/21	**4.65** [3.76-5.76]/ **5.91** [4.57-7.65]	5.31/5.42	<LOD-13.8/<LOD-43.9	<LOD/<LOD	<LOD/<LOD	10.4/19.4	11.0/25.4
POMURJE (MS) [b]	89	19	**7.98** [6.95-9.15]	8.15	<LOD-40.3	<LOD	<LOD	17.6	20.4
Men/Women	40/49	3/16	**9.19** [7.59-11.1]/ **7.10** [5.84-8.64]	10.5/6.64	<LOD-27.4/<LOD-40.3	<LOD/<LOD	5.29/<LOD	16.4/17.8	19.2/22.4
MEŽICA VALLEY (RA) [b,c]	78	15	**6.99** [6.09-8.03]	7.33	<LOD-43.9	<LOD	<LOD	14.0	18.9
Men/Women	48/30	6/9	**7.41** [6.38-8.61]/ **6.38** [4.84-8.41]	7.85/6.28	<LOD-25.8/<LOD-43.9	<LOD/<LOD	<LOD/<LOD	13.5/15.8	16.7/41.5
SAVINJSKO-POSAVSKA (SP) [b]	81	10	**8.04** [6.96-9.29]	8.46	<LOD-31.9	<LOD	<LOD	16.4	19.5
Men/Women	41/40	5/5	**9.26** [7.76-11.1]/ **6.96** [5.54-8.73]	8.87/7.38	<LOD-29.2/<LOD-31.9	<LOD/<LOD	<LOD/<LOD	18.4/14.6	19.5/23.5
ZASAVJE (ZA) [a]	103	41	**4.01** [3.46-4.65]	4.65	<LOD-43.9	<LOD	<LOD	9.34	10.7
Men/Women	52/41	20/21	**4.44** [3.52-5.58]/ **3.62** [3.01-4.36]	5.07/4.05	<LOD-43.9/<LOD-15.1	<LOD/<LOD	<LOD/<LOD	10.4/8.86	13.6/9.55

S27. Levels of **zinc in urine** [µg/L] of Slovenian study population of men and lactating women living in 9 study areas. There were no statistical differences between the study areas (indicated by [a]).

Population group	N	N < LOD	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [a]	79	0	**267** [226-314]	271	59.3-1829	76.1	98.8	668	856
Men/Women	37/42	0/0	**242** [184-319]/ **290** [238-354]	226/286	59.3-1540/88.3-1829	68.6/99.9	76.1/125	761/581	912/668
POSOČJE and IDRIJA (GO) [a]	98	0	**247** [211-290]	245	32.4-1921	56.6	95.3	677	827
Men/Women	49/49	0/0	**251** [203-311]/ **244** [324-441]	244/245	39.2-1337/32.4-1921	51.5/71.9	113/86.5	572/779	708/827
KOPER (KP) [a]	102	0	**321** [271-381]	335	13.0-2213	80.4	106	916	1222
Men/Women	50/52	0/0	**365** [292-457]/ **284** [219-369]	356/323	78.2-2213/13.0-1885	108/52.0	126/96.7	1037/788	1310/1077
JESENICE (KR) [a]	81	0	**230** [189-281]	258	25.8-1855	54.2	80.2	714	968
Men/Women	42/39	0/0	**257** [188-352]/ **204** [160-260]	306/236	25.8-1855/52.2-940	79.5/53.3	84.8/63.0	968/536	1280/635
MARIBOR (MB) [a]	98	0	**297** [259-340]	316	65.1-1045	83.5	103	644	789
Men/Women	42/56	0/0	**318** [264-382]/ **282** [231-344]	329/283	68.7-805/65.1-1045	103/80.1	127/94.2	603/668	649/912
POMURJE (MS) [a]	90	0	**285** [240-338]	282	34.4-1515	55.7	96.0	743	962
Men/Women	40/50	0/0	**311** [249-389]/ **265** [206-342]	302/252	49.7-1479/34.4-1515	89.8/53.2	120/72.5	743/824	892/962
MEŽICA VALLEY (RA) [a]	79	0	**259** [211-317]	268	20.7-1549	49.3	80.5	659	1108
Men/Women	49/30	0/0	**339** [264-434]/ **116** [123-225]	427/186	22.2-1549/20.7-520	54.4/20.7	114/57.0	1080/460	1191/483
SAVINJSKO-POSAVSKA (SP) [a]	81	0	**309** [266-357]	326	49.5-1739	110	120	635	791
Men/Women	41/40	0/0	**292** [234-364]/ **327** [267-400]	310/340	49.5-1739/70.9-1506	110/105	116/128	564/649	806/734
ZASAVJE (ZA) [a]	103	0	**274** [235-320]	294	17.3-1550	73.5	98.8	668	824
Men/Women	52/51	0/0	**306** [241-388]/ **246** [203-298]	353/244	17.3-1550/40.6-1003	60.3/84.5	100/98.8	748/639	1117/799
Lower Mežica valley [a]	57	0	**247** [197-311]	240	20.7-1196	54.4	96.1	617	870
Men/Women	31/26	0/0	**355** [270-465]/ **161** [117-222]	482/164	54.4-1196/20.7-520	96.9/25.5	134/64.8	790/446	1108/474
Upper Mežica valley [a]	22	0	**290** [183-460]	308	22.2-1549	49.3	50.2	1080	1191
Men/Women	18/4	0/0	**313** [185-532]/ **204** [41.5-1007]	308/277	22.2-1549/49.3-483	22.2/49.3	50.2/49.3	1191/483	1549/483

S28. Levels of **zinc in urine** [µg/g creatinine] of Slovenian study population of men and lactating women living in 9 study areas. There were no statistical differencse between the study areas (indicated by [a]).

Population group	N	N < LOD	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [a]	79	0	**278** [239-325]	293	24.2-2133	76.4	140	554	911
Men/Women	37/42	0/0	**185** [150-227]/ **399** [338-472]	205/426	24.2-570/116-2133	48.7/162	76.4/196	349/736	467/972
POSOČJE and IDRIJA (GO) [a]	98	0	**265** [233-302]	279	38.7-1159	90.7	109	610	697
Men/Women	49/49	0/0	**186** [159-218]/ **378** [324-441]	185/392	38.7-811/90.7-1159	87.3/127	91.5/140	320/672	443/824
KOPER (KP) [a]	102	0	**325** [285-370]	311	28.7-1423	127	147	754	880
Men/Women	50/52	0/0	**242** [209-279]/ **431** [358-520]	222/444	105-1283/28.7-1423	127/127	138/231	504/856	528/1184
JESENICE (KR) [a]	81	0	**264** [223-312]	316	36.8-1108	59.7	93.5	533	667
Men/Women	42/39	0/0	**200** [157-255]/ **357** [293-434]	236/421	36.8-618/94.3-1108	40.2/96.6	59.7/147	439/723	456/748
MARIBOR (MB) [a]	98	0	**283** [250-320]	309	40.8-1264	103	120	521	752
Men/Women	42/56	0/0	**221** [184-265]/ **340** [292-397]	232/337	40.8-753/70.6-1264	103/123	112/171	446/695	500/1089
POMURJE (MS) [a]	90	0	**328** [283-380]	329	24.3-3371	122	155	802	1166
Men/Women	40/50	0/0	**211** [174-256]/ **466** [396-550]	201/453	24.3-841/160-3371	86.1/215	120/239	400/1097	446/1213
MEŽICA VALLEY (RA) [a]	79	0	**285** [246-330]	290	22.1-933	110	131	651	712
Men/Women	49/30	0/0	**276** [228-334]/ **300** [237-379]	297/286	22.1-651/102-933	112/110	123/132	540/722	587/801
SAVINJSKO-POSAVSKA (SP) [a]	81	0	**300** [260-347]	316	23.1-1562	128	157	578	704
Men/Women	41/40	0/0	**239** [201-285]/ **379** [306-469]	264/401	34.4-581/23.1-1562	111/155	128/197	438/726	467/868
ZASAVJE (ZA) [a]	103	0	**283** [248-322]	303	19.7-901	109	130	614	644
Men/Women	52/51	0/0	**234** [193-284]/ **343** [292-402]	265/411	19.7-901/68.5-880	73.3/109	124/147	510/633	616/701
Lower Mežica valley [a]	57	0	**304** [261-352]	297	102-933	117	132	690	732
Men/Women	31/26	0/0	**292** [243-351]/ **318** [245-411]	297/295	112-651/102-933	117/131	156/132	519/732	619/801
Upper Mežica valley [a]	22	0	**242** [167-349]	278	22.1-587	31.7	108	486	575
Men/Women	18/4	0/0	**250** [160-391]/ **206** [95.8-445]	308/232	22.1-587/110-321	22.1/110	31.7/110	575/321	587/321

S29. Levels of **zinc in urine, SG normalized** [µg/g SG] of Slovenian study population of men and lactating women living in 9 study areas. There were no statistical differences between the study areas (indicated by [a]).

Population group	N	N < LOD	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [a]	77	0	**292** [257-333]	304	40.2-923	117	148	603	806
Men/Women	36/41	0/0	**274** [216-347]/ **310** [270-355]	283/308	40.2-923/114-820	54.3/147	136/180	799/513	825/686
POSOČJE and IDRIJA (GO) [a]	96	0	**275** [241-313]	285	59.0-3457	84.2	122	612	790
Men/Women	48/48	0/0	**246** [206-294]/ **306** [252-372]	257/299	59.0-1058/78.0-3457	93.1/84.2	110/144	494/663	762/909
KOPER (KP) [a]	101	0	**361** [322-406]	360	56.3-1322	154	178	778	913
Men/Women	49/52	0/0	**371** [314-438]/ **353** [298-418]	355/366	134-1322/56.3-1130	165/147	178/177	801/729	976/907
JESENICE (KR) [a]	80	0	**284** [237-339]	308	15.8-1175	56.8	85.9	653	861
Men/Women	41/39	0/0	**273** [203-367]/ **296** [240-365]	288/352	15.8-1175/52.2-940	54.0/88.7	77.1/114	715/580	869/737
MARIBOR (MB) [a]	97	0	**311** [278-348]	331	62.1-933	125	158	618	733
Men/Women	42/55	0/0	**319** [268-380]/ **305** [262-355]	348/326	62.1-933/69.4-847	147/120	160/157	576/686	625/737
POMURJE (MS) [a]	90	0	**341** [301-386]	361	39.4-1790	125	180	719	969
Men/Women	40/50	0/0	**318** [263-385]/ **361** [306-426]	353/365	39.4-1041/112-1790	133/125	180/179	628/746	853/969
MEŽICA VALLEY (RA) [a]	78	0	**321** [273-378]	359	35.2-1400	89.9	128	702	938
Men/Women	48/30	0/0	**394** [320-484]/ **232** [183-294]	443/246	35.2-1400/45.8-730	128/89.9	192/108	901/525	978/730
SAVINJSKO-POSAVSKA (SP) [a]	81	0	**322** [289-359]	306	77.1-1101	141	188	550	676
Men/Women	41/40	0/0	**322** [274-378]/ **323** [277-375]	310/306	77.1-1101/120-881	148/137	188/188	541/578	612/783
ZASAVJE (ZA) [a]	103	0	**296** [259-338]	311	20.6-1010	91.5	120.3	647	814
Men/Women	52/51	0/0	**327** [269-398]/ **267** [222-320]	356/288	20.6-982/55.8-1010	113/75.4	186/99.4	796/593	887/647
Lower Mežica valley [a]	56	0	**326** [276-384]	346	89.9-1400	110	154	668	901
Men/Women	30/26	0/0	**405** [329-499]/ **253** [200-320]	426/266	128-1400/89.9-730	159/105	195/110	801/565	938/639
Upper Mežica valley [a]	22	0	**310** [202-478]	437	35.2-1015	39.8	45.8	838	978
Men/Women	18/4	0/0	**376** [236-597]/ **132** [41.4-420]	455/170	35.2-1015/45.8-232	35.2/45.8	39.8/45.8	978/232	1015/232

S30. Levels of **cadmium in breast milk** [µg/L] in lactating women from 12 study areas in Slovenia. Due to different LOD, results are presented separately for each of the sampling period. RV=reference value (1 µg/L) (Iyengar, 1998).

Population group	N	N < LOD	N > RV	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
Sampling period 2011-13	353	246	0	- [-]	**<0.10**	<0.05-0.34	<0.05	<0.10	0.14	0.17
CELJE (CE)	37	22	0	- [-]	**<0.10**	<0.05-0.34	<0.05	<0.10	0.18	0.26
POSOČJE and IDRIJA (GO)	28	19	0	- [-]	**<0.10**	<0.10-0.22	<0.10	<0.10	0.14	0.21
KOPER (KP)	49	44	0	- [-]	**<0.10**	<0.10-0.18	<0.10	<0.10	<0.10	0.11
JESENICE (KR)	26	19	0	- [-]	**<0.10**	<0.10-0.20	<0.10	<0.10	0.15	0.17
MARIBOR (MB)	55	48	0	- [-]	**<0.10**	<0.05-0.24	<0.10	<0.10	0.11	0.18
POMURJE (MS)	41	31	0	- [-]	**<0.10**	<0.10-0.33	<0.10	<0.10	0.14	0.18
MEŽICA VALLEY (RA)	29	25	0	- [-]	**<0.10**	<0.10-0.11	<0.10	<0.10	0.10	0.10
SAVINJSKO-POSAVSKA (SP)	38	22	0	- [-]	**<0.10**	<0.10-0.19	<0.10	<0.10	0.16	0.17
ZASAVJE (ZA)	50	16	0	- [-]	**<0.10**	<0.05-0.21	<0.05	<0.05	0.12	0.18
Sampling period 2008-10	117	22	0	0.09 [0.08-0.10]	**0.09**	<0.05-0.34	<0.05	<0.05	0.16	0.23
LJUBLJANA (LJ)	49	9	0	0.09 [0.08-0.11]	**0.09**	<0.05-0.34	<0.05	<0.05	0.16	0.27
BELA KRAJINA (BK)	43	5	0	0.09 [0.08-0.10]	**0.09**	<0.05-0.22	<0.05	<0.05	0.15	0.16
KOČEVJE IN CERKNICA (KO)	25	8	0	0.09 [0.07-0.11]	**0.09**	<0.05-0.32	<0.05	<0.05	0.20	0.24

Remark: Statistical comparison between all study areas was not performed due to different LOD levels between the pilot (2008-09) and the second phase (2011-13). Within each of the phases, no statistical difference between the areas was observed.

S31. Levels of **lead in breast milk** [µg/L] in lactating women from 12 study areas in Slovenia. Due to different LOD, results are presented separately for each of the sampling period. Different letters indicate that the study groups (according to the study area) differed statistically significantly. RV=reference value (5 µg/L) (WHO, 1989).

Population group	N	N < LOD	N > RV	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
Sampling period 2011-12	353	170	1	**0.23** [0.21-0.25]	0.21	<0.20-5.42	<0.20	<0.20	0.72	0.99
Study area										
CELJE (CE) [a]	37	13	0	**0.26** [0.20-0.34]	0.26	<0.20-1.39	<0.20	<0.20	0.79	1.33
POSOČJE and IDRIJA (GO) [b]	28	23	0	- [-]	<0.20	<0.20-1.23	<0.20	<0.20	0.28	0.69
KOPER (KP) [a]	49	14	0	**0.30** [0.24-0.39]	0.35	<0.20-1.07	<0.20	<0.20	0.97	1.02
JESENICE (KR) [a,b]	26	11	0	**0.25** [0.17-0.37]	0.25	<0.20-2.22	<0.20	<0.20	1.03	2.06
MARIBOR (MB) [a]	55	24	0	**0.25** [0.20-0.31]	0.24	<0.20-1.82	<0.20	<0.20	0.73	1.51
POMURJE (MS) [a,b]	41	18	0	**0.24** [0.18-0.32]	0.23	<0.20-2.97	<0.20	<0.20	0.86	1.64
MEŽICA VALLEY (RA) [a]	29	8	1	**0.32** [0.22-0.46]	0.29	<0.20-5.42	<0.20	<0.20	0.72	1.19
SAVINJSKO-POSAVSKA (SP) [a,b]	38	18	0	**0.22** [0.17-0.30]	0.20	<0.20-1.00	<0.20	<0.20	0.74	0.99
ZASAVJE (ZA) [a,b]	50	41	0	- [-]	<0.15	<0.30-0.66	<0.30	<0.30	0.41	0.55
Sampling period 2008-10	117	83	1	- [-]	<1.00	<0.20-10.6	<1.00	<1.00	2.08	2.71
LJUBLJANA (LJ)	49	35	1	- [-]	<1.00	<1.00-10.6	<1.00	<1.00	2.43	3.97
BELA KRAJINA (BK)	43	30	0	- [-]	<1.00	<1.00-2.71	<1.00	<1.00	1.66	1.89
KOČEVJE and CERKNICA (KO)	25	18	0	- [-]	<1.00	<0.20-2.29	<1.00	<1.00	1.83	1.84

S32. Levels of **mercury in breast milk** [µg/L] in lactating women from 12 study areas in Slovenia. Different letters indicate that the study groups (according to the study area or sampling period) differed statistically significantly. RV=reference value (3 µg/L, population based) (Iyengar, 1998).

Population group	N	N < LOD	N > RV	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [b]	37	4	0	**0.06** [0.03-0.12]	0.12	<0.001-1.16	<0.001	<0.001	0.34	0.52
POSOČJE and IDRIJA (GO) [a,c]	28	1	1	**0.21** [0.11-0.37]	0.24	<0.001-3.39	0.04	0.06	1.18	1.25
KOPER (KP) [a]	49	0	0	**0.21** [0.17-0.25]	0.19	0.03-0.83	0.07	0.09	0.50	0.64
JESENICE (KR) [a,c]	26	0	0	**0.17** [0.12-0.25]	0.19	0.03-0.96	0.04	0.04	0.55	0.78
MARIBOR (MB) [b,c]	55	4	0	**0.08** [0.05-0.13]	0.15	<0.001-0.72	<0.001	0.01	0.40	0.57
POMURJE (MS) [a,b]	41	1	0	**0.10** [0.07-0.16]	0.14	<0.001-1.01	0.01	0.02	0.46	0.69
MEŽICA VALLEY (RA) [a,b]	29	0	0	**0.13** [0.09-0.19]	0.13	0.01-1.05	0.03	0.04	0.51	0.68
SAVINJSKO-POSAVSKA (SP) [a,b]	38	0	0	**0.13** [0.09-0.17]	0.14	0.03-1.81	0.03	0.04	0.40	0.78
ZASAVJE (ZA) [a]	50	0	0	**0.22** [0.18-0.27]	0.24	0.05-1.41	0.07	0.09	0.53	0.61
LJUBLJANA (LJ) [a]	49	2	0	**0.22** [0.17-0.28]	0.23	<0.05-1.31	<0.05	0.06	0.52	0.57
BELA KRAJINA (BK) [a,c]	43	5	0	**0.15** [0.12-0.20]	0.16	<0.05-0.58	<0.05	<0.05	0.43	0.52
KOČEVJE and CERKNICA (KO) [a,c]	25	1	0	**0.19** [0.13-0.28]	0.21	<0.05-0.90	0.04	0.06	0.52	0.65
Sampling period 2008-2010 [a]	117	8	0	**0.19** [0.16-0.22]	0.20	<0.05-1.31	<0.05	0.05	0.52	0.57
Sampling period 2011-2014 [b]	353	10	1	**0.13** [0.11-0.15]	0.15	<0.001-3.39	0.02	0.04	0.48	0.68

S33. Levels of **arsenic in breast milk** [µg/L] in lactating women from 12 study areas in Slovenia. Different letters indicate that the study groups (according to the study area or sampling period) differed statistically significantly. RV=reference value (3 µg/L) (Iyengar, 1998).

Population group	N	N < LOD	N > RV	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [b]	37	2	0	**0.15** [0.11-0.20]	0.14	<0.05-1.52	<0.05	0.07	0.44	1.27
POSOČJE and IDRIJA (GO) [b]	28	5	0	**0.12** [0.08-0.18]	0.12	<0.05-0.78	<0.05	<0.05	0.48	0.60
KOPER (KP) [a]	49	0	3	**0.36** [0.27-0.47]	0.31	0.08-3.70	0.12	0.13	1.44	3.01
JESENICE (KR) [b]	26	0	0	**0.15** [0.12-0.19]	0.15	0.06-0.48	0.07	0.07	0.38	0.39
MARIBOR (MB) [b]	55	0	0	**0.16** [0.14-0.18]	0.15	0.07-0.68	0.07	0.08	0.31	0.57
POMURJE (MS) [b]	41	1	0	**0.14** [0.12-0.17]	0.13	<0.05-0.91	0.06	0.08	0.29	0.44
MEŽICA VALLEY (RA) [b]	29	0	0	**0.16** [0.12-0.21]	0.13	0.06-1.07	0.06	0.07	0.66	0.81
SAVINJSKO-POSAVSKA (SP) [b]	38	1	0	**0.16** [0.12-0.22]	0.12	<0.05-0.93	0.06	0.07	0.72	0.88
ZASAVJE (ZA) [a,b]	50	0	0	**0.23** [0.19-0.27]	0.21	0.10-1.02	0.11	0.12	0.54	0.65
LJUBLJANA (LJ) [b]	49	0	1	**0.20** [0.16-0.25]	0.16	0.06-3.68	0.07	0.09	0.46	0.79
BELA KRAJINA (BK) [b]	43	0	0	**0.15** [0.12-0.18]	0.13	0.05-0.98	0.07	0.07	0.35	0.39
KOČEVJE and CERKNICA (KO) [b]	25	0	0	**0.18** [0.14-0.24]	0.16	0.07-1.44	0.08	0.09	0.33	0.62
Sampling period 2008-2010 [a]	117	0	1	**0.18** [0.16-0.20]	0.15	0.05-3.68	0.07	0.09	0.43	0.72
Sampling period 2011-2014 [a]	353	9	3	**0.18** [0.16-0.19]	0.16	<0.05-3.70	0.07	0.08	0.52	0.84

S34. Levels of **selenium in breast milk** [µg/L] in lactating women from 12 study areas in Slovenia. Different letters indicate that the study groups (according to the study area or sampling period) differed statistically significantly. LRV=lower reference value (10 µg/L); URV=upper reference value (25 µg/L) (Iyengar, 1998).

Population group	N	N < LOD	N<LRV	N>URV	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [b,c]	37	0	12	1	**11.7** [10.6-12.8]	11.2	7.32-28.8	7.72	8.71	15.9	23.2
POSOČJE and IDRIJA (GO) [a,b]	28	0	3	0	**13.8** [12.3-15.6]	13.5	5.36-24.9	9.20	9.68	20.7	21.2
KOPER (KP) [b,c]	49	0	7	0	**12.0** [11.3-12.7]	11.9	7.39-18.6	7.80	8.45	15.6	16.1
JESENICE (KR) [a,b]	26	0	3	1	**13.8** [12.2-15.5]	13.2	8.75-38.1	8.98	9.03	19.2	24.0
MARIBOR (MB) [b,c]	55	0	10	0	**12.3** [11.6-13.0]	12.2	7.13-19.0	8.66	9.70	16.1	18.1
POMURJE (MS) [b,c]	41	0	11	1	**11.9** [10.8-13.1]	12.0	6.42-26.7	7.24	7.97	17.6	19.2
MEŽICA VALLEY (RA) [b,c]	29	0	5	0	**11.7** [10.9-12.6]	11.4	7.63-17.2	8.82	9.45	15.0	16.1
SAVINJSKO-POSAVSKA (SP) [b,c]	38	0	10	0	**11.7** [10.9-12.6]	12.5	7.78-16.0	7.84	8.58	15.4	15.9
ZASAVJE (ZA) [c]	50	0	19	0	**10.8** [10.2-11.5]	10.9	7.20-24.6	7.58	8.07	13.5	14.1
LJUBLJANA (LJ) [a]	49	1	5	3	**15.2** [14.0-16.6]	15.1	6.23-26.5	9.43	10.1	22.4	24.1
BELA KRAJINA (BK) [a,b]	43	2	5	1	**13.5** [12.5-14.6]	13.2	6.98-29.3	9.81	9.97	17.5	18.4
KOČEVJE and CERKNICA (KO) [a]	25	3	1	1	**15.5** [14.1-17.2]	16.0	9.64-29.5	11.2	12.1	19.1	23.4
Sampling period 2008-2010 [a]	117	6	11	5	**14.6** [13.9-15.4]	14.7	6.23-29.5	9.64	10.1	20.3	23.4
Sampling period 2011-2014 [b]	353	0	80	3	**12.0** [11.7-12.4]	12.0	5.36-38.1	7.93	8.83	16.0	18.1

S35. Levels of **copper in breast milk** [µg/L] in lactating women from 12 study areas in Slovenia. Different letters indicate that the study groups (according to the study area or sampling period) differed statistically significantly. LRV=lower reference value (200 µg/L); URV=upper reference value (400 µg/L) (Iyengar, 1998).

Population group	N	N < LOD	N<LRV	N>URV	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [b,c]	37	0	1	9	**332** [300-368]	327	166-707	222	241	534	667
POSOČJE and IDRIJA (GO) [b,c]	28	0	1	9	**331** [285-386]	308	139-612	201	202	560	604
KOPER (KP) [b,c]	49	0	1	6	**316** [299-335]	313	167-460	222	256	422	427
JESENICE (KR) [b,c]	26	0	1	9	**353** [317-392]	365	141-525	275	276	473	518
MARIBOR (MB) [b,d]	55	0	0	20	**370** [352-388]	370	235-560	264	283	460	501
POMURJE (MS) [b,c]	41	0	2	12	**333** [299-371]	334	179-621	191	210	552	590
MEŽICA VALLEY (RA) [c]	29	0	1	5	**295** [261-335]	297	99-485	206	209	434	465
SAVINJSKO-POSAVSKA (SP) [b,c]	38	0	2	12	**324** [287-365]	318	114-579	118	220	525	578
ZASAVJE (ZA) [b,c]	50	0	0	16	**351** [328-375]	375	201-521	235	249	460	493
LJUBLJANA (LJ) [b,d]	49	0	2	21	**367** [340-397]	379	169-582	245	257	496	548
BELA KRAJINA (BK) [a,d]	43	0	0	30	**444** [414-476]	453	275-655	295	309	619	627
KOČEVJE and CERKNICA (KO) [a]	25	0	0	20	**523** [467-586]	523	337-954	354	358	702	838
Sampling period 2008-2010 [a]	117	0	2	71	**423** [401-446]	431	169-954	257	277	619	655
Sampling period 2011-2014 [b]	353	0	9	190	**336** [326-346]	337	99.3-707	210	238	475	534

S36. Levels of **zinc in breast milk** [µg/L] in lactating women from 12 study areas in Slovenia. Different letters indicate that the study groups (according to the study area or sampling period) differed statistically significantly. LRV=lower reference value (1000 µg/L); URV=upper reference value (2000 µg/L) (Iyengar, 1998).

Population group	N	N < LOD	N<LRV	N>URV	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [b,c]	37	0	4	18	**1875** [1551-2267]	1983	513-5288	590	937	3943	4819
POSOČJE and IDRIJA (GO) [b,c]	28	0	5	14	**1813** [1391-2362]	2002	235-4467	617	644	3886	4458
KOPER (KP) [b,c]	49	0	3	25	**1878** [1692-2085]	2063	710-3663	984	1126	2855	2998
JESENICE (KR) [c]	26	0	6	13	**1468** [1142-1885]	1724	285-3271	294	739	2805	2964
MARIBOR (MB) [a,b,c]	55	0	3	29	**2004** [1800-2231]	2019	492-4360	999	1222	3365	3767
POMURJE (MS) [b,c]	41	0	4	21	**1961** [1667-2307]	2064	610-5860	760	1048	3624	4407
MEŽICA VALLEY (RA) [b,c]	29	0	8	11	**1481** [1150-1906]	1664	207-3644	428	613	2812	3108
SAVINJSKO-POSAVSKA (SP) [b,c]	38	0	0	21	**2218** [1930-2548]	2088	1048-5731	1135	1250	4403	4742
ZASAVJE (ZA) [b,c]	50	0	4	17	**1666** [1482-1873]	1781	513-3461	664	1045	2664	2837
LJUBLJANA (LJ) [b,c]	49	0	4	27	**1968** [1687-2295]	2119	263-6094	817	1130	3281	4056
BELA KRAJINA (BK) [b,c]	43	0	5	31	**2360** [1960-2842]	2664	335-5394	795	985	4176	4613
KOČEVJE and CERKNICA (KO) [b,c]	25	0	1	17	**2987** [2321-3843]	3315	905-7904	1014	1508	5914	7559
Sampling period 2008-2010 [a]	117	0	10	75	**2289** [2054-2551]	2417	263-7904	817	1045	4421	5280
Sampling period 2011-2014 [b]	353	0	37	261	**1831** [1736-1932]	1965	207-5860	729	997	3153	3767

S37. Levels of **mercury in scalp hair** [ng/g] of Slovenian study population of men and lactating women living in 12 study areas. Different letters indicate that the study groups (according to the study area or sampling period) differed statistically significantly. RV=reference value (1000 ng/g) (US EPA).

Population group	N	N < LOD	N > RV	GM [CI95%]	MED	MIN-MAX	P5	P10	P90	P95
CELJE (CE) [c,d]	71	0	3	**223** [174-286]	261	12-1396	26	46	724	898
Men/Women	30/41	0/0	2/1	**198** [126-310]/ **244** [183-327]	217/298	12-1396/22-1312	13/34	37/66	763/578	1133/844
POSOČJE and IDRIJA (GO) [a,b]	77	0	12	**460** [383-552]	474	35-5735	118	158	1189	1560
Men/Women	35/42	0/0	8/4	**489** [365-656]/ **437** [344-554]	429/502	118-5735/35-1685	124/107	195/158	1461/955	1670/1148
KOPER (KP) [a]	100	0	29	**653** [550-776]	668	19-7068	191	284	1665	2560
Men/Women	49/51	0/0	18/11	**702** [515-957]/ **609** [514-723]	777/612	19-7068/151-1947	102/213	304/281	2993/1316	3697/1652
JESENICE (KR) [c,d]	69	0	1	**216** [177-263]	234	38-1524	48	65	592	656
Men/Women	31/38	0/0	0/1	**194** [145-261]/ **235** [179-310]	207/292	38-993/39-1524	45/48	82/56	511/622	592/767
MARIBOR (MB) [c,d]	97	0	6	**239** [196-291]	267	16-1999	32	60	901	1122
Men/Women	41/56	0/0	4/2	**264** [187-372]/ **223** [175-283]	278/265	25-1999/16-1220	38/32	57/79	984/700	1447/746
POMURJE (MS) [c,d]	78	0	3	**224** [184-273]	261	26-1434	47	56	598	969
Men/Women	30/48	0/0	2/1	**286** [211-388]/ **192** [149-231]	294/231	52-1434/26-1198	69/46	81/48	822/538	1004/598
MEŽICA VALLEY (RA) [c,d]	64	0	3	**241** [195-299]	245	13-3448	86	93	590	873
Men/Women	37/27	0/0	3/0	**257** [185-358]/ **222** [171-287]	225/261	13-3448/50-549	54/91	88/93	873/456	1383/537
SAVINJSKO-POSAVSKA (SP) [c,d]	67	0	0	**207** [173-248]	208	34-860	53	64	567	610
Men/Women	28/39	0/0	0/0	**200** [150-267]/ **212** [167-270]	196/212	41-860/34-674	59/40	64/53	610/567	658/596
ZASAVJE (ZA) [c]	94	0	3	**289** [248-338]	334	13-2220	97	116	633	748
Men/Women	49/45	0/0	2/1	**301** [237-383]/ **277** [226-339]	343/299	13-2220/48-1281	113/97	127/100	671/551	832/699
LJUBLJANA (LJ) [b,c]	77	0	5	**319** [252-404]	372	11-1660	27	78	885	1360
Men/Women	32/45	0/0	2/3	**332** [228-484]/ **310** [226-424]	365/382	11-1541/11-1660	43/27	98/78	885/831	1360/1129
BELA KRAJINA (BK) [d]	93	0	1	**168** [142-200]	174	30-1790	42	48	456	634
Men/Women	49/44	0/0	0/1	**149** [117-191]/ **192** [152-244]	137/194	30-746/42-1790	41/57	47/72	459/403	634/535
KOČEVJE and CERKNICA (KO) [c,d]	58	0	2	**233** [177-307]	251	10-4232	21	64	779	948
Men/Women	33/25	0/0	2/0	**247** [164-373]/ **216** [148-314]	253/249	13-4232/10-853	21/59	64/89	842/572	1420/608
Sampling period 2008-2010 [a]	228	0	8	**227** [199-258]	275	10-4232	42	59	746	885
Sampling period 2011-2014 [b]	717	0	60	**291** [271-312]	317	12-7068	52	88	911	1261

Table S38. Multiple linear regression: determinants of Pb exposure in the Mežica Valley study population.

		Estimate of change (95% CI), multiplicative factor		
		Total population	**Women, lactating**	**Men**
Pb in blood (µg/L)	*Model R^2*	*0.35 (p<0.001)*	*0.02 (p=0.400)*	*0.33 (p=0.002)*
		n=75	*n=29*	*n=46*
Sex		ns	-	-
Game consumption		ns	ns	ns
Smoking	No	1.00		1.00
	Yes	1.57 (0.92-2.70)#	ns	2.53 (0.92-6.95)#
Alcohol consumption		ns	ns	ns
Type of water supply		ns	ns	ns
Pb moss concentration (2-fold change)		**1.18 (1.11-1.26)****	**1.19 (1.02-1.38)***	**1.16 (1.07-1.27)***
Pb in urine (µg/L SG)	*Model R^2*	*0.25 (p<0.001)*	*0.10 (p=0.240)*	*0.33 (p=0.002)*
		n=73	*n=29*	*n=44*
Sex	Men	1.00	-	-
	Women	**0.68 (0.48-0.96)***	-	-
Age (years)	≤ 25	**0.52 (0.32-0.85)***		**0.34 (0.18-0.65)***
	25-35	1.00	ns	ns
	> 35	ns	ns	ns
Game consumption	No			1.00
	Yes	ns		1.53 (0.93-2.52)#
Type of water supply	Public	**2.22 (1.08-4.57)***		**2.12 (1.01-4.48)***
	Bottled	1.00	ns	1.00
	Private	2.26 (0.97-5.28)#	ns	**4.50 (1.57-12.9)***
Pb moss concentration (2-fold change)		**1.15 (1.03-1.27)***	1.18 (0.98-1.44)#	1.17 (0.98-1.40)#

Remark: Margin of statistical significance #p<0.1, *p<0.05, **p≤0.001, ns – not significant. The sex-related difference may (also) be on account of the lactating status of women.

S39. Levels of **Cd in soil, moss** *Hypnum cupressiforme* and **tap water** in the 12 study areas in Slovenia. Soil data represents geometric mean (GM) with 95% confiedence interval (CI95%), median (MED), minimum and maximum (MIN-MAX) concentrations of individual data interpolated from geochemical map of Slovenia; moss and tap water data represent MED (MIN-MAX) concentrations at sampling points in specific study areas.

	Cd in soil [mg/kg]				**Cd in moss** *Hypnum cupressiforme* [µg/g d.w.]			**Cd in tap water** [µg/L]		
Study area	**N**	**GM [CI95%]**	**MED**	**MIN-MAX**	**N**	**MED**	**MIN-MAX**	**N**	**MED**	**MIN-MAX**
Total	1076	**0.62** [0.60-0.65]	0.56	0.24-6.13	81	**0.29**	0.11-1.05	86	<LOD	<LOD-0.421
CELJE (CE)	79	**0.45** [0.42-0.49]	0.39	0.33-2.04	2	**0.58**	0.28-0.88	2	<LOD	<LOD-<LOD
POSOČJE and IDRIJA (GO)	96	**0.71** [0.66-0.76]	0.68	0.40-2.72	12	**0.27**	0.13-0.86	9	<LOD	<LOD-0.091
KOPER (KP)	102	**0.42** [0.41-0.43]	0.42	0.29-0.87	2	**0.18**	0.14-0.22	4	<LOD	<LOD-<LOD
JESENICE (KR)	80	**2.06** [1.82-2.33]	2.23	0.41-6.13	6	**0.33**	0.22-0.83	6	<LOD	<LOD-<LOD
MARIBOR (MB)	98	**0.46** [0.43-0.49]	0.44	0.28-1.00	7	**0.32**	0.21-0.43	5	<LOD	<LOD-<LOD
POMURJE (MS)	90	**0.35** [0.33-0.37]	0.32	0.26-0.89	7	**0.25**	0.11-0.51	14	<LOD	<LOD-0.421
MEŽICA VALLEY (RA)	79	**0.81** [0.76-0.86]	0.86	0.33-1.26	4	**0.48**	0.39-1.05	2	<LOD	<LOD-<LOD
Lower Mežica Valley	57	0.78 [0.72-0.84]	0.76	0.33-1.26	3	0.43	0.39-0.50	1	<LOD	-
Upper Mežica Valley	22	0.91 [0.87-0.94]	0.88	0.84-1.08	1	1.05	-	1	<LOD	-
SAVINJSKO-POSAVSKA (SP)	80	**0.71** [0.65-0.79]	0.74	0.28-2.55	15	**0.32**	0.14-0.89	15	<LOD	<LOD-<LOD
ZASAVJE (ZA)	104	**1.07** [1.01-1.13]	1.10	0.41-1.76	4	**0.45**	0.36-1.03	2	<LOD	<LOD-<LOD
LJUBLJANA (LJ)	100	**0.56** [0.52-0.60]	0.55	0.27-1.23	8	**0.28**	0.18-0.38	14	<LOD	<LOD-<LOD
BELA KRAJINA (BK)	98	**0.55** [0.49-0.60]	0.63	0.24-1.49	5	**0.23**	0.22-0.27	9	<LOD	<LOD-<LOD
KOČEVJE and CERKNICA (KO)	70	**0.43** [0.40-0.48]	0.37	0.31-1.33	9	**0.27**	0.14-0.41	4	<LOD	<LOD-<LOD

S40. Levels of **Pb in soil, moss** *Hypnum cupressiforme* and **tap water** in the 12 study areas in Slovenia. Soil data represents geometric mean (GM) with 95% confiedence interval (CI95%), median (MED), minimum and maximum (MIN-MAX) concentrations of individual data interpolated from geochemical map of Slovenia; moss and tap water data represent MED (MIN-MAX) concentrations at sampling points in specific study areas

	Pb in soil [mg/kg]				**Pb in moss** *Hypnum cupressiforme* [µg/g d.w.]			**Pb in tap water** [µg/L]		
Study area	**N**	**GM [CI95%]**	**MED**	**MIN-MAX**	**N**	**MED**	**MIN-MAX**	**N**	**MED**	**MIN-MAX**
Total	1084	**49.1** [46.3-52.1]	39.9	7.02-9393	81	**5.17**	2.71-304	86	**0.41**	<LOD-10.9
CELJE (CE)	79	**45.5** [43.4-47.7]	43.8	28.2-86.8	2	**3.87**	3.62-4.12	2	**0.81**	0.58-1.04
POSOČJE and IDRIJA (GO)	96	**39.5** [37.3-41.9]	42.7	21.8-81.1	12	**7.20**	4.09-11.9	9	**0.40**	0.08-0.58
KOPER (KP)	102	**18.3** [17.1-19.5]	17.8	7.02-40.3	2	**4.89**	4.62-5.17	4	**0.19**	0.15-0.34
JESENICE (KR)	80	**260** [209-332]	296	15.6-1629	6	**6.51**	4.06-15.9	6	**0.32**	0.10-1.28
MARIBOR (MB)	98	**50.3** [44.6-56.8]	47.0	10.1-148	7	**6.17**	4.06-8.65	5	**0.70**	0.37-0.72
POMURJE (MS)	90	**23.0** [21.9-24.1]	23.9	11.3-34.1	7	**4.89**	2.71-6.66	14	**0.34**	0.05-10.9
MEŽICA VALLEY (RA)	79	**284** [215-376]	202	41.5-9393	4	**26.7**	7.99-304	2	**0.16**	<LOD-0.32
Lower Mežica Valley	57	155 [134-180]	149	40.4-549	3	17.7	7.99-35.7	1	0.32	-
Upper Mežica Valley	22	1368 [821-2279]	1357	322-9393	1	304	-	1	<LOD	-
SAVINJSKO-POSAVSKA (SP)	81	**28.3** [29.5-34.4]	28.4	20.0-108	15	**4.90**	2.98-8.65	15	**0.58**	0.16-4.66
ZASAVJE (ZA)	104	**45.0** [38.3-52.8]	30.3	22.7-566	4	**5.00**	4.23-5.44	2	**0.62**	0.19-1.04
LJUBLJANA (LJ)	100	**57.8** [52.2-64.0]	50.1	25.5-216	8	**4.75**	2.96-14.6	14	**0.55**	0.06-6.45
BELA KRAJINA (BK)	98	**43.8** [42.2-45.4]	45.3	31.6-74.5	5	**4.34**	2.94-8.92	4	**0.45**	0.14-0.88
KOČEVJE and CERKNICA (KO)	70	**29.5** [27.5-31.7]	28.2	17.5-66.7	9	**4.76**	2.72-11.9	9	**0.62**	0.03-2.27

S41. Levels of **Hg in soil, moss** *Hypnum cupressiforme* and **tap water** in the 12 study areas in Slovenia. Soil data represents geometric mean (GM) with 95% confidence interval (CI95%), median (MED), minimum and maximum (MIN-MAX) concentrations of individual data interpolated from geochemical map of Slovenia; moss and tap water data represent MED (MIN-MAX) concentrations at sampling points in specific study areas. *Analysis of Hg in 29 of 81 moss samples has not been performed.

	Hg in soil [mg/kg]				**Hg in moss** *Hypnum cupressiforme* [µg/g d.w.]*			**Hg in tap water** [µg/L]		
Study area	**N**	**GM [CI95%]**	**MED**	**MIN-MAX**	**N**	**MED**	**MIN-MAX**	**N**	**MED**	**MIN-MAX**
Total	1084	**176** [166-185]	161	25.2-45494	52	**0.050**	0.040-0.160	83	**0.91**	0.25-9.78
CELJE (CE)	79	**139** [134-144]	139	71.2-196	1	**0.060**	0.060-0.060	2	**1.18**	0.91-1.45
POSOČJE and IDRIJA (GO)	95	**527** [403-690]	384	139-45494	7	**0.070**	0.060-0.160	9	**0.99**	0.52-1.94
Town of Idrija	11	16573 [9737-28209]	20305	2056-45494	1	0.070	-	2	1.51	1.08-1.94
KOPER (KP)	102	**277** [274-281]	279	227-315	1	**0.060**	0.060-0.060	4	**0.93**	0.68-1.29
JESENICE (KR)	80	**756** [670-841]	765	203-2266	5	**0.060**	0.050-0.080	6	**0.87**	0.35-2.10
MARIBOR (MB)	98	**106** [98.4-114]	115	48.1-171	6	**0.045**	0.040-0.090	5	**0.53**	0.25-0.64
POMURJE (MS)	90	**41.5** [38.7-44.6]	38.2	25.2-96.2	4	**0.055**	0.040-0.060	14	**1.04**	0.30-1.98
MEŽICA VALLEY (RA)	79	**236** [226-246]	253	154-296	3	**0.060**	0.050-0.060	2	**0.48**	0.38-0.58
SAVINJSKO-POSAVSKA (SP)	81	**129** [115-146]	138	54.3-423	11	**0.050**	0.040-0.060	15	**0.91**	0.46-1.98
ZASAVJE (ZA)	104	**154** [148-159]	159	109-237	3	**0.050**	0.050-0.060	2	**0.85**	0.79-0.91
LJUBLJANA (LJ)	100	**230** [204-260]	201	103-1257	4	**0.045**	0.040-0.060	13	**0.92**	0.40-9.78
BELA KRAJINA (BK)	98	**75.5** [72.5-78.7]	70.3	63.3-124	2	**0.050**	0.050-0.050	4	**1.08**	0.50-1.69
KOČEVJE and CERKNICA (KO)	70	**175** [162-190]	152	113-435	5	**0.040**	0.040-0.070	7	**0.94**	0.58-4.51

S42. Levels of **As in soil, moss** *Hypnum cupressiforme* and **tap water** in the 12 study areas in Slovenia. Soil data represents geometric mean (GM) with 95% confidence interval (CI95%), median (MED), minimum and maximum (MIN-MAX) concentrations of individual data interpolated from geochemical map of Slovenia; moss and tap water data represent MED (MIN-MAX) concentrations at sampling points in specific study areas.

	As in soil - *not available* [mg/kg]				**As in moss** *Hypnum cupressiforme* [µg/g d.w.]			**As in tap water** [µg/L]		
Study area	**N**	**GM [CI95%]**	**MED**	**MIN-MAX**	**N**	**MED**	**MIN-MAX**	**N**	**MED**	**MIN-MAX**
Total	-	-	-	-	81	**0.26**	0.13-0.83	86	**0.14**	0.06-1.64
CELJE (CE)	-	-	-	-	2	**0.25**	0.21-0.29	2	**0.32**	0.11-0.53
POSOČJE and IDRIJA (GO)	-	-	-	-	12	**0.31**	0.19-0.83	9	**0.16**	0.07-0.45
KOPER (KP)	-	-	-	-	2	**0.21**	0.20-0.23	4	**0.15**	0.10-0.39
JESENICE (KR)	-	-	-	-	6	**0.26**	0.16-0.44	6	**0.22**	0.06-0.38
MARIBOR (MB)	-	-	-	-	7	**0.27**	0.17-0.41	5	**0.08**	0.07-0.24
POMURJE (MS)	-	-	-	-	7	**0.38**	0.24-0.44	14	**0.13**	0.07-0.64
MEŽICA VALLEY (RA)	-	-	-	-	4	**0.36**	0.25-0.68	2	**0.09**	0.08-0.09
SAVINJSKO-POSAVSKA (SP)	-	-	-	-	15	**0.24**	0.14-0.40	15	**0.16**	0.07-0.53
ZASAVJE (ZA)	-	-	-	-	4	**0.27**	0.25-0.28	2	**0.50**	0.46-0.53
LJUBLJANA (LJ)	-	-	-	-	8	**0.23**	0.13-0.34	14	**0.16**	0.09-0.54
BELA KRAJINA (BK)	-	-	-	-	5	**0.27**	0.16-0.37	4	**0.10**	0.08-0.51
KOČEVJE and CERKNICA (KO)	-	-	-	-	9	**0.22**	0.13-0.44	9	**0.17**	0.08-1.64

S43. Levels of **Mn in soil, moss** *Hypnum cupressiforme* and **tap water** in the 12 study areas in Slovenia. Soil data represents geometric mean (GM) with 95% confidence interval (CI95%), median (MED), minimum and maximum (MIN-MAX) concentrations of individual data interpolated from geochemical map of Slovenia; moss and tap water data represent MED (MIN-MAX) concentrations at sampling points in specific study areas.

	Mn in soil [mg/kg]				**Mn in moss** *Hypnum cupressiforme* [µg/g d.w.]			**Mn in tap water** [µg/L]		
Study area	**N**	**GM [CI95%]**	**MED**	**MIN-MAX**	**N**	**MED**	**MIN-MAX**	**N**	**MED**	**MIN-MAX**
Total	1084	**874** [849-900]	861	94.1-3584	81	**151**	18.1-1175	86	**0.30**	<LOD-10.5
CELJE (CE)	79	**560** [519-603]	558	175-1227	2	**143**	124-162	2	**2.32**	0.67-3.97
POSOČJE and IDRIJA (GO)	96	**1348** [1275-1424]	1420	729-2339	12	**78.0**	41.4-322	9	**0.25**	0.06-1.94
KOPER (KP)	102	**743** [718-770]	757	384-1193	2	**73.4**	72.8-73.9	4	**0.06**	0.02-0.24
JESENICE (KR)	79	**1276** [1124-1450]	1503	94.1-2604	6	**216**	38.9-488	6	**0.78**	0.11-3.07
MARIBOR (MB)	98	**840** [804-878]	855	470-1356	7	**193**	103-552	5	**0.37**	0.03-1.90
POMURJE (MS)	90	**929** [899-960]	963	470-1443	7	**193**	43.9-1175	14	**0.17**	<LOD-10.5
MEŽICA VALLEY (RA)	79	**958** [887-1033]	1064	470-2445	4	**368**	240-756	2	**0.16**	<LOD-0.31
SAVINJSKO-POSAVSKA (SP)	81	**767** [703-836]	747	370-2847	15	**212**	18.1-714	15	**0.67**	0.10-6.81
ZASAVJE (ZA)	104	**493** [465-522]	487	306-978	4	**149**	18.1-714	2	**2.03**	0.10-3.97
LJUBLJANA (LJ)	100	**726** [668-789]	787	135-1611	8	**172**	18.1-499	14	**0.89**	<LOD-3.83
BELA KRAJINA (BK)	98	**1820** [1725-1922]	1957	795-3584	5	**109**	43.2-221	4	**1.42**	0.02-3.41
KOČEVJE and CERKNICA (KO)	70	**737** [685-794]	693	483-2020	9	**78.1**	43.4-352	9	**0.28**	<LOD-3.93

S44. Levels of **Se in soil, moss** *Hypnum cupressiforme* and **tap water** in the 12 study areas in Slovenia. Soil data represents geometric mean (GM) with 95% confidence interval (CI95%), median (MED), minimum and maximum (MIN-MAX) concentrations of individual data interpolated from geochemical map of Slovenia; moss and tap water data represent MED (MIN-MAX) concentrations at sampling points in specific study areas.

	Se in soil - *not available* [mg/kg]				**Se in moss** *Hypnum cupressiforme* [µg/g d.w.]			**Se in tap water - *not available***		
Study area	**N**	**GM [CI95%]**	**MED**	**MIN-MAX**	**N**	**MED**	**MIN-MAX**	**N**	**MED**	**MIN-MAX**
Total	-	-	-	-	81	**0.21**	0.13-0.42	-	-	-
CELJE (CE)	-	-	-	-	2	**0.17**	0.15-0.18	-	-	-
POSOČJE and IDRIJA (GO)	-	-	-	-	12	**0.32**	0.23-0.42	-	-	-
KOPER (KP)	-	-	-	-	2	**0.22**	0.22-0.22	-	-	-
JESENICE (KR)	-	-	-	-	6	**0.23**	0.15-0.29	-	-	-
MARIBOR (MB)	-	-	-	-	7	**0.18**	0.15-0.21	-	-	-
POMURJE (MS)	-	-	-	-	7	**0.18**	0.14-0.22	-	-	-
MEŽICA VALLEY (RA)	-	-	-	-	4	**0.19**	0.13-0.25	-	-	-
SAVINJSKO-POSAVSKA (SP)	-	-	-	-	15	**0.18**	0.15-0.24	-	-	-
ZASAVJE (ZA)	-	-	-	-	4	**0.24**	0.21-0.28	-	-	-
LJUBLJANA (LJ)	-	-	-	-	8	**0.21**	0.13-0.36	-	-	-
BELA KRAJINA (BK)	-	-	-	-	5	**0.18**	0.15-0.29	-	-	-
KOČEVJE and CERKNICA (KO)	-	-	-	-	9	**0.22**	0.16-0.33	-	-	-

S45. Levels of **Cu in soil, moss** *Hypnum cupressiforme* and **tap water** in the 12 study areas in Slovenia. Soil data represents geometric mean (GM) with 95% confidence interval (CI95%), median (MED), minimum and maximum (MIN-MAX) concentrations of individual data interpolated from geochemical map of Slovenia; moss and tap water data represent MED (MIN-MAX) concentrations at sampling points in specific study areas.

	Cu in soil [mg/kg]				**Cu in moss** *Hypnum cupressiforme* [µg/g d.w.]			**Cu in tap water** [mg/L]		
Study area	**N**	**GM [CI95%]**	**MED**	**MIN-MAX**	**N**	**MED**	**MIN-MAX**	**N**	**MED**	**MIN-MAX**
Total	1084	**27.6** [26.8-28.4]	26.5	4.92-137	81	**5.63**	3.66-11.4	86	**0.007**	<LOD-0.057
CELJE (CE)	79	**15.3** [14.6-16.0]	15.1	8.09-24.7	2	**5.41**	5.18-5.65	2	**0.015**	0.007-0.023
POSOČJE and IDRIJA (GO)	96	**40.1** [37.8-42.5]	39.3	19.1-87.7	12	**7.11**	5.05-11.4	9	**0.008**	<LOD-0.018
KOPER (KP)	102	**39.9** [38.7-41.0]	40.7	26.9-55.6	2	**5.19**	5.00-5.37	4	**0.002**	<LOD-0.007
JESENICE (KR)	80	**34.6** [31.1-38.5]	34.7	5.30-77.2	6	**5.97**	4.46-7.44	6	**0.010**	0.002-0.010
MARIBOR (MB)	98	**37.0** [34.7-39.4]	38.7	16.9-77.3	7	**5.98**	4.13-7.95	5	**0.009**	0.002-0.012
POMURJE (MS)	90	**24.1** [22.5-25.9]	24.1	12.5-61.9	7	**5.75**	5.01-6.39	14	**0.006**	<LOD-0.026
MEŽICA VALLEY (RA)	79	**38.4** [33.7-43.9]	32.3	17.4-137	4	**5.49**	4.56-6.65	2	**0.006**	0.002-0.009
SAVINJSKO-POSAVSKA (SP)	81	**21.8** [20.1-23.7]	21.8	4.92-68.6	15	**5.18**	4.26-6.89	15	**0.008**	0.002-0.039
ZASAVJE (ZA)	104	**19.0** [18.2-19.9]	18.5	10.1-30.3	4	**5.64**	5.17-6.89	2	**0.014**	0.005-0.023
LJUBLJANA (LJ)	100	**30.9** [28.4-33.7]	30.7	11.9-83.9	8	**5.38**	3.96-9.59	14	**0.007**	<LOD-0.057
BELA KRAJINA (BK)	98	**24.8** [23.7-26.0]	24.9	15.5-42.3	5	**4.40**	4.22-7.08	4	**0.005**	0.002-0.30
KOČEVJE and CERKNICA (KO)	70	**17.1** [15.6-18.7]	17.2	10.4-34.8	9	**5.43**	3.66-6.94	9	**0.008**	<LOD-0.022

S46. Levels of **Zn in soil, moss** *Hypnum cupressiforme* and **tap water** in the 12 study areas in Slovenia. Soil data represents geometric mean (GM) with 95% confidence interval (CI95%), median (MED), minimum and maximum (MIN-MAX) concentrations of individual data interpolated from geochemical map of Slovenia; moss and tap water data represent MED (MIN-MAX) concentrations at sampling points in specific study areas.

	Zn in soil [mg/kg]				**Zn in moss** *Hypnum cupressiforme* [µg/g d.w.]			**Zn in tap water** [µg/L]		
Study area	**N**	**GM [CI95%]**	**MED**	**MIN-MAX**	**N**	**MED**	**MIN-MAX**	**N**	**MED**	**MIN-MAX**
Total	1084	**122** [118-126]	108	24.0-1843	81	**28.7**	16.0-66.7	86	**54.8**	5.8-676
CELJE (CE)	79	**83.0** [77.3-89.1]	74.0	67.0-297	2	**29.5**	27.0-32.0	2	**206**	128-283
POSOČJE and IDRIJA (GO)	96	**101** [96.5-105]	97.5	64.1-199	12	**27.4**	19.7-64.5	9	**33.4**	7.4-99.8
KOPER (KP)	102	**76.6** [75.2-78.0]	76.6	58.0-91.5	2	**26.6**	17.8-35.3	4	**25.3**	21.9-72.2
JESENICE (KR)	80	**338** [285-402]	418	24.0-1180	6	**31.6**	20.8-62.5	6	**71.6**	22.8-133
MARIBOR (MB)	98	**141** [132-150]	139	67.4-340	7	**30.3**	23.9-42.9	5	**108**	53.9-166
POMURJE (MS)	90	**94.3** [91.0-97.7]	97.3	60.8-125	7	**27.5**	20.7-40.1	14	**56.6**	27.8-338
MEŽICA VALLEY (RA)	79	**221** [195-251]	197	100-1843	4	**62.2**	43.1-66.7	2	**57.4**	32.8-82.0
Lower Mežica Valley	57	182 [170-194]	184	99.6-293	3	62.3	43.1-66.7	1	82.0	-
Upper Mežica Valley	22	367 [255-528]	297	172-1843	1	62.0	-	1	32.8	-
SAVINJSKO-POSAVSKA (SP)	81	**115** [109-122]	115	70.9-369	15	**27.0**	18.8-48.4	15	**68.6**	5.8-676
ZASAVJE (ZA)	104	**152** [134-174]	114	79.9-1156	4	**31.2**	26.3-48.4	2	**144**	5.8-283
LJUBLJANA (LJ)	100	**127** [119-136]	121	56.1-285	8	**26.4**	18.7-40.5	14	**59.2**	11.1-237
BELA KRAJINA (BK)	98	**107** [104-111]	109	70.7-199	5	**20.8**	17.6-27.7	4	**32.3**	5.9-101
KOČEVJE and CERKNICA (KO)	70	**77.3** [70.9-83.2]	78.7	50.0-130	9	**30.0**	16.0-37.7	9	**51.4**	9.4-648

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3.4 Prenatal Mercury Exposure and Child Neurodevelopment Outcomes at 18 months: Results from the Mediterranean PHIME cohort

The paper is authored by F. Barbone and his colleagues from the Institute for Maternal and Child Health – "Burlo Garofolo", Trieste, University of Udine, and Academic Hospital of Udine Italy; D. Mazej, J. Snoj Tratnik, A. Miklavčič Višnjevec and M. Horvat from the JSI; K. Sofianou from the Institute of Child Health, Athens, Greece; M. Krsnik, J. Osredkar, D. Neubauer, J. Kodrič, and S. Stropnik from the University Medical Center in Ljubljana; I. Prpić, O. Petrović, and I. Vlašić-Cicvarić from the Clinical Hospital Centre of Rijeka, Croatia, and Z. Špirić from the Green Infrastructure Ltd, Zagreb, Croatia. It has been published in the International Journal of Hygiene and Environmental Health.

The paper assessed the association between prenatal exposure to Hg from fish consumption during pregnancy and the development of the children's nervous system. The study was set within the EU 6th framework project PHIME to address the uncertainty associated with the potential health effects of low-level exposure to metals, including Hg. The study population included mother-child pairs residing in different regions of the Mediterranean area: Northern Adriatic area (Italy, Slovenia, Croatia) and Aegean area (Greece), therefore representing consumption of different amounts and different species of fish from different origins. Prenatal exposure was assessed through cord blood Hg concentrations, which represents the exposure in the last trimester of the pregnancy. Neurodevelopment was assessed at 18 months of age using standardized assessment kit Bayley Scales of Infant and Toddler Development, 3rd Edition (BSID-III or Bayley-III), which enabled to assess cognitive, language and motor development of children. Assessment of association between internal exposure and development accounted for all potential non-genetic confounders of neurodevelopment that were available, and were selected from demographic and socioeconomic information, lifestyles and nutritional habits.

The total Hg concentrations observed in the study population were much lower than those reported in the Faroe study and also some others (Seychelles, New Zealand), but were similar to those found in most of the European population. At these concentrations, the main results of the meta-analysis did not show any overall negative associations with cognitive and language scales of development. However, although weak, a significant inverse relation between total Hg levels and developmental motor scores at 18 months was observed in Croatian and Slovenian study populations.

The paper provides the overall assessment of association between low-level Hg exposure due to fish consumption and neurodevelopment in the Northern Adriatic (Slovenia, Croatia, Italy) and Aegean area (Greece).

I conducted a part of total Hg analysis in maternal and cord blood and maternal hair of mother-child pairs from all participating countries, participated in interviewing the mothers 6-8 weeks after birth in Slovenia, and partly managed the Slovenian database.

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Prenatal mercury exposure and child neurodevelopment outcomes at 18 months: Results from the Mediterranean PHIME cohort

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ABSTRACT

Introduction: Neurotoxicity due to acute prenatal exposure to high-dose of mercury (Hg) is well documented. However, the effect of prenatal exposure to low Hg levels on child neurodevelopment and the question about "safety" of fish-eating during pregnancy remain controversial. International comparisons of Hg concentrations in mother-child biological samples and neurodevelopmental scores embedded in birth cohort studies may provide useful evidence to explore this issue.

Materials and methods: The Mediterranean (Italy, Slovenia, Croatia, and Greece) cohort study included 1308 mother-child pairs enrolled in the Public Health Impact of long-term, low-level, Mixed Element exposure in a susceptible population EU Sixth Framework Programme (PHIME). Maternal hair and venous blood, cord blood and breast milk samples were collected, and total Hg (THg) levels were measured. Demographic and socio-economic information, lifestyles and nutritional habits were collected through questionnaires at different phases of follow-up. Children at 18 months of age underwent neurodevelopmental testing using the Bayley Scales of Infant and Toddler Development, Third Edition (BSID-III). Multivariate linear and logistic regressions were performed, for each country, to assess the association between THg and BSID-III scores, obtaining adjusted β coefficients and odds ratios (ORs). These values were used to conduct a meta-analysis, to explore possible heterogeneity among countries and to obtain combined estimates of the association between THg exposure and BSID-III scores.

Results: Median THg (ng/g) was: 704 in maternal hair, 2.4 in maternal blood, 3.6 in cord blood, and 0.6 in breast milk. THg concentrations were highest in Greece and lowest in Slovenia. BSID-III neurodevelopmental scores

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were higher in Croatia and Slovenia. The meta-analysis of multivariate linear models found an overall positive association between language composite score and receptive communication scaled score and increasing THg in maternal hair (n = 1086; β = 0.55; 95%CI: 0.05–1.05 and n = 1075; β = 0.12; 95%CI: 0.02–0.22, respectively). The meta-analysis of logistic regression models showed that the overall adjusted OR between THg in cord blood and suboptimal gross motor score was borderline significant (n = 882; OR = 1.03; 95%CI: 1.00–1.07). Heterogeneity was found across the four sub-cohorts for language composite score in maternal blood, and for fine motor scaled score in cord blood and breast milk. Language composite score and THg concentrations in maternal venous blood were positively related (n = 58; β = 4.29; CI95% (−0.02, 8.60)) in Croatia and an increase of 1 ng/g of THg in maternal venous blood was associated with a reduced risk for children to fall in the lowest quintile of language score by 31% (n = 58; OR = 0.69; CI 95%: 0.37, 1.01). The comparison of β coefficients obtained by multiple linear regression model showed an inverse association between fine motor score and THg concentrations in cord blood for Croatia (n = 54; β = −0.53; CI 95%: −1.10, 0.04) and Slovenia (n = 225; β = −0.25; CI 95%: −0.49, −0.01). In Slovenia THg level in breast milk was associated with suboptimal fine motor performance (n = 195; OR = 5.25; CI 95%: 1.36, 21.10).

Conclusions: This study showed an inverse relation between THg levels and developmental motor scores at 18 months, although the evidence was weak and partially internally and externally inconsistent. No evidence of detrimental effects of THg was found for cognitive and language outcomes at these concentrations and age.

1. Introduction

Neurotoxicity due to acute exposure to high-dose of mercury (Hg) is well known because of poisoning cases in Japan and Iraq (Amin-Zaki et al., 1974; Bose-O'Reilly et al., 2010; Counter and Buchanan, 2004; Diez, 2009). Children of asymptomatic mothers, exposed to high levels of methylmercury (MeHg) during pregnancy, showed several neurodevelopmental abnormalities including delayed onset of motor and language development, impairments of sensory functions, severe mental retardation, seizures and in some cases coma and death (Bose-O'Reilly et al., 2010; Counter and Buchanan, 2004; Davidson et al., 2004; Diez, 2009; Myers and Davidson, 1998; UNEP DTIE Chemicals Branch, 2008). The implications of these findings caused concern: Hg is an element that is ubiquitous in nature and is methylated in the aquatic environment by bacteria to form MeHg (National Research Council (US), Committee on the Toxicological Effects of Methylmercury (2000)). This organic compound bioaccumulates in marine organisms and is biomagnified through the food chain (Myers and Davidson, 1998). Therefore, the primary source of non-occupational Hg exposure is consumption of fish and other seafood, especially predatory species (e.g., tuna, swordfish, shark, bass, king mackerel, tilefish) (Bose-O'Reilly et al., 2010; Diez, 2009; Myers et al., 2007; Oken and Bellinger, 2008).

Several cohort studies have evaluated the effect of pre-natal exposure to MeHg on child neurodevelopment, with conflicting results. The Norwegian Mother and Child Cohort Study (MoBa) found at three years of age a significant association between prenatal MeHg exposure above the 90th percentile and delayed language and communication skills in a low exposed population (Vejrup et al., 2016). In this cohort, a positive association between low levels of prenatal mercury exposure and language and communication skills was found at five years. However, in the highest exposure group a matched sibling analyses suggested that mercury was negatively associated with language skills (Vejrup et al., 2018). Cohort studies conducted in New Zealand (Kjellstrom et al., 1989) and the Faroe Islands, reported in children (6–7 years of age) dose-related neurological deficits associated with prenatal MeHg exposure (by maternal fish intake), such as impairments of attention, language, verbal memory, motor (speed) and visuospatial function and loss of intelligence quotient (IQ) points (Debes et al., 2006; Grandjean et al., 1998, 1997; Holmes et al., 2009). Only weaker deficits were identified concerning motor speed, attention, and language in adolescents of a mean age of 14 years (Debes et al., 2006; Dovydaitis, 2008; Holmes et al., 2009). On the other hand, the Seychelles study did not find alterations of neuropsychological development and IQ at each studied age between 6 months and 24 years, although the Hg/MeHg levels of exposure were similar to those found in the Faroe Islands study (Bose-O'Reilly et al., 2010; Counter and Buchanan, 2004; Davidson et al., 2011; Davidson et al., 2010; Davidson et al., 2008a, 2008b; 2006a, 2006b; 1998; Dovydaitis, 2008; Myers et al., 2007; Strain et al., 2015, 2008; Van Wijngaarden et al., 2017).

In 2005 Cohen et al., 2005 aggregated results from the Faroe Islands study, the Seychelles Child Development Study, and the New Zealand study showing that a prenatal MeHg exposure sufficient to increase the concentration of mercury in maternal hair at delivery by 1 μg/g decreases intelligence quotient by 0.7 points.

A birth cohort study carried out in Japan suggested that, in neonates of 3 days, Hg levels in maternal hair (median 1.96 μg/g) affect early motor function (Suzuki et al., 2010). In a cohort of Spanish children of 4 years, total mercury concentrations (THg) in hair greater than 1 μg/g were associated with deficits in cognitive abilities. However, in the larger Spanish Infancia y Medio Ambiente (INMA) cohort, the relation between cord blood level of THg and mental and psychomotor development were by and large negative (Llop et al., 2012). Inconsistent results were shown in Boucher et al. (2010), on Canadian Inuit children while no detrimental effects of Hg low level were found in the Avon Longitudinal Study of Parents and Children (ALSPAC). In this cohort maternal and infants' fish intakes resulted related to higher mean developmental scores, suggesting a beneficial effect of fish intake on neurodevelopment (Hibbeln et al., 2007).

The evaluation of the toxic effects of contaminants in fish-eating populations is complicated by the fact that fish is an important source of beneficial nutrients, like polyunsaturated fatty acids (PUFAs), lean protein, iron, iodine, selenium, vitamins, and choline. These nutrients are transferred from the mother to the fetus via placenta and play a positive role in fetal neurodevelopment (Genuis, 2008; Gil and Gil, 2015; Starling et al., 2015). This might explain why the effects on child neurodevelopment of prenatal exposure to low Hg levels, are still unclear, although it has been demonstrated that mercury can cross both the placenta and the blood-brain barrier and it can be measured in umbilical cord blood (Al-Saleh et al., 2016; Grandjean et al., 2005).

The uncertainty about "safety" of fish intake during pregnancy represents an important issue of public health (Genuis, 2008). The Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JECFA) in 2004 had fixed a maximum acceptable intake for MeHg of 1.6 μg/kg body weight per week for the general population and for pregnant women to protect the fetus from neurotoxic effects (JECFA, 2004). In the same year, the U.S. Food and Drug Administration (FDA) recommended limiting intake of fish to no more than 12 oz (340 gr) per week. In 2014 the FDA evaluated benefits and concerns of eating fish during pregnancy and breastfeeding and suggested that children of pregnant women who consumed two seafood meals per week (8–12 oz, 227–340 g) could have an additional 3.3 IQ points at 9 years of age (FDA Food and Drug Administration, 2014; McGuire et al., 2016).

To address the uncertainty associated with the health effects of exposure to low-level metals including mercury, the EU Commission Sixth Framework Programme for Research and Technological Development funded the project entitled 'Public health impact of long-term, low-level mixed element exposure in susceptible population strata' (PHIME) (PHIME, 2011). Within the PHIME framework, in 2007, partners from Italy, Slovenia, Croatia and Greece established a prospective mother-child cohort aimed to assess the association between mercury exposure from food consumption during pregnancy and development of the children nervous system. Preliminary results from the Italian PHIME sub-cohort, published by Valent et al. (2013a), showed no association between child neurodevelopment at 18 months of age and prenatal Hg exposure. BSID-III scores were influenced directly by higher child intake of fish, higher maternal IQ, and female child's sex. Also, maternal fish intake and maternal serum LCPUFAs were not associated with neurocognitive abilities. On the other hand, results from the Croatian PHIME sub-cohort, published by Prpić et al. (2017) showed a negative correlation at 18 months between cord blood THg concentration and fine motor skills (rho = −0.22, p = 0.01).

This study aimed to assess at 18 months of age the association of low-level prenatal mercury exposure on neurodevelopment among residents in the four Mediterranean coastal regions of Italy, Slovenia, Croatia and Greece where pregnant women consume different amounts and different species of fish from different origins (Miklavčič et al., 2013). A specific primary objective of this prospective cohort study was to compare the impact of different levels of mercury exposure while taking into account potential confounders or effect modifiers.

2. Material and methods

2.1. Study population

A detailed description of the study protocol has been published previously (Valent et al., 2013b).

In brief, recruitment took place at the Institute for Maternal and Child Health IRCCS Burlo Garofolo in Trieste, Italy (I); at the Maternity Hospital of the University Medical Centre of Ljubljana, Slovenia (S); at the University Hospital of Rijeka, Croatia (C); and at the general regional hospitals of Mytilini (Lesvos), Chios, Samos, and Leros in Greece (G). At recruitment, eligible women were approached for consent after their routine morphologic ultrasound scan between 20 and 22 gestational weeks (I), at routine visits between 34 and 38 gestational weeks (C), or during their hospital stay for delivery (S, C, G). Times for enrollment were chosen according to logistic considerations in each country. In Italy and Croatia, the eligible women provided a maternal urine sample and underwent the Raven's Progressive Matrices Test, a test of general intelligence. At different phases, three questionnaires were administered to mothers: (a) a short questionnaire to identify any excluding conditions and to provide some brief information on family and lifestyles during pregnancy; (b) a long questionnaire to collect information on demographic, socioeconomic and health status, on pregnancy and delivery, on lifestyles and dietary habits; in the end (c) a supplementary questionnaire to update information on the family and child. Biological samples (maternal hair, maternal venous blood, cord blood and breast milk) were collected in each country of the study cohort during different phases. Children at 18 (range 16–20) months of age underwent neurodevelopmental evaluation.

Only children born during or after week 37 of gestation who had at least one measure of THg exposure and underwent the Bayley Scales of Infant and Toddler Development at 18 ± 2 months were included in the analysis of the present paper.

2.2. Ethics

The research protocol was approved by the Ethics Committees of the University of Udine, of the Institute for Maternal and Child Health IRCCS Burlo Garofolo, of the Clinical Center of Rijeka, of the Institute of Child Health of Athens and by the National Ethics Committee of the Republic of Slovenia. All aspects of the study, including ethics, were monitored annually by the European Commission.

2.3. Mercury exposure

In the present study, THg concentration in biological samples was the main exposure of interest. THg concentrations were measured in maternal hair, maternal blood, umbilical cord blood and/or cord tissue and breast milk from lactating women. Hair samples, approximately 1 g of hair cut close to the occipital area, were stored in transparent mercury-free plastic bags in a dark and uncontaminated place and periodically sent to Jozef Stefan Institute (JSI) of Ljubljana for analysis. Aliquots of blood, cord tissue, and breast milk samples were stored in freezers (below −24 °C) and then sent or transported in a frozen state (on dry ice) to the laboratories for analysis.

THg in biological samples was determined by cold vapor atomic absorption spectrometry (CVAAS) using three different analytical procedures, depending on matrix type and the study group (period of sampling). All measurements were made under strict quality control procedures and gave comparable results. In addition, the JSI laboratory participated in a series of inter-laboratory comparisons organized within the PHIME project. Three inter-comparisons used lyophilized samples of human blood from non-exposed persons, people occupationally exposed to elemental Hg, fish eaters while the fourth study used fresh blood from the general population. The obtained values were in good agreement with the assigned values (Miklavčič et al., 2013).

The analytical procedures used are described in detail elsewhere (Akagi, 1997; Horvat et al., 1991; Miklavčič et al., 2013, 2011). The estimated analytical precision of the measurements was less than 10%, the limit of detection (LOD) of the procedures was < 0.1 ng/mL in blood and cord blood and < 1 ng/g in hair.

MeHg in hair was measured by gas chromatography–electron capture detection (GC-ECD), whereas MeHg in cord blood was measured by a cold vapor atomic fluorescence detector (CVAFS). The estimated analytical precision of the measurements was 12%, the LODs were 0.2 ng/g in hair, and 0.02 ng/g in blood. The analytical procedures are described in detail by Miklavčič et al. (2013). MeHg measurements were performed on a restricted group of subjects, however, since among subjects with MeHg data, the ratio of MeHg to THg was very high (median, 0.99 in hair and 0.90 in cord blood), we assumed that in the majority of cohort members most Hg was methylated and, therefore, THg could be used as a proxy for MeHg.

2.4. Evaluation of child neurodevelopment (study outcome)

Child neurodevelopment was assessed at 18 months of age (range, 16–20 months) by using the Bayley Scales of Infant and Toddler Development, Third Edition (BSID-III) which measures the major areas of child development. The testing was conducted in each study centers by pediatricians or psychologists who had attended the same BSID-III Accreditation Workshop. In Italy, inter-rater reliability was very high for the BSID-III cognitive score (intraclass correlation coefficient [ICC] 0.98, 95% CI: 0.97–0.99), language score (ICC 0.99, 95% CI: 0.99–1.00), and motor score (ICC 0.93, 95% CI: 0.90–0.97) (Valent et al., 2013a).

As outcomes, we considered the composite scores - cognitive (COG), language (LAN) and motor (MOT) - and the scaled scores receptive communication (RC), expressive communication (EC), fine motor (FM) and gross motor (GM). The scaled scores represent a child's performance on a subtest relative to his or her same age peers and were derived from the standardization of total raw score (the sum of the number of points earned for a subtest) for child's age in days at test administration. For each composite score, the distribution of scaled score (or the sum of scaled scores, RC plus EC and FM plus GM) was

Table 1
General characteristics of 1308 mothers and their children.

	Total (n = 1308)	Croatia (n = 127)	Greece (n = 302)	Italy (n = 607)	Slovenia (n = 272)	*p*-value[a]
Mother's age at delivery, mean ± std (median)	31.4 ± 5.1 (32)	30.0 ± 4.5 (30)	28.9 ± 6.3 (29)	33.3 ± 4.3 (33)	30.4 ± 4.1 (30)	< 0.001
Maternal BMI before pregnancy, mean ± std (median)	23.2 ± 4.5 (22.5)	23.2 ± 4.5 (22.2)	23.5 ± 5.2 (22.9)	22.8 ± 3.8 (22.2)	23.8 ± 4.8 (22.1)	< 0.001
Weight gain during pregnancy, mean ± std (median)	13.9 ± 5.4 (14)	16.1 ± 5.7 (16)	13.5 ± 6.7 (13)	13.5 ± 4.4 (13)	13.9 ± 5.2 (14)	< 0.001
Mother's occupation, n (%):						
Employed on maternity	880 (68.0)	113 (91.1)	68 (55.6)	458 (76.5)	241 (88.9)	
Employed worker	130 (10.0)	–	77 (25.6)	52 (8.7)	1 (0.4)	
Housewife	184 (14.2)	3 (2.4)	133 (44.2)	47 (7.8)	1 (0.4)	
Other condition	101 (7.8)	8 (6.5)	23 (7.6)	42 (7.0)	28 (10.3)	< 0.001
Mothers' marital status, n (%):						
Married/Living together	1227 (94.4)	124 (97.6)	298 (98.7)	542 (90.3)	263 (97.1)	
Widow/single/never married/Separated/divorcing	73 (5.6)	3 (2.4)	4 (1.3)	58 (9.7)	8 (2.9)	< 0.001
Mother's educational level, n (%):						
Elementary and middle school	265 (20.3)	74 (58.7)	73 (24.2)	100 (16.5)	18 (6.6)	
High school	560 (43.0)	17 (13.5)	166 (55.2)	293 (48.4)	84 (31.0)	
University degree	478 (36.7)	35 (27.8)	62 (20.6)	212 (35.0)	169 (62.4)	< 0.001
Home size, n (%):						
< 50 mq	108 (8.3)	15 (12.2)	12 (4.0)	41 (6.8)	40 (14.7)	
50–100 mq	812 (62.7)	73 (59.3)	175 (58.1)	406 (67.7)	158 (58.1)	
> 100 mq	376 (29.0)	35 (28.5)	114 (37.9)	153 (25.5)	74 (27.2)	< 0.001
Number of cigarettes smoked, by mother, during pregnancy, mean ± std (median)	162.4 ± 591.4 (0)	150.0 ± 477.7 (0)	210.6 ± 683.2 (0)	144.2 ± 559.6 (0)	154.7 ± 596.4 (0)	0.127
Alcoholic drinks per week during pregnancy, mean ± std (median)	1.5 ± 2.8 (0.5)	1.2 ± 2.2 (0.3)	1.6 ± 2.7 (0.6)	1.5 ± 2.8 (0.3)	0.8 ± 1.4 (0.3)	0.013
Fish consumption of mother per week during pregnancy, mean ± std (median)	1.4 ± 1.2 (1)	1.8 ± 1.1 (1.5)	1.7 ± 1.3 (1.5)	1.5 ± 1.2 (1.1)	0.9 ± 0.9 (0.6)	< 0.001
Children's sex, n (%):						
Male	634 (48.6)	69 (54.3)	130 (43.5)	306 (50.4)	129 (47.4)	0.124
Female	671 (51.4)	58 (45.7)	169 (56.5)	301 (49.6)	143 (52.6)	
Birth weight (g), mean ± std (median)	3418.9 ± 458.5 (3410)	3583.7 ± 385.7 (3580)	3317 ± 418.3 (3300)	3418.9 ± 452.5 (3400)	3450.8 ± 516.3 (3475)	< 0.001
Breastfeeding months, mean ± std (median)	8.4 ± 6 (8)	5.8 ± 5.3 (4)	4.2 ± 5 (2)	10.1 ± 5.9 (10)	8.9 ± 5.3 (9)	< 0.001
Child intake of homogenized fish (number of months with at least one portion per week), mean ± std (median)	2.1 ± 4.1 (0)	1.3 ± 3.4 (0)		2.7 ± 4.5 (0)	0.9 ± 2.5 (0)	< 0.001
Child intake of fresh fish (number of months with at least one portion per week), mean ± std (median)	7.7 ± 4.2 (8)	6.4 ± 4.9 (7.0)	7.6 ± 2.2 (7)	9.1 ± 4 (10)	4.7 ± 4.4 (5)	< 0.001
Number of children living in home (excluding the newborn), n (%):						
None	689 (52.7)	71 (55.9)	116 (38.4)	355 (58.5)	147 (54.0)	
One or more	619 (47.3)	56 (44.1)	186 (61.6)	252 (41.5)	125 (46.0)	< 0.001
Daycare attendance at 18 month, n (%):						
Member of the family or other people not included in the family	970 (74.2)	116 (91.3)	293 (97.0)	378 (62.3)	183 (67.3)	
Kindergarden	338 (25.8)	11 (8.7)	9 (3.0)	229 (37.7)	89 (32.7)	< 0.001

[a] Differences among countries were assessed by the Chi Square test (for categorical variables) and the Kruskall Wallis test (for continuous variables).

used to derive corresponding percentiles which were converted to composite scores. Higher are the composite or the scaled scores, and better is the child's performance (neurodevelopment). Scaled scores range from 1 to 19 and composite scores from 40 to 160.

2.5. Potential explanatory variables

The potential explanatory variables considered in this study were: mother's age at delivery, maternal body mass index before pregnancy; weight gain, smoking habits, alcohol and fish consumption of mother during pregnancy; as socio-economic indicator we used occupational and marital status, educational level, home size (surface in m^2 of the house in which mother lived) of mother during pregnancy; child's gender and birth weight in grams, child's fresh and homogenized fish consumption until 18 months of age, breastfeeding history until 18 months of age, number of children living in home and daycare attendance at age 18 months. Fish consumption during pregnancy was collected soon after delivery using a detailed 138-item questionnaire adapted from a validated food frequency questionnaire (FFQ) (Valent et al., 2013b). The questionnaire included seven quantitative questions on fish, which addressed the frequency of consumption of 150-g servings of fish, crustaceans, and mollusks (cooked according to different recipes), and fish in oil. For each fish item, conversion from categories of consumption into continuous intakes of fish servings was done by assigning to each category a consumption level equal to the median value for that category (e.g., 2–4 times/week became three times/

Table 2
Distributions of THg concentrations (ng/g) in different biological samples and BSID-III composite and scaled score of children assessed at 18 months.

	N	Mean	SD	Min	20th percentile	40th percentile	Median	60th percentile	80th percentile	Max	*p*-value[a]
THg in maternal hair during pregnancy (ng/g)	1282	997.0	1035.1	16.9	337	566	704	881	1456	13520.0	
Croatia	127	967.9	1176.6	24	288	555	662	782.6	1175	8710	
Greece	295	1408.5	1146.9	55	550	960	1097	1371.5	2003	8973	
Italy	605	1062.1	1029.0	16.9	436	641.5	788	938.5	1475	13520	
Slovenia	255	381.0	297.1	24	127.5	251.5	317	378.5	565.5	1880	< 0.001
THg in maternal blood during pregnancy (ng/g)	733	3.2	3.4	0.1	1.0	2.0	2.4	2.8	4.4	39.6	
Croatia	126	3.2	2.9	0.6	1.2	1.9	2.3	3.0	4.5	20.5	
Italy	607	3.2	3.5	0.1	1.0	2.0	2.4	2.8	4.4	39.6	0.355
THg in cord blood (ng/g)	1078	5.2	5.0	0.1	1.5	2.8	3.6	4.6	7.8	33.1	
Croatia	122	5.1	5.1	0.5	1.4	2.7	3.7	4.7	7.7	32.3	
Greece	247	7.7	6.0	0.5	2.8	4.8	6.2	7.4	11.4	33.1	
Italy	458	5.6	4.8	0.1	2.1	3.4	4.0	5.2	7.9	32.8	
Slovenia	251	2.1	1.6	0.2	0.8	1.3	1.7	1.9	3.0	10.0	< 0.001
THg in breast milk (ng/g)	819	0.4	1.2	0.0	0.1	0.2	0.2	0.2	0.4	28.3	
Croatia	75	0.3	0.4	0.0	0.1	0.1	0.2	0.2	0.3	2.4	
Greece	36	1.7	2.9	0.0	0.2	0.4	0.7	1.1	2.0	11.5	
Italy	492	0.3	1.3	0.0	0.1	0.2	0.2	0.2	0.3	28.3	
Slovenia	216	0.3	0.3	0.0	0.1	0.2	0.2	0.2	0.4	2.9	< 0.001
Cognitive composite score	1302	106.9	11.3	65	100	105	105	110	115	145	
Croatia	126	107.5	13.2	80	95	100	105	110	120	145	
Greece	300	101.4	10.9	65	95	100	100	105	110	135	
Italy	604	106.3	8.2	75	100	105	105	110	115	130	
Slovenia	272	114.1	12.7	70	105	110	115	115	125	145	< 0.001
Language composite score	1302	100.3	12.0	47	91	97	100	103	109	141	
Croatia	126	108.0	14.2	65	97	103	109	112	118	141	
Greece	300	97.2	12.5	47	86	94	97	100	109	132	
Italy	604	97.8	8.6	47	91	97	97	100	106	121	
Slovenia	272	105.5	13.6	56	94	103	106	109	115	141	< 0.001
Receptive Communication scaled score	1300	11.1	2.5	1	9	10	11	11	13	19	
Croatia	126	12.2	2.7	3	10	12	12	13	14	19	
Greece	300	10.0	2.5	1	8	9	10	11	12	16	
Italy	604	10.6	1.6	1	9	10	11	11	12	14	
Slovenia	270	12.7	2.8	5	10	12	13	14	15	19	< 0.001
Expressive Communication scaled score	1301	9.0	2.2	1	7	8	9	9	11	18	
Croatia	126	10.4	2.9	4	8	10	10	11	13	17	
Greece	299	9.0	2.2	1	7	8	9	10	11	17	
Italy	604	8.6	1.8	1	7	8	9	9	10	14	
Slovenia	272	9.2	2.4	3	7	8	9	9	11	18	< 0.001
Motor composite score	1302	102.6	8.5	61	97	100	103	103	110	142	
Croatia	126	108.3	10.1	85	100	107	107	110	115	142	
Greece	300	98.8	8.4	61	94	97	100	100	107	121	
Italy	604	101.5	5.8	67	97	100	100	103	107	115	
Slovenia	272	106.5	9.6	61	100	103	107	110	112	136	< 0.001
Fine Motor scaled score	1302	11.5	1.8	3	10	11	11	12	13	19	
Croatia	126	11.8	2.1	8	10	11	12	12	14	19	
Greece	300	10.9	2.0	3	10	10	11	11	13	18	
Italy	604	11.4	1.4	6	10	11	11	12	13	16	
Slovenia	272	12.3	2.1	4	11	12	12	13	14	19	< 0.001
Gross Motor scaled score	1298	9.3	1.5	1	8	9	9	10	10	19	
Croatia	126	10.8	2.2	4	9	10	10	11	12	19	
Greece	299	8.7	1.4	3	8	9	9	9	10	12	
Italy	603	9.0	1.0	1	8	9	9	9	10	12	
Slovenia	270	9.9	1.6	4	9	10	10	10	11	15	< 0.001

[a] Differences among Countries were assessed by the Kruskall Wallis test.

week). Overall fish intake was calculated by summing the estimated weekly intake of all fish types (Valent et al., 2013b).

2.6. Statistical analysis

THg concentrations in different biological samples and BSID-III scores were described, for each country, by arithmetic means and standard deviations (SDs), quintiles, median and minimum-maximum ranges. The other continuous variables are presented as means, medians, and SDs. Categorical variables are presented as number and percentages. Differences among countries for general characteristics of the enrolled population, for distributions of THg concentrations and for the BSID-III scores were assessed by the Kruskal Wallis test (for continuous variables) and the $\chi 2$ test (for categorical variables). The relation between THg concentrations and BSID-III scores was explored with linear regression analysis. Logistic regression analysis was conducted as sensitivity analysis. Linear regression models included BSID-III scores as dependent continuous variables assuming that the neurodevelopmental scores decreased proportionally with increasing THg concentrations. THg was $\log_2$ transformed because of its skewed distribution. However, models with no logarithmic transformation were also built for sensitivity analysis (data not shown). Logistic regression was conducted to evaluate the hypothesis that the probability of suboptimal development (Golding et al., 2016), measured as a dichotomous variable derived from the distribution of the BSID-III score, depended on the increasing THg concentration. Given that the Italian sub-cohort was the largest, the lower end of each Italian BSID-III score distribution closest to 20% was defined as suboptimal development (Golding et al., 2016), and considered as a cut-off to dichotomize the corresponding scores in all countries. Separate models were built for each neurodevelopmental

Table 3
Meta-analysis of the association between BSID-III scores and THg concentrations in maternal hair, maternal blood, cord blood and breast milk, when no heterogeneity by country was found.

	Meta-analysis Linear regression			Meta-analysis Logistic regression		
	N	Overall β[a]	95%CI	N	Overall OR[c]	95%CI
Cognitive composite score:						
THg in maternal hair (ng/g)	1083	0.20[a]	(-0.29, 0.69)	1081	0.99[a]	(0.97, 1.00)
THg in maternal blood[b] (ng/g)	636	−0.09[a]	(-0.61, 0.43)	634	0.98[a]	(0.93, 1.03)
THg in cord blood (ng/g)	892	0.13[a]	(-0.39, 0.64)	891	0.98[a]	(0.95, 1.01)
THg in breast milk (ng/g)	734	−0.01[a]	(-0.53, 0.50)	738	1.07[a]	(0.90, 1.28)
Language composite score:						
THg in maternal hair (ng/g)	1086	0.55[b]	(0.05, 1.05)	1084	0.99[g]	(0.98, 1.01)
THg in cord blood (ng/g)	896	0.25[b]	(-0.29, 0.78)	895	0.98[g]	(0.95, 1.02)
THg in breast milk (ng/g)	735	0.26[b]	(-0.26, 0.79)	739	0.94[g]	(0.74, 1.19)
Motor composite score:						
THg in maternal hair (ng/g)	1083	−0.12[c]	(-0.47, 0.22)	1074	1.01[h]	(0.99, 1.02)
THg in maternal blood[b] (ng/g)	636	0.11[c]	(-0.25, 0.48)	628	0.99[h]	(0.94, 1.06)
THg in cord blood (ng/g)	892	−0.11[c]	(-0.47, 0.25)	848	1.00[h]	(0.97, 1.04)
THg in breast milk (ng/g)	734	−0.01[c]	(-0.38, 0.36)	733	1.09[h]	(0.86, 1.39)
Receptive Communication scaled score:						
THg in maternal hair (ng/g)	1075	0.12[d]	(0.02, 0.22)	1083	0.99[b]	(0.97, 1.00)
THg in maternal blood[b] (ng/g)	628	−0.02[d]	(-0.12, 0.08)	635	1.00[b]	(0.94, 1.07)
THg in cord blood (ng/g)	887	0.12[d]	(-0.08, 0.32)	861	0.99[b]	(0.96, 1.02)
THg in breast milk (ng/g)	728	0.08[d]	(-0.02, 0.19)	732	0.96[b]	(0.74, 1.23)
Expressive Communication scaled score:						
THg in maternal hair (ng/g)	1272	0.04[e]	(-0.06, 0.13)	1084	1.00[i]	(0.98, 1.01)
THg in maternal blood[b] (ng/g)	727	0.13[e]	(-0.22, 0.48)	635	0.98[i]	(0.92, 1.04)
THg in cord blood (ng/g)	1070	0.01[e]	(-0.09, 0.11)	895	1.01[l]	(0.98, 1.04)
THg in breast milk (ng/g)	809	0.01[e]	(-0.09, 0.10)	739	0.90[l]	(0.68, 1.20)
Fine Motor scaled score						
THg in maternal hair (ng/g)	1082	−0.03[f]	(-0.11, 0.06)	985	1.01[l]	(0.99, 1.02)
THg in maternal blood[h] (ng/g)	636	0.05[f]	(-0.04, 0.15)	542	1.01[l]	(0.96, 1.08)
Gross Motor scaled score:						
THg in maternal hair (ng/g)	1081	−0.01[a]	(-0.07, 0.05)	1072	1.00[m]	(0.99, 1.01)
THg in maternal blood[b] (ng/g)	635	−0.02[a]	(-0.08, 0.05)	627	1.01[m]	(0.95, 1.07)
THg in cord blood (ng/g)	890	−0.03[a]	(-0.09, 0.03)	882	1.03[m]	(1.00, 1.07)
THg in breast milk (ng/g)	732	0.09[a]	(-0.07, 0.24)	692[d]	0.95[m]	(0.75, 1.20)

[a]Adjusted for mother's educational level, child's gender, child's birth weight (grams), child's consumption of fresh fish.
[b]Adjusted for mother's educational level, child's gender, child's consumption of fresh fish, number of children living in home.
[c]Adjusted for child's gender, child's birth weight (grams), breastfeeding history.
[d]Adjusted for mother's educational level, child's gender, child's consumption of fresh fish, number of children living in home, home size.
[e]Adjusted for mother's educational level, child's gender, number of children living in home.
[f]Adjusted for mother's educational level, child's gender, child's birth weight (grams), breastfeeding history, mother's fish consumption during pregnancy.
[g]Adjusted for mother's educational level, child's gender, child's consumption of fresh fish.
[h]Adjusted for mother's occupation, child's gender, child's birth weight (grams), child's consumption of fresh fish, breastfeeding history, day care attendance at 18 months.
[i]Adjusted for mother's educational level, child's gender, child's consumption of fresh fish, breastfeeding history.
[l]Adjusted for child's gender, mother's smoking habits, mother's weight gain during pregnancy, breastfeeding history, day care attendance at 18 months.
[m]Adjusted for child's gender, child's birth weight (grams), child's consumption of fresh fish.

[a] THg concentrations (ng/g) were $\log_2$ transformed in multiple linear regression.
[b] Meta-analysis was conducted between Italy and Croatia, the only countries that had maternal blood samples.
[c] The ORs were estimated each 100 ng/g for THg concentrations in maternal hair.
[d] Meta-analysis was conducted only among Italy, Greece, and Slovenia. In Croatia only 4 children were in the lowest quintile and had valid THg measurements in breast milk, therefore the logistic model was not applied.

score and stratified by country. Multivariate linear and logistic regression models were built to assess the association between THg and BSID-III score, adjusting for potential confounding variables. Among the covariates considered as potential confounders, only those associated with at least one BSID-III outcome ($p < 0.15$), were included in the final models (data not shown). Given that THg concentrations were $\log_2$ transformed, in linear regression models the β coefficient corresponded to the variation in BSID-III score associated with the doubling in THg levels (i.e., the lower the β coefficient, the worse the effect of THg concentration). In logistic regression models, the odds ratio (OR) corresponded to the risk of a suboptimal development associated with the THg levels (i.e., the higher the OR, the worse the effect of THg concentration). Meta-analysis procedures were performed to assess combined estimates of the association between each of the seven BSID-III scores and the pre and post-natal mercury exposure from the four countries. Adjusted β coefficients and ORs obtained by multivariate models were used. The presence of heterogeneity among the four countries was explored. Estimates of association were computed using weighted regression, and the weights were the inverse of the local variances, i.e., according to the fixed-effect model. The heterogeneity, I-squared (I2), was calculated under the fixed-effect hypothesis and if the heterogeneity was detected (I2 > 50%), the random-effect was applied. When no heterogeneity was found, only the overall estimates were shown. In case of heterogeneity, the adjusted β coefficients and ORs for each country were shown in forest plots. SAS (version 9.4 SAS Institute INC., Cary, N.C., USA) and STATA (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP) were used for the statistical analysis.

3. Results

3.1. Descriptive analyses

In total, 2189 (C = 233; G = 466; I = 900; S = 590) pregnant women were enrolled in the cohort, while 1308 subjects remained in the study and provided information for this analysis. Compared with those who remained under observation, mothers withdrawn from the study were more likely to hold an "Elementary and Middle school degree" (28.0% vs 20.8%), less likely to hold a "University degree", (31.3% vs 36.5%, p = 0.013) and less likely to live in a house they

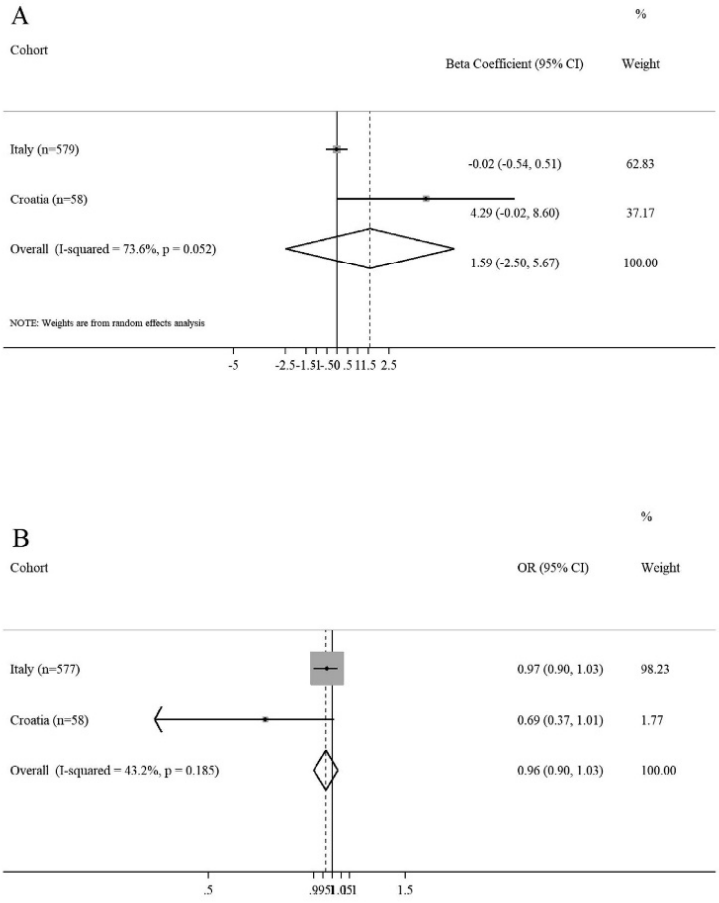


Fig. 1. Meta-analysis of the multiple linear regression (A) and of the multiple logistic regression (B) between LAN composite score and THg concentrations in maternal blood.

For multiple linear regression THg concentrations are $\log_2$ transformed.

A. Adjusted for mother's educational level, child's gender, child's consumption of fresh fish, number of children living in home.

B. Adjusted for mother's educational level, child's gender, child's consumption of fresh fish.

owned (64.1% vs 71.5%, p = 0.008). On the other hand, marital status, employment status, and age at delivery were similar.

The characteristics of mother-child pairs in the four countries are shown in Table 1. Mother's age at delivery, employment and marital status, and educational level were significantly different among the four countries. In Slovenia, the maternal intake of fish (servings/week) during pregnancy was the lowest. Italian mothers introduced fresh fish consumption in the diet of their children earlier than the mothers of the other three countries. Duration of breastfeeding (number of months of breastfeeding up to 18 months) appears more prolonged in I and S than in C and G.

The distribution of THg concentrations and the BSID-III composite and scaled scores by country is presented in Table 2. Mean concentrations of THg in maternal hair, cord blood, and breast milk were higher in the Greek sub-cohort. The lowest mean levels of THg in maternal hair and cord blood were found in Slovenia. Mean composite and scaled score were higher in Croatian and Slovenian children. The distribution of BSID-III scores, as continuous and dichotomous variables, and unadjusted comparisons of differences by mother's and child's characteristics are shown in Appendix.

3.2. Meta-analysis

Table 3 shows the overall (all countries combined) results of the meta-analysis, using both β coefficients and ORs obtained by the fully adjusted multivariate models, between each BSID-III score and pre- and post-natal THg exposure, when no heterogeneity by country was found. Considering the meta-analysis of multivariate linear models, an overall positive association was found between LAN and RC and increasing THg in maternal hair (n = 1086; β = 0.55; 95%CI: 0.05–1.05 and n = 1075; β = 0.12; 95%CI: 0.02–0.22, respectively). The overall adjusted OR between THg in cord blood and suboptimal GM score was borderline significant (n = 882; OR = 1.03; 95%CI: 1.00–1.07). In details the number of children with a suboptimal GM score was: 2 in Croatia (n = 53), 72 in Greece (n = 180), 98 in Italy (n = 424) and 34 in Slovenia (n = 225).

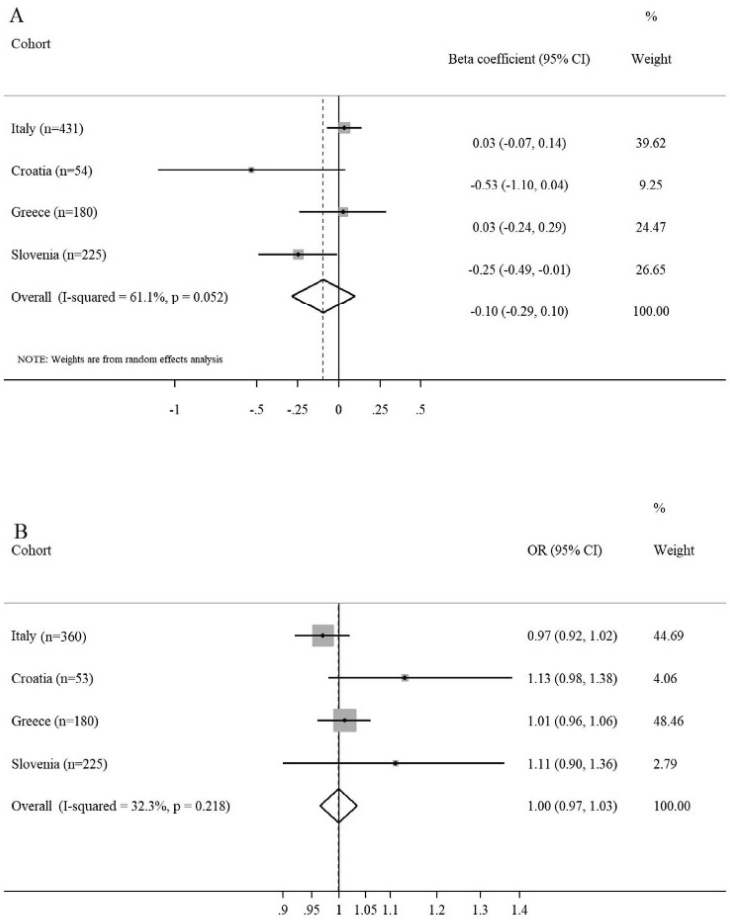


Fig. 2. Meta-analysis of the multiple linear regression (A) and of the multiple logistic regression (B) between FM scaled score and THg concentrations in cord blood.

For multiple linear regression THg concentrations are $\log_2$ transformed.

A. Adjusted for mother's educational level, child's gender, child's birth weight (grams), breastfeeding history, mother's fish consumption during pregnancy.

B. Adjusted for child's gender, mother's smoking habits during pregnancy, mother's weight gain during pregnancy, breastfeeding history, day care attendance at 18 months.

Heterogeneity by country was found for the relation between LAN and THg in maternal blood (Fig. 1), and for FM and THg in cord blood (Fig. 2) and breast milk (Fig. 3). The association, measured by the multivariate linear regression, between LAN and $\log_2$(THg) concentrations in maternal blood, is shown in Fig. 1A. Samples of maternal venous blood were collected only in Italy and Croatia. In Croatia, such an association was positive and borderline significant (n = 58; β = 4.29; 95%CI: −0.02, 8.60). Consistent results are also obtained in Croatia by multivariate logistic regression (Fig. 1B), where the THg concentrations in maternal venous blood were associated with a reduced risk for children to fall in the lowest quintile of LAN of 31% (n = 58; OR = 0.69; 95%CI: 0.37, 1.01). Instead, in Italy LAN was not associated with THg concentrations in maternal venous blood neither in linear nor in logistic regression models. A borderline statistically significant, inverse association between FM and $\log_2$(THg) concentrations in cord blood is shown in Fig. 2A for Croatia (n = 54, β = −0.53; 95%CI: −1.10, 0.04) and Slovenia (n = 225; β = −0.25; 95%CI: −0.49, −0.01). The associations of THg in breast milk with FM are displayed in Fig. 3A (linear regression) and Fig. 3B (logistic regression). The linear regression models did not show a significant association for any country (Fig. 3A). From the logistic regression models (Fig. 3B), for Slovenia, higher THg levels in breast milk were associated with higher risk of suboptimal FM (n = 195; OR = 5.25; IC95%: 1.36, 21.10).

4. Discussion

THg concentrations found in biological samples of our Eastern Mediterranean study were much lower than those reported in the Faroe and Seychelles studies (Myers et al., 2007; Grandjean et al., 1992; Cernichiari et al., 1995) but also rather lower than in previous studies conducted in New Zealand (Kjellström et al., 1989) and in the Spanish INMA study (Llop et al., 2012). Nevertheless, the mean and median THg in maternal hair, maternal blood, and cord blood in our overall cohort and the sub-cohorts from Greece, Italy, Croatia and Slovenia were not very different from those measured in populations of most European countries, US regions and the reference dose (RfD) of 5,5 ng/g for Hg in

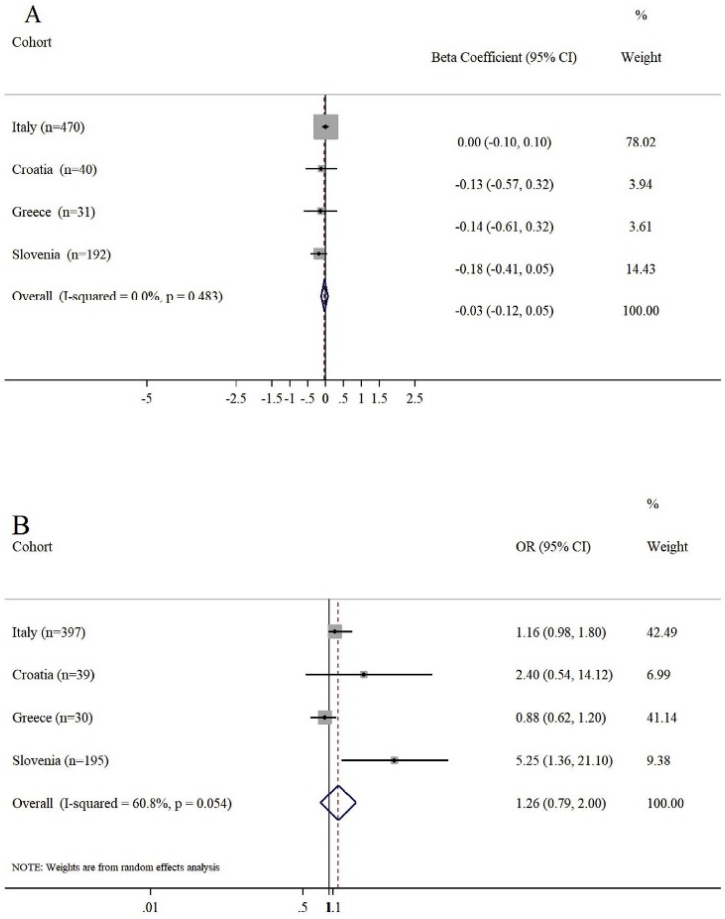


Fig. 3. Meta-analysis of the multiple linear regression (A) and of the multiple logistic regression (B) between FM scaled score and THg concentrations in breast milk.

For multiple linear regression THg concentrations are $\log_2$ transformed.

A. Adjusted for mother's educational level, child's gender, child's birth weight (grams), breastfeeding history, mother's fish consumption during pregnancy.

B. Adjusted for child's gender, mother's smoking habits during pregnancy, mother's weight gain during pregnancy, breastfeeding history, day care attendance at 18 months.

cord blood, calculated by EPA (Gao et al., 2007; Mahaffey et al., 2009). For this reason, we believe that our results may be relevant, especially when extrapolated to most western populations.

In our study, the main results of the meta-analysis do not show overall negative associations with cognitive and language scales. More complex is the interpretation of the results found in relation to motor scales. In addition, although differences exist by country in terms of mother's age at delivery, employment and marital status, educational level, maternal intake of fish during pregnancy, child's intake of fish up to 18 months and mean BSID-III composite and scaled scores, the results of the meta-analysis focused on the effects of THg on the BSID-III do not show a significant heterogeneity by country, except for some THg measurements and LAN and FM.

4.1. Cognitive scales

Combined analyses did not find convincing associations between THg and cognitive outcomes.

In our study THg levels in cord blood do not correlate better with BSID-III composite scores compared to THg levels in mother's hair. This observation differs with some evidence of the literature (Barregård, 2005). According to some authors, THg concentration in cord blood is the preferred biomarker for prenatal exposure. Hg concentrations in hair can be affected by several factors, including hair color and variable growth rates, limiting its usefulness as an indicator of Hg concentrations in the body (Budtz-Jørgensen et al., 2004).

Although a large number of covariates were considered to adjust β coefficients and ORs used in the meta-analysis, neurodevelopment might be influenced by variables that are still not known or not well measured. In our study, we did not take into account some pollutants in fish that might affect neurodevelopment and also the genetic susceptibility was not considered. MeHg metabolism differs largely between individuals and may explain differences in MeHg susceptibility, i.e., individuals with a slower MeHg metabolism accumulate MeHg and develop more toxic effects. For the developing child, this means that the slower the metabolism in the mother, the higher the MeHg dose in the fetus; also the elimination half-time of MeHg varies greatly between individuals, ranging from 45 to 70 days, but extreme values, up to almost 190 days have also been reported (Clarkson, 2002). One explanation for this variation might be hereditary differences in MeHg metabolism. The genetic background can result in individual differences in MeHg body retention despite similar fish intake. Some evidence suggests that genetic factors are very important for MeHg toxicity and several key genes for Hg toxicity have already been identified (Custodio et al., 2004; Engström et al., 2013; Gundacker et al., 2009; Schläwicke Engström et al., 2008). For example, there is evidence that variants of genes associated with glutathione (GSH) metabolism are determinants of MeHg retention in the body. Some evidence from two Mediterranean cohorts supports a role of ABC transporter genes in the accumulation of MeHg during early development (Llop et al., 2014). In addition, recent analyses conducted in subjects enrolled in the INMA and in the Italian and Greek components of this PHIME study showed that the BSID mental scale improved with the increase of cord blood Hg concentrations for children carrying a high activity allele of CYP3A, while for children carrying low activity CYP3A alleles the association was near null (Llop et al., 2017). Results from the Croatian and Slovenian cohorts of the PHIME study indicates the importance of the genetic polymorphism apolipoprotein E (APOE). Carriers of the APOE ε4 allele showed a negative association between hair or cord blood THg levels and cognitive scores of the BSID III test, while this was not observed with ε2 or ε3 carriers (Snoj Tratnik et al., 2017).

Despite inconsistent results found in different studies, in an overview Karagas et al. (2012) concluded that the recent evidence suggests that low-level MeHg exposure might affect fetal growth, and evidence exists that low levels of prenatal MeHg exposure may cause neurocognitive effects in early childhood. Our results do not support the conclusions by Karagas and are consistent with those from the INMA cohort (Llop et al., 2012; Ramon et al., 2011).

4.2. Language scales

In the overall Mediterranean PHIME cohort, the meta-analysis carried out using the adjusted β coefficients for the 4 countries showed that higher maternal hair THg levels were associated with better LAN scores. This positive association with maternal hair THg is explained by the RC component, within the LAN scale. The relations between LAN scale and THg measured in cord blood and breast milk do not show a statistically significant positive association. For THg measured in maternal blood heterogeneous results were observed given that only for Croatia a positive, borderline significant, association was seen (Fig. 1). Due to the limited size of the Croatian sub-cohort, the interpretation of these results should be considered with caution. Also, although we adjusted all our analyses for several potential confounders, residual confounding may still be present (Genuis, 2008; Gil and Gil, 2015; Starling et al., 2015).

Although these findings appear to be in contrast with the results conducted in 3 years old children in Taiwan (His et al., 2014), they are consistent with those from ALSPAC (Iles-Caven et al., 2016).

4.3. Motor scales

We found no evidence of adverse associations between THg exposure, measured in all the biological samples, and MOT score. These results are consistent with those from the ALSPAC study (Golding et al., 2016) conducted in England, with the New England Children's Amalgam Trial (Bellinger et al., 2007) and partially with the Spanish INMA study that found that increasing THg levels were associated with significantly lower psychomotor scores only among females (Ramon et al., 2011). On the other hand, in the Japanese Tohoku Study of Child Development, The Psychomotor Development Index (PDI) of BSID-II was significantly correlated with cord-blood THg only in boys, and significance of the association remained unchanged after adjusting for possible confounders (Tatsuta et al., 2017).

The interpretation of our study is more complex when the two motor components (FM and GM) of BSID-III are analyzed separately. For the whole PHIME cohort, results of the meta-analysis using adjusted ORs show that THg is associated with a slightly increased risk of a suboptimal GM score only in the cord blood. Heterogeneity by country was found for the association between THg cord blood and FM score, with a borderline statistically significant negative association for the Croatian and Slovenian sub-cohorts (Fig. 2A). These results confirm what was recently published restricted to the Croatian PHIME sub-cohort (Prpić et al., 2017). Furthermore, in the Slovenian sub-cohorts (Fig. 3B) THg concentrations in breast milk appeared to be a risk factor for an FM performance within the lowest quintile. Some evidence has emerged on the interaction effect between gene polymorphisms of APOE and Hg levels on fine motor scores, as in a Taiwanese cohort examined at two years with Hg measured in the cord blood (His et al., 2014).

On the other hand, Hg (with and without eating fish) was associated neither with fine nor with gross motor skills in the ALSPAC study (Iles-Caven et al., 2016). In the Seychelles Child Development Study, there were no adverse associations between prenatal MeHg and any of the measured endpoints, including fine motor skills (Van Wijngaarden et al., 2017).

Finally, a recent review related to the causes of impairment in fine motor development conclude that there is conflicting evidence regarding prenatal and early child effects of Hg. However, the evidence from primate studies and the Minimata accident raises the possibility of an adverse effect many years after exposure (Golding et al., 2014). Therefore, special effort should be devoted to the long-term follow-up of mother-child cohorts especially as it refers to measuring

psychomotor outcomes.

5. Methodological issues

Results from our study must be evaluated bearing in mind the reasons that lead to conflicting evidence concerning prenatal and early child effects of mercury (Starling et al., 2015). Main differences by study protocol may include: type of biological samples used to measure Hg levels (maternal hair, blood and urine; cord blood; breast milk; child hair and urine; etc.), timing of sampling (first, second or third trimester of pregnancy, birth, postnatal, etc.); laboratory methods in Hg measurements; proportion of Hg species; comparability of neurodevelopment testing (Bayley I, II, III, others); child age at follow-up; confounding factors (e.g., maternal age and intelligence, maternal smoke or use of alcohol, breastfeeding, family socioeconomic status, co-exposure to other toxic elements or to beneficial nutrients) and effect modifiers such as child's gender, ethnicity and genetic variations. On this respect, our study was able to measure Hg from several biological samples, but the timing of data collection varied by country. The same lab (Institute Josef Stefan, Ljubljana, Slovenia) measured Hg for all samples but not all measurements included a complete Hg speciation, i.e., THg values from this study were assumed to be representative of a correct MeHg measure. Unmeasured or poorly measured determinants of neurodevelopmental outcomes may have left residual confounding in the measures of association. Additionally, a thorough assessment of interaction was beyond the scope of this general paper. Finally, this article focuses on a neurodevelopment assessment conducted at 18 months of age. Therefore, the full effects of prenatal Hg exposure may not be measurable yet.

Additionally, it cannot be excluded that mother's and child's fish intake could be misreported on the questionnaires and the estimates of weekly servings could be affected by errors leading to residual confounding of Hg by fish intake. Maternal IQ during pregnancy was tested only in the Italian and Croatian sub-cohorts, and measurements of PUFAs were available only for Italy. Finally, our study may not have had sufficient statistical power to detect the effects of Hg if they were very subtle.

Even if we were aware that by dichotomizing the Bayley scores we would have lost valuable information, we ran logistic regression models anyway for the purpose of sensitivity analyses, as also done in the ALSPAC study (Golding et al., 2016). With the odds ratio could be in some conditions (for some outcomes and some cohorts) an overestimate of the relative risk because the outcome (the cut-off was the lowest 20% in Italian subcohort) is not a rare event and in these cases log-binomial regression could have been the most appropriate model.

6. Conclusions

We found some evidence that THg was associated with decreased developmental motor scores at 18 months. However, these results, which describe both fine and gross motor development and were measured in different biological samples and in four Mediterranean populations, have only partial internal and external consistency and should be confirmed by further testing conducted at an older age. No detrimental effects of THg were demonstrated for cognitive and language outcomes so far at these low levels. Follow-up activities within this Mediterranean PHIME cohort are ongoing to elucidate further the long-term neurological effects of prenatal mercury exposure.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ijheh.2018.07.011.

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3.5 Prenatal Mercury Exposure, Apolipoprotein E and Neurodevelopment

The paper was authored by J. Snoj Tratnik, I. Falnoga, A. Trdin, D. Mazej, V. Fajon, A. Miklavčič, and M. Horvat from the JSI; A. B. Kobal, J. Osredkar, A. Sešek-Briški, M. Krsnik, D. Neubauer, J. Kodrič, S. Stropnik, D. Gosar, and P. Lešnik Musek from the University Medical Center Ljubljana; J Marc and S. Jurkovič Mlakar from the Faculty of Pharmacy Ljubljana; O. Petrović, I. Vlašić-Cicvarić, I. Prpić, A. Milardović, J. Radić Nišević, D. Vuković, E. Fišić from the Medical Center in Rijeka; and Z. Špirić from the Institute for Applied Ecology, OIKON, Zagreb. It has been published in the Environmental Research.

This paper presents an extension of the work presented in the previous Section (3.4), which continued within the CROME-LIFE project (Cross-Mediterranean Environment and Health Network). The present work further assessed the association between prenatal exposure to Hg and neurodevelopment, taking into account co-exposure to Pb, essential element status and the genetic background of children. Polymorphism of apolipoprotein E (APOE) gene was selected as a susceptibility biomarker which could have a role in toxicokinetics of Hg and/or synaptogenesis and neurite growth during the development of a child's nervous system. The associations were evaluated separately for carriers of different alleles of the *APOE*. The genotyping analyses have been done within the CROME-LIFE project for the two neighboring population groups of mother-child pairs – Slovenian and Croatian.

The paper is important because it demonstrates the importance of identifying genotypes associated with increased or decreased susceptibility of individuals. We showed that even at low to moderate exposure to Hg, which was mainly through fish consumption, carriers of variant alleles showed negative association between Hg in cord blood and cognitive development, while the carriers of the common alleles showed no statistically significant association. Such studies contribute to the exposome approach, which aims to reveal not only the totality of exposures, but also the internal chemical environment of individuals and its role in the exposure.

The paper fulfilled the following aims of the thesis:

- Construction of statistical model to assess association between low-level prenatal Hg exposure and neurodevelopment (cognitive, language and motor), including all potential factors that could influence exposure and/or neurodevelopmental outcome (co-exposure, essential elements status, life-style habits).
- Evaluation of the role of *APOE* genetic polymorphism in association between the Hg exposure and neurodevelopment.

Since the paper has been published, the work has continued to include additional polymorphic genes, namely *BDNF*, *PON1*, and *CPOX*. In all cases, carriers of variant alleles showed a more negative association between prenatal Hg exposure and cognitive scores in comparison to carriers of common alleles. The work has been presented at the PPTOX VI conference in the Faroe Islands (Snoj Tratnik et al., 2018).

I conducted a part of Hg analysis in cord blood and maternal hair, participated in interviewing the mothers 6-8 weeks after birth, managed the database, performed the statistical analysis and wrote the manuscript.

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Prenatal mercury exposure, neurodevelopment and apolipoprotein E genetic polymorphism

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ABSTRACT

The aim of the present study was to evaluate the association between prenatal exposure to mercury (Hg) and neurodevelopment of the child, taking into account genetic polymorphism of apolipoprotein E (*Apoe*) and other relevant confounders. Six hundred and one mother-child pairs were recruited from the central Slovenia region and 243 from Rijeka, on the Croatian coast of the northern Adriatic. The total Hg in cord blood, Bayley Scales of Infant and Toddler Development, Third Edition (Bayley-III) assessment at 18 months of age and *Apoe* genotyping was performed on 361 children; 237 of them were from Slovenia and 124 from Croatia. The results showed negative association between low-to-moderate Hg exposure in children with normal neurodevelopmental outcome and cognitive and fine motor scores at 18 months of age as assessed by Bayley III. The Hg-related decrease in cognitive score was observed only in children carrying at least one *Apoe* ε4 allele, while the decrease in fine motor scores was independent of the *Apoe* genotype. Adjusting for selenium (Se) and lead (Pb) levels, a positive association between Se and the language score and a negative association between Pb and the motor score was observed, but not in the subgroup of children carrying the ε4 allele.

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1. Introduction

Mercury (Hg) is among the environmental chemicals which are known to have adverse impacts on human health via various toxicological mechanisms. The general population is exposed to methyl Hg through the diet (mostly through fish consumption) and to elemental Hg (Hg^0) vapour through its release from dental amalgam fillings (UNEP/WHO, 2008). The central nervous system is the most sensitive target of methyl Hg and is also a target of Hg^0 vapour exposure, the neurotoxicity being prominent especially during the developmental stage of the nervous system (Andersen et al., 2000). While there is no clear evidence, that exposure to small amounts of Hg^0 vapour from dental amalgams in either prenatal or early postnatal exposure may induce sub-clinical changes in children (Davidson et al., 2004; Yoshida, 2002), methyl Hg can cause neurodevelopmental disorders and sub-clinical brain dysfunction at doses much lower than those affecting adult brain functions (Grandjean and Landrigan, 2006). Chronic exposure to low-to-moderate levels of methyl Hg (median level of 4.5 μg/g

Abbreviations: APOE, Apolipoprotein E; *Apoe*, apolipoprotein E gene; BDNF, brain-derived Neurotrophic Factor; BSID-II, Bayley Scales of Infant Development, Second Edition; Bayley-III, Bayley Scales of Infant and Toddler Development, Third Edition; CVAAS, Cold Vapour Atomic Absorption Spectrometry; GM, geometric mean; Max, maximum; Min, minimum; LOD, limit of detection; PHIME, Public Health Impact of Long-term Low-level Mixed Element Exposure in Susceptible Population Strata; PGR, progesterone receptor; PON1, Paraoxonase 1; PUFA, poly-unsaturated fatty acids; RfD, Reference Dose; SNP, single nucleotide polymorphism; TF, transferrin

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total Hg in maternal hair, 13 % of mothers exceeding 10 μg/g) can lead to neuropsychological dysfunctions in the domains of language, attention, and memory, and to a lesser extent in visual-spatial and motor functions as observed in a Faroe Islands birth cohort (Grandjean et al., 1997), where besides Hg, high exposure to polybrominated biphenyls was evident. A number of cross-sectional and prospective cohort studies assessed the neurodevelopmental effects of chronic low-to-moderate prenatal methyl Hg exposure (with maximum level of 26 μg/g total Hg in maternal hair) from maternal fish consumption (reviewed by Valent et al., 2013). However, the findings were inconsistent, possibly because of differences in outcomes, biomarkers, confounders, study populations, Hg concentrations, and other contaminants in fish (Valent et al., 2013).

One of the most recognized confounders in assessing neurodevelopmental effects are beneficial nutrients contained in fish: proteins, macroelements, microelements (selenium, zinc), fat-soluble vitamins and unsaturated fatty acids (PUFAs). Beside the nutritional and other confounders mentioned above, an increasing amount of evidence demonstrates that (epi)genetic variability of proteins involved in absorption, transport, elimination or distribution of toxic metals in the human body modifies (either protects or increases) the risk of toxic effects in an individual, especially at low exposures (Basu et al., 2014). There is evidence that gene polymorphisms for enzymes involved in glutathione and metallothionein metabolism can affect retention of methyl Hg in the human body (Barcelos et al., 2013; Gundacker et al., 2010; Schläwicke Engström et al., 2008; Wang et al., 2012) and that polymorphisms of ABC transporter genes can modify the transport of methyl Hg across the placenta and the accumulation of methyl Hg during early development (Llop et al., 2014). Apart from the genes involved in the absorption-distribution-metabolism-elimination pathways, there are certain other genotypes that can increase susceptibility to toxic effects. As recently observed by Julvez and Grandjean (2013) in a longitudinal cohort study ALSPAC (Julvez et al., 2013), any overall methyl Hg toxicity was not detectable at the low exposure levels, even when adjustments for beneficial dietary factors from maternal seafood intake and social class were included in the models. Adverse associations among genetically susceptible groups were discovered in analyses that were stratified by the single nucleotide polymorphism (SNP) allelic variants: 4 SNPs functionally related to the *BDNF, PON1, TF* and *PGR* genes appeared to modify the methyl Hg-outcome associations with cognitive deficits in children with the minor alleles (mutations) (Julvez et al., 2013). Gene polymorphism of apolipoprotein E (*Apoe*), which codes for the major lipid-transporting protein APOE expressed in the brain (in astrocytes), has been the subject of a small number of studies investigating the effects of methyl Hg on neurodevelopment. Three common *Apoe* alleles, epsilon 2 (ε2), epsilon 3 (ε3) and epsilon 4 (ε4) (Zannis and Breslow, 1981), give rise to three homozygous (ε2/ε2, ε3/ε3, and ε4/ε4) and three heterozygous phenotypes (ε3/ε2, ε4/ε3, and ε4/ε2) (Mahley, 1988). According to experimental studies on animals, the *Apoe* ε4 allele is associated with poor neural repair function and is one of the risk factors associated with Alzheimer disease (Buttini et al., 1999), but the role of APOE genetic variants has become increasingly apparent also in neurodevelopment (Tuminello and Han, 2011; Wright et al., 2003). Being involved in metabolism of cholesterol, which has a major role in neurite outgrowth and synaptogenesis, APOE is capable of influencing either neurodevelopment or modifying the response to maternal diet or maternal exposure to environmental pollutants (Wright et al., 2003). The authors suggested that ε4 allele, normally associated with higher cholesterol levels, has a beneficial effect on neurodevelopment because higher scores were observed among ε4 carriers in compare to either ε2 or ε3 carriers at 24 month of age. In addition, the Bayley mental development score of ε4 carriers had a less steep dose-response to cord lead (Pb) levels than the response in subjects with either the ε2 or ε3 allele (Wright et al., 2003). On the other side, Ng et al. (2013) reported, that the ε4 carriers prenatally exposed to methyl Hg (median cord blood total Hg was 12.2 μg/L) had poorer neurodevelopment at 2 years of age as assessed by Comprehensive Developmental Inventory for Infants and Toddlers (CDIIT) than ε2 and ε3 carriers.

The aim of the present study was to evaluate the association between prenatal exposure to Hg *in utero* due to fish consumption and amalgam fillings of the mother and the neurodevelopment of the child, taking into account genetic polymorphism of *Apoe* (Trdin, 2015) and other relevant confounders. Since fish from Mediterranean have higher levels of Hg compared to the same species from other parts of the world such as the Atlantic Ocean (Renzoni et al., 1998), more than one third of pregnant women in this study were selected from the area of the Northern Adriatic Sea and the rest from areas in close proximity. To account for the beneficial effect of fish consumption, the analysis of association between Hg exposure and neurodevelopment included also micronutrient selenium (Se), a well-known Hg antagonist, which is also present in fish. It protects cells against the toxic effects of Hg in a number of different organisms (Chen et al., 2006; Falnoga and Tušek-Žnidarič, 2007). Lead is a well-known neurotoxicant and exposure of newborns to low levels of Pb is associated with decreasing central neurologic function during childhood (Landrigan and Todd, 1994). Since developmental effects of Pb occur during a critical time window, i.e. <2 years of age (Sanders et al., 2009), level of Pb in cord blood was considered as a confounder in the statistical assessment of Hg-neurodevelopment association, as it can significantly influence the association.

2. Methods

2.1. Study design and subjects

This prospective cohort study was set within a 5-year integrated project on the public health impact of long-term, low-level, mixed element exposure in susceptible population strata (PHIME). The presented study includes two recruitment areas: 1) the city of Ljubljana, Slovenia and its surroundings (up to 50 km) and 2) the coastal city of Rijeka, Croatia and its county (Primorsko-goranska). Women eligible for recruitment (explained by Valent et al. (2013)) were approached for consent during their hospital stay for delivery (Slovenia) or during routine visits between 34 and 38 gestational weeks (Croatia). Recruitment took place at the Maternity Hospital of the University Medical Centre of Ljubljana (Slovenia) and at the University Hospital of Rijeka (Croatia). In Slovenia, maternal hair and cord blood were collected at or immediately after birth, in Croatia maternal hair, maternal blood and maternal urine were collected in the 34th week of pregnancy, and cord blood at birth. In both study populations, breast milk was sampled one month after delivery and at the same time mothers were asked to complete a questionnaire. The study design, including the setting, recruitment, criteria for exclusion, questionnaires, biological sampling and outcome assessment is described in detail by Valent et al. (2013).

The National Ethics Committees of Slovenia and Croatia approved the study.

2.2. Biological sampling

In the third trimester or at delivery, venous blood of pregnant women was collected into a 7-mL tube containing sodium heparin for total Hg, Pb and Se determinations; and in a 3-mL tube (K_3EDTA) for the determination of genetic polymorphisms. Maternal urine (50 mL) was collected by the mothers at home for

total Hg and creatinine determination. Maternal hair (1–3 cm closest to scalp) were collected by hospital personnel and stored in transparent Hg-free PVC bags in the dark and periodically sent to the Jožef Stefan Institute (JSI) for total Hg analysis. At delivery, mixed umbilical cord blood was collected in 7-mL tube (NaH) for total Hg, Pb and Se determinations, 20 mL of child's urine (optional) was collected for total and creatinine determinations and 3–5 cm of cord tissue was retained for determination of genetic polymorphisms at the Faculty of Pharmacy, University in Ljubljana. Aliquots of blood, urine, milk, and cord tissue samples were frozen (below −24 °C) and then transported on dry ice to the JSI, for the determination of total Hg, methyl Hg in all types of samples and Se and Pb in whole blood; and to the University Clinical Centre of Ljubljana, for the determination of Se in plasma and creatinine in urine.

2.3. Questionnaire

One month after delivery, mothers were given a questionnaire to obtain information about their socio-demographic and health status, nutritional habits (detailed food frequency questionnaire, particularly for fish consumption), life-style (smoking), potential exposure and residential and occupational history. A supplementary questionnaire was administered approximately 18 months after delivery. It assessed changes in residence, maternal marital and occupational status, anthropometric measures and developmental milestones of the child, breastfeeding history, child intake of fish, diseases, and day care attendance. Data obtained from the questionnaires was used as covariates assessing the association between Hg exposure and neuropsychological outcome.

2.4. Total mercury in biological samples

Total Hg in biological samples was determined at the JSI by cold vapour atomic absorption spectrometry (CVAAS) using three different analytical procedures, depending on matrix type. All measurements were made under strict quality control procedures and gave comparable results. In addition, the JSI laboratory participated in a series of inter-laboratory comparisons organised within the PHIME project. Three inter-comparisons used lyophilised samples of human blood from non-exposed persons, people occupationally exposed to elemental Hg, fish eaters while the fourth study used fresh blood from the general population. The obtained values were in good agreement with the assigned values (Miklavčič et al., 2013).

The analytical procedures used are described in detail elsewhere (Akagi, 1997; Horvat et al., 1991a; Miklavčič et al., 2011, 2013). The estimated analytical precision of the measurements was less than 10 %, the limit of detection (LOD) of the procedures was <0.1 ng/mL in blood, cord blood and urine and <1 ng/g in hair. Concentrations of total Hg in the urine were normalized by urinary creatinine.

2.5. Lead in cord blood

An aliquot of blood (0.3 mL) was diluted ten times with an alkaline solution (ammonia, Merck, Suprapur) containing Triton X-100 (Sigma-Aldrich, SigmaUltra) and ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA, Fisher Scientific, analytical reagent grade) (Barany et al., 1997). Measurements were made using an Octapole Reaction System (ORS) Inductively Coupled Plasma Mass Spectrometer (7500ce, Agilent) equipped with an ASX-510 Autosampler (Cetac). The LOD, calculated as three times the standard deviations of the blank sample, was 1 ng/mL blood. Reproducibility of measurements was 6 %. A reference material Seronorm trace elements whole blood L-1 (Lot.MR4206, Sero) was used to check the accuracy of the results.

2.6. Selenium in plasma

Selenium in plasma was determined by Zeeman graphite furnace atomic absorption spectrometry (GF AAS) using a palladium chemical modifier. The methods used were presented in a previous publication (Kobal et al., 2004). The standard was a Merck selenium Tritisol standard 9948. Quality control included Seronorm Trace Elements in Serum. The detection limit for plasma was 1.9 μg/L, within-run imprecision was ± 3.2 % and day to day imprecision ± 5.6 %.

2.7. Apoe genotyping

Children's DNA was isolated from cord tissue using a QIAamp DNA Mini Kit, QIAGEN, US, following the instructions for DNA purification from tissues provided in the QIAamp DNA Mini and Blood Mini Handbook 04/2010. Maternal DNA was isolated from leukocytes in peripheral blood using the High Pure PCR Template Preparation Kit (Roche), following the manufacturer's instructions. Samples were diluted (1:20) with ultrapure water (UltraPure DNase/RNase-Free Distilled Water, Sigma-Aldrich).

Apolipoprotein E genotyping was performed using TaqMan® pre-designed SNP genotyping assay small scale with C_3084793_20 for rs429358 and C_904973_10 for rs7412 (Applied biosystems, Foster City, Ca, ZDA). Apolipoprotein E genotyping was carried out using the Roche LightCycler® 480 II (Trdin, 2015).

2.8. Neurodevelopmental assessment

Child neurodevelopment was assessed at 18 (range 16–20) months of age using a standardised individually administered developmental assessment instrument the Bayley Scales of Infant and Toddler Development, Third Edition (Bayley-III) (Bayley, 2006). Cognitive, Language (Receptive and Expressive), Motor (Fine and Gross) Scales were administered according to the standardised procedure. Both the scaled and composite scores were calculated. As suggested by Aylward (Aylward, 2013), composite scores <85 or scaled sores <7 should be considered indicative of disabilities when categorizing the results at Bayley III. The tests were conducted by trained psychologists (Croatia) and by trained psychology students (Slovenia); all of them were certified after attending a two-day workshop on the administration and scoring of Bayley III. In Slovenian group the interrater concordance was calculated for 39 children. Two raters rated each child during their assessment session. The Krippendorf's Alpha coefficients were calculated for each scale. They were 0.984 for Cognitive Scale, 0.988 and 0.961 for Receptive Language and Expressive Language Scale, respectively and 0.943 and 0.954 for Fine Motor and Gross Motor Scale, respectively.

2.9. Statistical analysis

Mercury levels were log transformed to normalize the distribution of the data. Wilcoxon sum-rank or Kruskal-Wallis tests were used to compare the Hg levels between the stratified population groups. Correlations between continuous and between continuous and categorical variables were assessed using either Spearman correlation coefficients, Pearson's correlation coefficients or ANOVA. Multiple linear regression models were used to evaluate the association between the Hg exposure levels and outcome (Bayley-III score). The regression models were adjusted for covariates that were correlated to the outcome with the level of significance $p<0.25$. Model 1 was adjusted for the genotype (carrier of ε4 or not), model 2 was adjusted for the genotype and

mothers' age and education, gender of the child, birth weight, smoking during pregnancy and country (Slovenia vs. Croatia). Model 3 was additionally adjusted for Se and Pb levels in cord blood. Highly correlated variables were not included in the same model to avoid the effect of multi-collinearity. *P* values <0.05 were considered statistically significant and *p* values <0.1 marginally significant. Statistical analyses were performed using STATA (version 12, Statacorp, TX, USA).

3. Results

3.1. General characteristics of the study population

Six hundred and one pregnant women were recruited from the central Slovenia region and 243 from Rijeka on the Croatian coast of the northern Adriatic. During pregnancy no adverse health effects were observed in either group of pregnant women. Determination of total Hg in cord blood, Bayley-III assessment at 18 months of age and *Apoe* genotyping was performed on 361 children; 237 of them were from Slovenia and 124 from Croatia. Table 1 presents the general socio-demographic characteristics of the studied populations.

Among the children, 51 % were boys and 49 % girls. They were born with a gestational age of between 28 and 42 weeks, in Slovenia 5 children were born before the 36th week of pregnancy and 2 in Croatia. Mean birthweight was comparable between the study groups, as was the Body Mass Index (BMI) of the mothers before pregnancy. The education level of the participating mothers was unequally distributed between the Slovenian and Croatian population – most of the mothers in Slovenia had tertiary education, while mothers in Croatia mostly had secondary education. In both study groups, approx. half of the women lived on the periphery of the town, approx. 30 % in the city centre and the remainder were from rural areas. In the first weeks of life, children of participating mothers were mostly breastfed; in the 1st week the percentage of exclusively and predominantly breastfed infants was 85 % and in the 6th week about 70 %. During pregnancy, 8 % of the mothers smoked cigarettes in both study groups. Most of the mothers from Slovenia had between 0 and 9 amalgam dental fillings, 24 % of the mothers had 10 or more. Unfortunately, in Croatia only 29 women reported the number of amalgam fillings, therefore this data is not representative. Among the mothers from Slovenia, 63 % of them had work done on their amalgam fillings by a dentist. However, this question was answered by only 50 % of the participants. The frequency of sea fish consumption was higher in the group of Croatian mothers, with almost 70 % of mothers consuming fish at least once per week, while in Slovenia it was only 25 % (Table 1).

The data from the Table 1 were used as co-variables in the assessment of association between Hg exposure and neurodevelopment, but only if they were significantly associated with the neuropsychological outcome at a margin of significance of $p<0.25$. The marital status of the participating mothers was not considered as a socio-demographic factor since 98 % of the participating women were either married or living with a partner.

3.2. Exposure assessment, Apoe genotypes and neurodevelopmental performance

The overall geometric mean (GM) of total Hg in cord blood was 2.05 ng/g (Table 2). The levels were significantly higher in the Croatian (GM=3.41 ng/g, 95 % CI 2.96–3.94 ng/g) than in the Slovenian (GM=1.58 ng/g, 95 % CI 1.42–1.74 ng/g) mother-child pairs ($p<0.001$), as was Hg in maternal hair (Croatia: GM=598 ng/g, 95 % CI 505–708 ng/g; Slovenia: GM=273 ng/g, 95 % CI 244–306 ng/g; $p<0.001$). Maternal blood Hg levels were measured in 139 mothers and the levels were 1.6 times lower than in the corresponding cord blood samples (Table 2). One mother had an extreme value of total Hg in urine (32.3 ng/mL), which meant that this mother-child pair was excluded from the statistical analysis.

Table 1
General characteristics of the study populations of mother-child pairs.

	Slovenia	Croatia	Total
n	237	124	361
Boys, n (%)	117 (49%)	69 (56%)	186 (52%)
Girls, n (%)	120 (51%)	55 (44%)	175 (48%)
Gestational age (weeks)			
Mean (min–max)	39.5 (28–42)	39.3 (34–41)	39.4 (28–42)
Birthweight (g)			
Mean (min–max)	3459 (2009–4750)	3591 (2400–4820)	3502 (2009–4820)
Mother's age at delivery (years)			
Mean (min–max)	31 (22–44)	30 (19–42)	30.5 (19–44)
BMI (kg/m^2) before pregnancy			
Mean (min–max)	24.0 (17.1–44.5)	22.8 (16.9–40.7)	23.9 (16.9–44.5)
Education			
Primary school, n (%)	1 (0.4%)	2 (2%)	3 (1%)
Middle school, n (%)	14 (6%)	71 (61%)	85 (24%)
High school, n (%)	75 (32%)	11 (9%)	86 (24%)
University degree, n (%)	146 (62%)	32 (28%)	178 (51%)
Residential environment			
Centre, n (%)	66 (28%)	27 (24%)	93 (27%)
Periphery, n (%)	99 (42%)	58 (58%)	165 (47%)
Rural, n (%)	71 (30%)	21 (18%)	92 (26%)
Breastfeeding in the 6th weeks of life			
Exclusive or predominant, n (%)	123 (75%)	50 (71%)	173 (74%)
Partial or formulae, n (%)	42 (25%)	20 (29%)	62 (26%)
Cigarette smoke during pregnancy			
Yes	18 (8%)	12 (10%)	30 (8%)
No	219 (92%)	110 (90%)	329 (92%)
No. of amalgam fillings			
up to 3, n (%)	84 (38%)	28 (97%)	112 (44.5%)
3–9, n (%)	84 (38%)	1 (3%)	85 (34%)
10 or more, n (%)	54 (24%)	0 (0%)	54 (21.5%)
Work on amalgam fillings by dentist			
Yes, n (%)	78 (63%)	29 (41%)	107 (55%)
No, n (%)	45 (37%)	42 (59%)	108 (45%)
Consumption of sea fish			
Less than once per month, n (%)	89 (38%)	7 (5.5%)	96 (27%)
1–3 times per month, n (%)	88 (37%)	32 (26%)	120 (33%)
At least once per week, n (%)	59 (25%)	85 (68.5%)	144 (40%)
Consumption of alcoholic beverages			
Never, n (%)	126 (53%)	65 (54%)	191 (53.5%)
At least occasionally, n (%)	111 (47%)	55 (46%)	166 (46.5%)

Cord blood total Hg levels strongly correlated with maternal hair levels ($r=0.86$, $p<0.001$), but less strongly with maternal urinary levels in a subgroup of the population ($r=0.41$, $p<0.001$). Cord blood total Hg levels gave a positive correlation with the frequency of fish consumption during pregnancy ($r_s=0.45$, $p<0.001$), but did not correlate with the number of dental amalgam fillings in mothers ($r_s=-0.08$, $p=0.224$).

Most children had the $\varepsilon3/\varepsilon3$ genotypes, considered as a wild (reference) genotype (72 %); the frequency of genotypes $\varepsilon2/\varepsilon2$ and $\varepsilon2/\varepsilon3$ was 9 %, and the frequency of susceptible genotypes $\varepsilon3/\varepsilon4$ and $\varepsilon4/\varepsilon4$ was 18 % (Trdin, 2015). The respective frequencies were similar between Slovenian (73 %, 10 % and 17 %) and Croatian study population (70 %, 9 % and 21 %). Two children, one from Slovenia and one from Croatia, had $\varepsilon2/\varepsilon4$ genotype, which was not considered in the statistical analysis due to the functional discrepancy

Table 2
Total mercury (Hg) in mother-child pairs from Slovenia and Croatia, stratified by Apoe genotypes - by the presence of ε4 allele. n – sample number, GM-geometric mean, 95 % CI-95 % confidential interval, min - minimum value, max - maximum value.

Biomarker	ε2 and ε3 carriers GM (95% CI) min–max, n	ε4 carriers GM (95% CI) min–max, n	All GM (95% CI) min–max, n	p-value
Cord blood Hg (ng/g)	1.98 (1.80–2.19) 0.22–32.3, n=292	2.37 (1.90–2.94) 0.16–26.3, n=68	2.05 (1.87–2.25) 0.16–32.3, n=360	0.079
Maternal hair Hg (ng/g)	359 (320–402) 24–8710, n=279	373 (288–483) 19–4623, n=68	361 (326–401) 19–8710, n=347	0.369
Matched pairs of mothers and children:				
Cord blood Hg (ng/g)	3.34 (2.82–3.95) 0.46–32.3, n=111	3.80 (2.95–4.91) 0.82–11.3, n=28	3.43 (2.97–3.95) 0.46–32.3, n=139	0.329
Maternal hair Hg (ng/g)[a]	559 (455–685) 19–8710, n=111	687 (516–913) 80–2080, n=28	582 (490–691) 19–8710, n=139	0.380
Maternal blood Hg (ng/g)[a]	2.33 (2.03–2.67) 0.55–20.5, n=111	2.49 (1.96–3.16) 0.59–7.10, n=28	2.36 (2.10–2.66) 0.55–20.5, n=139	0.376
Maternal urine Hg (μg/g creatinine)[a]	0.74 (0.60–0.91) <LOD–7.06, n=94	0.94 (0.61–1.45) <LOD–9.72, n=24	0.78 (1.03–1.61) <LOD–9.72, n=118	0.396

[a] Stratification was done by maternal genotypes.

between the ε2 and ε4 isoforms. Table 2 shows the levels of total Hg stratified by the *Apoe* genotypes. Marginally significantly higher Hg levels in cord blood were observed in children carrying at least one ε4 allele than in non-carriers. The cord blood levels stratified by maternal genotype did not differ significantly (p=0.268), with GM=3.63 ng/g in ε4 carriers and 3.12 ng/g in ε4 non-carriers. Mercury levels in maternal hair, maternal blood and maternal urine showed no significant differences between the genotypes.

Among the Bayley-III scores (Table 3), cognitive score differed between the Slovenian and Croatian population significantly ($p < 0.001$), Slovenian children having an average score of 114.3 ($\pm$ 13.0) and Croatian 106.7 ($\pm$ 13.2). Language and motor scores did not differ significantly between the studied populations. Cognitive and motor composite scores were marginally significant while the fine motor scaled score was significantly lower in ε4 carriers than in ε2 and ε3 carriers (ε4 non-carriers). The language composite score did not differ significantly between the genotypes. The majority of the children had assessment scores within the normal limits for cognitive, language and motor scale. Six, 20 and 5 children had delayed performance in cognitive, language and motor composite scales, respectively. None of these babies was premature (gestational age of the babies was 38–42).

Table 4 and Table 5 show associations between exposure to Hg during pregnancy (levels of Hg in cord blood and maternal hair) and neurodevelopment of children for three main scales – cognitive, language and motor, and for fine and gross motor subtests.

Table 3
Bayley-III scores for children of 18 months of age, stratified by *Apoe* genotype (presence of ε4 allele). n – sample number, SD - standard deviation, min - minimum value, max - maximum value.

Bayley-III score	ε2 and ε3 carriers mean ± SD	ε4 carriers mean ± SD	All mean ± SD	p-value
Cognitive	112.2 ± 13.5 60–145 (n=292)	109.6 ± 14.1 75–145 (n=68)	111.7 ± 13.6 60–145 (n=360)	0.096
Language	105.9 ± 14.6 50–144 (n=292)	106.1 ± 14.6 77–141 (n=68)	106.0 ± 14.4 50–144 (n=360)	0.885
Motor	107.8 ± 10.5 58–142 (n=292)	105.8 ± 9.2 82–130 (n=68)	107.4 ± 10.3 58–142 (n=360)	0.098
Fine-motor	12.3 ± 2.2 4–19 (n=292)	11.7 ± 2.0 8–16 (n=68)	12.2 ± 2.2 4–19 (n=360)	0.044
Gross-motor	10.3 + 1.97 2–19 (n=291)	10.1 + 2.1 5–16 (n=68)	10.3 + 1.99 2–19 (n=359)	0.504

The models were constructed for the total population adjusted for *Apoe* genotype and separately for ε4 allele carriers and those without the ε4 allele (ε2, ε3 carriers). Outliers (subjects that did not fit the regression line between cord blood and maternal hair total Hg levels, n=3) were excluded from the multiple regression analysis. The Pearson coefficient for correlation between cord blood and hair Hg levels was 0.92 ($p < 0.001$), with all subjects included, the coefficient was 0.86 ($p < 0.001$).

Hg in cord blood showed a negative correlation with cognitive composite score in ε4 carriers, which was significant in the fully adjusted model (Model 3) and marginally significant in the model with no adjustment for Se and Pb cord blood levels (Model 2) (Table 4). Correlations between maternal hair Hg levels and the cognitive score were similar, but with lower significance (Table 5). A marginally significant (cord blood Hg as a predictor) and significant (hair Hg as a predictor) interaction between the genotype and Hg levels (*Apoe* × Hg) was observed for this domain in the fully adjusted model (Model 3) (Table 4 and Table 5). In the motor domain, increasing cord blood or hair Hg levels were significantly associated with a decrease in the fine motor scaled score. The decrease was less significant in the models additionally adjusted for Se and Pb (Model 3). Beta estimates were similar among the genotypes (Table 4 and Table 5). Composite motor scores were significantly or marginally significantly correlated with cord blood or hair Hg levels in the models adjusted for socio-demographic characteristics (Model 2). Beta estimates showed more negative effects in ε4 carriers than in non-carriers, but no significant interaction *Apoe* × Hg was observed (Table 4 and Table 5). Gross motor scaled score showed no significant association with Hg levels in either genotype (Table 4 and Table 5). An association between cord blood or hair Hg levels and language domain showed no significant effect, but with β estimates being more negative in ε4 carriers than in non-carriers (Table 4 and Table 5). A marginally significant interaction *Apoe* × Hg was observed in the model using cord blood Hg as an exposure biomarker, which had been adjusted for socio-demographic characteristics (Model 2) (Table 4).

Regression models additionally adjusted for Se and Pb levels revealed a marginally significant positive association between serum Se levels and language composite score in all subjects (cord blood model β=7.6, p=0.094; hair model β=8.6, p=0.077), while the association was insignificant in ε4 carriers (cord blood model β=15.0, p=0.316; hair model β=11.3, p=0.416). A negative association between cord blood Pb levels and motor score was observed in ε2 and ε3 carriers (cord blood model β=−3.2, p=0.035; hair model β=−3.0, p=0.049), but not in ε4 carriers (cord blood model β=4.4, p=0.222; hair model β=3.89, p=0.242). The association was insignificant in all subjects (cord blood model

Table 4
Bayley-III score associations with total Hg in cord blood (ln ng/g) using multiple linear regression, β – estimate of change in neurodevelopment score, CI 95 – 95% confidence interval. Results of 45 models are presented.

Model	Subjects	n	β (CI 95%), p-value, dependant variable *model R^2, model p-value*				
			Cognitive	**Language**	**Motor**	**Fine motor**	**Gross motor**
Models 1	All	357	**−2.22 (−3.85, −0.60), 0.007**	0.40 (−1.34, 2.14), 0.650	−1.03 (−2.27, 0.20), 0.102	**−0.51 (−0.80, −0.26), < 0.001**	0.18 (−0.06, 0.42), 0.143
			model R^2=0.026, p=0.009	*model R^2=0.0006, p=0.900*	*model R^2=0.013, p=0.099*	*model R^2=0.051, p<0.001*	*model R^2=0.007, p=0.295*
	ε2, ε3 carriers	289	−1.77 (−3.58, 0.04), 0.055	0.67 (−1.32, 2.65), 0.508	−0.87 (−2.29, 0.55), 0.227	**−0.45 (−0.74, −0.15), 0.003**	0.17 (−0.10, 0.44), 0.210
			model R^2=0.013	*model R^2=0.002*	*model R^2=0.005*	*model R^2=0.031*	*model R^2=0.006*
	ε4 carriers	68	**−3.98 (−7.72, −0.25), 0.037**	−0.64 (−4.29, 3.02), 0.729	−1.65 (−4.13, 0.83), 0.189	**−0.76 (−1.28, −0.24), 0.005**	0.22 (−0.35, 0.78), 0.449
			model R^2=0.064	*model R^2=0.002*	*model R^2=0.026*	*model R^2=0.116*	*model R^2=0.009*
	Interaction p* *Apoe × Hg*	357	0.188	0.242	0.267	0.641	0.275
Models 2	All	346	−0.82 (−2.62, 0.97), 0.367	−0.32 (−2.42, 1.77), 0.762	**−1.74 (−3.14, −0.34), 0.015**	**−0.49 (−0.78, −0.21), 0.001**	−0.11 (−0.39, 0.17), 0.443
			model R^2=0.09, p<0.001	*model R^2=0.063, p=0.015*	*model R^2=0.051, p=0.023*	*model R^2=0.095, p<0.001*	*model R^2=0.092, p<0.001*
	ε2, ε3 carriers	281	−0.19 (−2.19, 1.81), 0.849	−0.07 (−2.48, 2.33), 0.951	−1.59 (−3.21, 0.03), 0.054	**−0.46 (−0.79, −0.13), 0.007**	−0.10 (−0.41, 0.20), 0.499
			model R^2=0.09, p<0.001	*model R^2=0.062, p=0.031*	*model R^2=0.036, p=0.175*	*model R^2=0.062, p=0.900*	*model R^2=0.092, p<0.001*
	ε4 carriers	65	−4.04 (−8.26, 0.19), 0.061	−1.91 (−6.43, 2.62), 0.400	−2.41 (−5.33, 0.51), 0.104	**−0.61 (−1.18, −0.03), 0.039**	−0.21 (−0.94, 0.52), 0.572
			model R^2=0.15, p=0.087	*model R^2=0.13, p=0.500*	*model R^2=0.133, p=0.295*	*model R^2=0.0006, p=0.001*	*model R^2=0.161, p=0.220*
	Interaction p* *Apoe × Hg*	346	0.075	0.092	0.338	0.541	0.281
Models 3	All	283	−1.41 (−3.47, 0.66), 0.181	−1.03 (−3.46, 1.40), 0.406	−1.16 (−2.76, 0.44), 0.153	**−0.33 (−0.66, −0.01), 0.043**	−0.10 (−0.41, 0.22), 0.553
			model R^2=0.12, p<0.001	*model R^2=0.091, p=0.016*	*model R^2=0.056, p=0.100*	*model R^2=0.103, p<0.001*	*model R^2=0.078, p=0.029*
	ε2, ε3 carriers	232	−0.49 (−2.76, 1.78), 0.949	−0.59 (−3.42, 2.45), 0.682	−0.84 (−2.68, 1.00), 0.369	−0.29 (−0.66, 0.08), 0.122	**−0.06 (−0.41, 0.29), 0.742**
			model R^2=0.12, p<0.001	*model R^2=0.074, p=0.0102*	*model R^2=0.049, p=0.253*	*model R^2=0.086, p=0.002*	*model R^2=0.093, p=0.017*
	ε4 carriers	51	**−5.44 (−10.7, 0.19), 0.043**	−2.61 (−7.41, 2.20), 0.276	−2.54 (−5.94, 0.86), 0.139	−0.51 (−1.16, 0.13), 0.117	−0.26 (−1.09, 0.57), 0.528
			model R^2=0.19, p=0.207	*model R^2=0.323, p=0.202*	*model R^2=0.224, p=0.258*	*model R^2=0.208, p=0.098*	*model R^2=0.136, p=0.793*
	Interaction p *Apoe × Hg*	283	0.052	0.181	0.275	0.353	0.418

Remark: Models[1]: crude, the 'All' model adjusted for *Apoe* genotype; Models[2]: adjusted for country and particular socio-demographic characteristics (mother's age at delivery, child's gender, birth weight, educational level of the mother, smoking during pregnancy) and *Apoe* genotype; Models[3]: Models[2] + adjusted for serum Se and cord blood Pb. Outliers (n = 3) were omitted from the regression models.
* p-value for interaction term (Apoe*Hg) in the models with all subjects.

Table 5
Bayley-III score associations with total Hg in maternal hair (ln ng/g) using multiple linear regression. β – estimate of change in neurodevelopment score. CI 95–95% confidence interval. Results of 45 models are presented.

Model	Subjects	n	β (CI 95%), p-value *model R^2*, *model p-value*				
			Cognitive	Language	Motor	Fine motor	Gross motor
Model 1	All	353	**−1.94 (−4.13, −0.74), 0.010**	0.63 (−0.96, 2.22), 0.436	−0.83 (−1.95, 0.29), 0.145	**−0.39 (−0.62, −0.16), 0.001**	0.14 (−0.08, 0.36), 0.211
			model R^2=0.024, p=0.014	*model R^2=0.002, p=0.733*	*model R^2=0.013, p=0.095*	*model R^2=0.039, p=0.001*	*model R^2=0.007, p=0.293*
	ε2, ε3 carriers	259	−1.25 (−2.94, 0.44), 0.147	1.08 (−0.77, 2.93), 0.251	−0.64 (−1.94, 0.67), 0.338	**−0.31 (−0.58, 0.04), 0.027**	0.13 (−0.12, 0.38), 0.292
			model R^2=0.007	*model R^2=0.005*	*model R^2=0.003*	*model R^2=0.017*	*model R^2=0.004*
	ε4 carriers	63	**−4.21 (−7.27, −1.15), 0.008**	−0.84 (−3.93, 2.25), 0.590	−1.47 (−3.62, 0.68), 0.177	**−0.66 (−1.09, −0.23), 0.003**	0.17 (−0.34, 0.67), 0.513
			model R^2=0.101	*model R^2=0.004*	*model R^2=0.027*	*model R^2=0.123*	*model R^2=0.006*
	Interaction p* *Apoe* × *Hg*	353	0.158	0.317	0.265	0.764	0.206
Model 2	All	353	−0.51 (−2.17, 1.15), 0.546	0.53 (−1.41, 2.47), 0.589	−1.19 (−2.40, 0.01), 0.052	**−0.36 (−0.63, −0.10), 0.008**	**−0.11** (−0.37, 0.16), 0.428
			model R^2=0.090, p < 0.001	*model R^2=0.065, p=0.073*	*model R^2=0.044, p=0.027*	*model R^2=0.080, p < 0.001*	*model R^2=0.084, p < 0.001*
	ε2, ε3 carriers	276	0.19 (−1.69, 2.07), 0.840	0.64 (−1.64, 2.91), 0.581	−0.97 (−2.39, 0.45), 0.178	**−0.32 (−0.63, −0.01), 0.043**	−0.14 (−0.43, 0.14), 0.323
			model R^2=0.092, p < 0.001	*model R^2=0.062, p=0.035*	*model R^2=0.028, p=0.241*	*model R^2=0.048, p=0.010*	*model R^2=0.097, p < 0.001*
	ε4 carriers	66	−3.23 (−6.89, 0.44), 0.083	0.05 (−3.82, 3.93), 0.979	−2.07 (−4.39, 0.26), 0.081	−0.48 (−0.98, 0.02), 0.061	0.02 (−0.68, 0.72), 0.962
			model R^2=0.134, p=0.116	*model R^2=0.142, p=0.402*	*model R^2=0.111, p=0.275*	*model R^2=0.209, p=0.006*	*model R^2=0.077, p=0.700*
	Interaction p* *Apoe* × *Hg*	353	0.064	0.112	0.390	0.716	0.135
Model 3	All	280	−1.12 (−3.08, 0.84), 0.262	−0.01 (−2.32, 2.30), 0.671	−1.04 (−2.43, 0.34), 0.139	−0.29 (−0.59, 0.01), 0.059	**−0.12** (−0.42, 0.18), **0.441**
			model R^2=0.118, p < 0.001	*model R^2=0.097, p=0.015*	*model R^2=0.055, p=0.081*	*model R^2=0.098, p < 0.001*	*model R^2=0.079, p=0.039*
	ε2, ε3 carriers	226	−0.15 (−2.37, 2.06), 0.893	0.26 (−2.52, 3.03), 0.856	−0.64 (−2.30, 1.02), 0.445	−0.23 (−0.59, 0.12), 0.197	−0.10 (−0.44, 0.25), 0.585
			model R^2=0.122, p < 0.001	*model R^2=0.082, p=0.086*	*model R^2=0.046, p=0.243*	*model R^2=0.080, p=0.006*	*model R^2=0.092, p=0.028*
	ε4 carriers	51	−4.02 (−8.54, 0.50), 0.080	**−0.50 (−4.75, 3.74), 0.810**	−2.35 (−4.84, 0.14), 0.063	**−0.40 (−0.95, 0.16), 0.157**	**−0.15** (−0.87, 0.58), 0.663
			model R^2=0.172, p=0.285	*model R^2=0.295, p=0.281*	*model R^2=0.244, p=0.099*	*model R^2=0.278, p=0.022*	*model R^2=0.130, p=0.817*
	Interaction p* *Apoe* × *Hg*	280	0.094	0.183	0.345	0.457	0.280

Remark: Model[1]: crude, the 'All' model adjusted for *Apoe* genotype; Model[2]: adjusted for country and particular socio-demographic characteristics (mother's age at delivery, child's gender, birth weight, educational level of the mother, smoking during pregnancy) and *Apoe* genotype; Model[3]: Model[2] + adjusted for serum Se and cord blood Pb. Outliers (n = 3) were omitted from the regression models.

* p-value for interaction term (Apoe*Hg) in the models with all subjects.

$\beta = -2.17$, $p = 0.111$; hair model $\beta = -1.54$, $p = 0.141$). The negative association between cord blood Pb levels and fine motor score was observed in all subjects (cord blood model $\beta = -0.64$, $p = 0.020$; hair model $\beta = -0.65$, $p = 0.019$), in $\varepsilon 2$ and $\varepsilon 3$ carriers (cord blood model $\beta = -0.82$, $p = 0.008$; hair model $\beta = -0.82$, $p = 0.008$), but not in $\varepsilon 4$ carriers (cord blood model $\beta = 0.73$, $p = 0.294$; hair model $\beta = 0.57$, $p = 0.408$).

4. Discussion

In the present study a negative association between low-to-moderate Hg exposure in a general population and neurodevelopmental scores for cognitive performance and fine motor skills of a child was observed. The neurodevelopmental scores observed in the study were still within the normal range of scores. The Hg-associated decrease in cognitive performance was significant only in the carriers of at least one *Apoe* $\varepsilon 4$ allele, while a decrease in fine motor skills was independent of the genotype.

4.1. Mercury exposure

Cord blood total Hg levels observed in the present study were 0.16–9.95 ng/g (GM 1.58 ng/g) in the Slovenian and 0.79–32.3 ng/g (GM 3.40 ng/g) in the Croatian population, while in maternal hair the total Hg levels were 24–2075 ng/g (GM 273 ng/g) in Slovenian and 19–8710 ng/g (mean 576 ng/g) in Croatian mothers. In total, 42 children (12 %) exceeded a cord blood total Hg level of 5.8 μg/L (Table 2), the corresponding equivalent to a Reference Dose (RfD) of 0.1 μg methyl Hg/kg body weight per day (US EPA, 2001). Among them, 8 children were from Slovenia and 34 from Croatia. Similarly, 8 mothers from Slovenia and 33 from Croatia exceeded a hair total Hg level of 1 μg/g, the corresponding equivalent of the RfD (US EPA, 2001). Higher Hg levels observed in Croatian mother-child pairs compared to those from Slovenia correspond to a higher frequency of fish consumption in Croatia as assessed from the questionnaire data. The data showed that the mean frequency of fresh fish consumption was 2.5 meals/month and 5 meal/month for Slovenian and Croatian mothers, respectively. The number of dental amalgam fillings was higher in Slovenian mothers, but unfortunately the data was provided by only 29 out of 124 Croatian mothers and inorganic Hg exposure from amalgam fillings could not be compared between the study populations. From the strong correlation that exists between cord blood and maternal hair total Hg and between cord blood total Hg and fish consumption, exposure in both study populations was characteristic for fish consumption. The number of amalgam fillings did not show any correlation with Hg levels in cord blood.

In general, cord blood levels of Hg in the Slovenian and Croatian study populations were above the average for Central Europe (Višnjevec et al., 2014), but lower than in other Mediterranean countries: e.g. Greece (median 5.8 ng/g), Italy (median 3.9 ng/g) (Miklavčič et al., 2013) and Spain (Višnjevec et al., 2014). Mean levels of Hg measured previously in cord blood of mother-child pairs from central Slovenia who did not consume seafood were on average 1.16 ± 0.80 μg/L ($\pm$ SD) (Horvat et al., 1991b). Levels reported for women from Croatia residing in the Islands of the Central Adriatic and consuming fish regularly were much higher (7.7 ± 5.1 ng/mL cord blood) (Horvat et al., 1988).

4.2. Mercury and neurodevelopment

The developing central nervous system is especially susceptible to toxic effects of methyl Hg and Hg° vapour (Andersen et al., 2000). Although both species of Hg readily pass through the placental and blood-brain barriers, there is a lack of evidence for subclinical developmental changes in children exposed *in utero* to a low dose of Hg° vapour (Yoshida, 2002), e.g. from amalgam fillings. The neurodevelopmental outcome of prenatally exposed children has been assessed in a number of studies involving populations exposed to environmentally relevant concentrations of total Hg. Apart from the three major longitudinal studies performed so far, among which the Faroe Island birth cohort is the only one that demonstrated negative effects of Hg from seafood consumption (population mean cord blood Hg was 23 μg/L, mean maternal hair Hg was 4.3 μg/g) (Grandjean et al., 1997), there is evidence that low levels of prenatal Hg exposure may cause early childhood neurocognitive effects (Karagas et al., 2012). In a US cohort with mean maternal hair Hg levels of 0.55 μg/g (10 % of samples was above 1.2 μg/g), higher fish intake in pregnancy was associated with higher infant cognition at 6 months of age, as assessed by Visual Recognition Memory (VRM), but higher Hg exposure levels were associated with lower cognition after adjusting for participants characteristics (Oken et al., 2005). The findings were confirmed by the Peabody Picture Vocabulary Test (PPVT) and Wide Range Assessment of Visual Motor Abilities (WRAVMA) at age 3 years (Oken et al., 2008). Mercury exposure of the Croatian birth cohort, described in the present study, has been analysed previously in relation to results of neurosonographic exam, and descriptive analyses showed maternal pregnancy hair Hg ≥ 1 μg/g was associated with a smaller cerebellar volume (Cace et al., 2011).

4.3. Mercury, neurodevelopment and APOE

Apoe polymorphism has been studied extensively in relation to neurodegenerative diseases and *Apoe* $\varepsilon 4$ allele has been recognized as one of the genetic risk factors associated with Alzheimer's disease (Buttini et al., 1999), albeit the role of APOE isoforms in early life is controversial (Tuminello and Han, 2011). APOE is believed to promote myelination, synaptogenesis or other fatty acid/cholesterol mediated processes associated with neurodevelopment (Mauch et al., 2001; Wright et al., 2003). In consistency with this, Wright et al. (2003) were the first to show improved Mental Development Index (MDI) of the Bayley Scale at 24 months of age in $\varepsilon 4$ carriers compared to $\varepsilon 2$ and $\varepsilon 3$ carriers. Similarly, $\varepsilon 4$ carriers cognitively outperformed non-carriers in several studies involving a young population (Tuminello and Han, 2011). The authors suggested that the *Apoe* $\varepsilon 4$ allele is beneficial in earlier ages and may only confer risk of cognitive decline later in life (Tuminello and Han, 2011). According to this theory, the beneficial effects of *Apoe* $\varepsilon 4$ allele in early years predict selection for this variant to a higher degree than do the detrimental effects that are manifested in the post-reproductive years (e.g. as AD).

There are only few studies addressing Hg exposure, APOE and a child's neurodevelopment. Ng et al. (2013) found that prenatal Hg exposure, with mean cord blood Hg level of 12.2 μg/L, was negatively associated with cognition, social and whole neurodevelopmental quotients as assessed by Comprehensive Developmental Inventory for Infants and Toddlers at age 2 years among subjects who had at least one *Apoe* $\varepsilon 4$ allele. Statistical interaction between cord blood Hg and *Apoe* polymorphism has been confirmed by the authors for cognition, language and fine motor tests. Their later studies revealed that elevated Hg in cord blood enhances the risk of deficit behaviour in pre-school children (2 years of age) who were $\varepsilon 4$ carriers (Ng et al., 2015). Another study of children (8–12 years of age) exposed to Hg from amalgam fillings (as indicated by urinary Hg levels) together with other sources, reported significant statistical interactions between *Apoe* and Hg in the learning and memory domain in boys, with impaired performance in $\varepsilon 4$ carriers, while in girls in the attention and motor domains, but with improved performance in $\varepsilon 4$ carriers (Woods et al., 2014).

In consistency with the findings of Ng et al. (2013), who reported a higher level of Hg exposure as we do, the present study

indicates a negative association between Hg and cognitition in children of 18 months of age, who were carriers of at least one ε4 allele. The statistical interaction between cord blood Hg and *Apoe* in this domain was confirmed with marginal significance (Table 4), while the interaction between maternal hair Hg and *Apoe* was significant in the fully adjusted model (Table 5). Within the motor domain, an increasing exposure to Hg during pregnancy was associated significantly with lower fine motor scores, but the association was not modified by *Apoe* polymorphism. Adjustment for Se and Pb levels revealed lower significance of the Hg negative association (Table 4 and Table 5). In a study where Hg exposure was investigated in relation to impaired neurodevelopmental outcome in the Croatian subpopulation without adjusting or stratifying for genotype, a negative association was found for the fine motor subtest as well. The results are reported by Prpic et al. (this issue).

The mechanism for the observed association is yet to be identified, however, the difference in Hg-associated decrease between the genotypes may be explained by the APOE role in Hg metabolism. The APOE ε4 isoform is without cysteins, whereas the ε3 isoform contains one cysteine and one arginine, and the ε2 isoform two cysteines. Cysteine contains one thiol (–SH) group which can conjugate with metals and help remove them from essential cellular binding sites. Our data showed that mean cord blood Hg levels were higher in ε4 carriers than in those without ε4 allele. However, the significance of this difference was marginal, and cord blood levels cannot be directly used to quantify levels in the brain (based on the blood-brain ratios documented by Björkman et al. (2007) and Vahter et al. (1994)). Based on the APOE functional differences, it would be worth stratifying for ε2 carriers separately in the regression modelling, but due to the low frequency of ε2 carriers (9 %) the statistical power to do this was insufficient. We constructed stratified models from which ε2 carriers were excluded, but the models did not differ markedly from those with ε2 carriers included (data not presented).

4.4. Association between neurodevelopment, APOE and other elements

In addition to the observed negative Hg-related associations, regression modelling also showed negative association between lower motor scores (composite and fine) and cord blood Pb levels (GM 8.48 ng/g, range 1.82–34.1 ng/g) in the subjects not carrying ε4 allele (data not presented). This is in consistency with the findings of a study by Wright et al. (2003), where a higher decrease in Mental Developmental Index (BSID-II) per unit change in cord blood Pb level (mean 6.6 ± 3.4 μL) was observed among ε4 non-carriers at 24 months of age, suggesting possible protection against Pb exposure by effect modification among ε4 carriers. Also in the study by Ng et al. (2013) where Pb cord blood levels were used as a confounder in the associations between Hg and the BSID score, a negative association with neurodevelopmental outcome was reported, but it was not indicated by the authors as to whether or not the effect was modified by the genotype.

A positive association, although marginally significant, between Se levels in cord blood serum (GM 40.1 ng/g, range 15–70 ng/g) and the language domain was observed among the ε4 non-carriers, but not in the ε4 carriers. An increase in children's language comprehension with increasing Se levels (as determined in maternal erythrocytes in gestational age of 30 weeks) was observed by Skröder et al. (2014) in Bangladesh where Se levels in the study population varied considerably (0.19–0.87 mg/g haemoglobin). Children in their study were tested at the same age as in our study (18 months) using the BSID-II.

4.5. Limitations and strengths of the study

The main limitation of the present study was the sample size, with a relatively low number of ε4 and ε2 carriers compared to the wildtype (ε3/ε3). The number of children with elevated Hg levels (> 5.8 ng/g) was relatively low: 11 among ε4 carriers and 37 among ε4 non-carriers. Although two different birth cohorts, one from Slovenia and one from Croatia, were merged for the purpose of this study, both followed the same study design and implementation which provided comparable datasets. In order to avoid false positive correlations in regression modelling, the models were adjusted for the location (country) of each subpopulation.

Variables that were obtained from the questionnaires and were used as confounders in the regression modelling introduced additional shortcomings into our study. For example, for a significant number of mothers data on the duration of breastfeeding was lacking and was omitted from the regression analysis since its inclusion yielded insignificant and illogical correlations.

Although Hg exposure in the present study is characterized mainly by fish consumption, a certain portion arises from amalgam fillings, which is expected to contribute more to overall exposure at lower Hg levels. Unfortunately, our database lacked information on the number of amalgam fillings for the majority of Croatian women, and therefore could not be included in the regression modelling. To specify this contribution, speciation analysis should be performed. The children with delayed performance were predominantly Slovenian, with relatively lower levels of cord blood Hg. By identifying the Hg species in the samples it should be possible to quantify Hg° vapour exposure in relation to neurodevelopment and/or neurobehaviour.

A larger sample size would enable us to stratify the regression models, not only according to specific genotype, but also according to gender, since neuropsychological performance may differ considerably between boys and girls (Karagas et al., 2012). The models in the present study indicated significantly higher language and motor scores in girls than in boys (data not presented).

The added value of the present study is undoubtedly the evaluation of association between Hg and neurodevelopmental performance using two different exposure biomarkers: cord blood and maternal hair Hg levels, which are both generally accepted as biomarkers of prenatal methyl Hg exposure. Total Hg levels in hair samples (length 1–3 cm) correlated well with cord blood levels ($r=0.92$), which was confirmed by the good agreement between the regression models (Tables 4 and 5). Regarding the critical windows of neurodevelopment (Rice and Barone, 2000), the 3rd trimester of pregnancy can be as equally important in neurodevelopmental outcomes as the 1st trimester, and it would also be worth measuring Hg levels in hair segments corresponding to this period of vulnerability.

Furthermore, taking into account the effects of beneficial/neurotoxic chemicals in relation to neurodevelopment is of crucial importance in such studies, since adjusting for Se and Pb levels in cord blood resulted in more negative β estimates on cognitive score and in less significant association between Hg and motor score (Model 3 compared to Model 2, Tables 4 and 5). In addition, determining PUFA levels in the blood of children, representing a beneficial nutritional cofounder in assessing Hg-related effects, would be equally important, especially as our regression models did not reveal any positive association between maternal fish consumption obtained from the questionnaire and neurodevelopmental scores (data not presented).

5. Conclusions

The present study indicates that even low-to-moderate Hg

exposure in children with normal neurodevelopmental outcome can be associated with lower cognitive and fine motor scores at 18 months of age. Despite the relatively low number of subjects carrying the *Apoe* ε4 allele, the study provided firm evidence of Hg-associated decrease in cognitive performance among ε4 carriers, while the decrease in fine motor scores was independent of the genotype. Gene-environment interaction was indicated for the cognitive score. We demonstrated that accounting for beneficial factors like Se and other potentially neurotoxic substances like Pb is crucial in assessing such associations. Re-evaluation of the obtained results is needed with higher predictive values of neuropsychological test used at later age and with consideration of other polymorphisms identified that can influence neurodevelopmental performance. In addition, the results do not make clear what is the contribution of inorganic Hg exposure to the overall negative association between Hg and neurodevelopment. Speciation analysis in biological samples would help reveal this and is planned for the follow-up.

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Declaration of interest

The authors report no declaration of interest.

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3.6 Exposure to Mercury in Susceptible Population Groups Living in the Former Mercury Mining Town of Idrija, Slovenia

The paper was authored by A. B. Kobal from the ex-Department of Occupational Health, Idrija Mercury Mine; J. Snoj Tratnik, D. Mazej, V. Fajon, D. Gibičar, A. Miklavčič, D. Kocman, J. Kotnik, and M. Horvat from the JSI; A. Sešek-Briški, J. Osredkar, M. Krsnik, M. Prezelj from the University Medical Center Ljubljana; Č. Knap and B. Križaj from the Idrija Health Service; and L. Liang from the Cebam Analytical, U.S. It has been published in the Environmental Research.

In this paper Hg exposure of the residents of the town of Idrija using HBM is evaluated. Contamination of environment due to over 500 years of Hg mining in the town and its surroundings persists, even after the closure of the mine in 1995. The study investigated if the most vulnerable population groups (pregnant women, newborns and children aged 6-11 years) living in the contaminated area are still at risk. Besides the internal exposure assessed through measurements of Hg in maternal, cord and child's blood, maternal and child's urine and hair, potential interaction of Hg with Se in plasma was assessed in the study groups of pregnant women and school-age children. In pregnant women antioxidative enzyme activity (glutathione peroxidase, superoxide dismutase and catalase) in erythrocytes of maternal and cord blood was also assessed to investigate possible interaction between Hg and oxidative stress as a consequence of elevated Hg exposure. The internal exposure and biological response was compared to the participants living in the Hg non-contaminated environment.

The town of Idrija has been thoroughly investigated in terms of environmental and biologic monitoring (Hg concentrations in soil, water, air, and biota) and monitoring of occupational exposure in the mineworkers. However, after the closure of the mine, none of the studies investigated the actual exposure in the residents of the town, non-occupationally exposed to Hg. The latter has been achieved through the present paper. Based on the findings, the paper also gives recommendations regarding the consumption of certain food items for the residents of the town, particularly pregnant women.

However, evaluation of the association between exposure due to contamination from past mining activities and neurodevelopment of children was not possible, as too few mother-child pairs participated in the PHIME study (n=10). Moreover, the samples for genotyping analysis were not available within the time frame of the present work. Continuation of the exposure assessment in the town of Idrija is planned within the second national HBM including children and adolescents. This will allow also an assessment of the distribution of frequencies of relevant genotypes that could influence the exposure to Hg. Due to a half century of increased exposure to inorganic Hg in this population, increased frequencies of alleles that may decrease susceptibility to Hg are hypothesized as an adaptation mechanism.

Within this paper, I contributed to the complete Hg analysis in the school-age children living in the town of Idrija, Ljubljana and Žužemberk, and part of Hg analysis in cord blood samples from mother-child pairs. I also performed the statistical analysis for all study groups and prepared a major part of the manuscript in consultation with Dr. Kobal.

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Exposure to mercury in susceptible population groups living in the former mercury mining town of Idrija, Slovenia

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ABSTRACT

500 years of mercury (Hg) mining in the town of Idrija has caused severe pollution in Idrija and its surroundings. Following the closure of the mine in 1995, the environment remains contaminated with Hg. Sources of elemental-, inorganic- and methyl Hg exposure were identified, potential environmental level of exposure to Hg was evaluated and actual internal exposure to Hg was assessed in selected susceptible population groups comprising school-age children and pregnant women living in Idrija and in control groups from rural and urban environments. The study of pregnant women ($n=31$) was conducted between 2003 and 2008, and the study of school-age children ($n=176$) in 2008. Potential interaction of Hg with selenium (Se) in plasma was assessed in both study populations, while in pregnant women antioxidative enzyme activity (glutathione peroxidase, superoxide dismutase and catalase) in erythrocytes of maternal and cord blood was also assessed. Actual exposure to Hg as indicated by levels of Hg in children's blood (geometric mean (GM) 0.92 μg/L), mother's blood (GM 1.86 μg/L), children's urine (GM 1.08 μg/g crea.), mother's urine (GM 2.51 μg/L), children's hair (GM 241 ng/g) and mother's hair (GM 251 ng/g) was higher in the two study groups from Idrija than in the control groups from rural areas, but was still at the level of a "normal" population and reflects mainly exposure to elemental Hg (Hg^0) from dental amalgam and, to a certain extent atmospheric Hg^0. Furthermore, the internal doses of Hg received during pregnancy did not decrease the bioavailability of Se. Based on observation in children, the increase in Se protein expression is suggested to be a consequence of moderately elevated exposure to Hg^0. The observed changes in activity of antioxidative enzymes, as biomarkers of oxidative stress, appear to be mainly associated with pregnancy *per se* and not with an increased exposure to Hg. In view of the continuing increased potential for Hg exposure and the low number of pregnant women studied, the results warrant a further longitudinal study of a larger group of pregnant women residing in the area of the former mercury mine.
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1. Introduction

Mercury (Hg) is a ubiquitous global pollutant that adversely affects humans and wild life. The general population is exposed to methyl Hg through the diet (mostly from consuming fish) and to elemental Hg (Hg^0) from dental amalgam fillings. Populations living in Hg contaminated sites, such as those located in areas near mercury mines and industrially polluted land, may also be exposed to Hg^0 through inhalation of contaminated ambient air. Over 500 years of mercury mining in the town of Idrija, Slovenia has

Abbreviations: CAT, catalase; CI, confidence interval; CV, coefficient of variation; GM, geometric mean; GPx, glutathione peroxidase; Hb, haemoglobin; HBM I, health-based value, below which no biological and/or adverse health effects are expected; HBM II, health-based value, which could be associated with biological and/or potential adverse health effects; Hg^{2+}, inorganic mercury; Hg^0, elemental mercury; Max, maximum; Min, minimum; P10, 10th percentile; P90, 90th percentile; PHIME, Public Health Impact of Long-term Low-level Mixed Element Exposure in Susceptible Population Strata; P-Se, plasma selenium; RfD, reference dose; SD, standard deviation; SOD, superoxide dismutase; THg, total mercury

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caused severe Hg pollution of the environment in the town and its surroundings (Byrne et al., 1995; Gnamuš et al., 2000; Gosar et al., 1997; Hess, 1991; Kavčič, 1974; Kosta et al., 1974; Lupšina et al., 1992; Palinkaš et al., 1995). In the period after the mine closed in 1995 the Hg concentrations in sediments, soil, vegetation, water, fish, and partly also in air, remained elevated (Gnamuš et al., 2000; Gosar and Šajn, 2003; Gosar et al., 1997; Grönlund et al., 2005; Horvat et al., 2002, 1999; Jereb et al., 2004; Kocman et al., 2011a, 2011b; Kotnik et al., 2005). However, in the period after the mine closed the extent of Hg exposure in the general non-occupationally exposed population living in Idrija has yet to be assessed. An exposure assessment was made possible through the two cross-sectional studies presented in this paper, where school-age children and pregnant women from contaminated and control areas were compared.

School-age children and pregnant women were chosen for the study since both are recognised as susceptible population groups. Exposure to methyl Hg, particularly in the prenatal period, has neurotoxic effects on the development of a child's central nervous system. Even low levels of exposure in pregnant women appear to have subtle persistent effects on children's mental development (Davidson et al., 2000; Grandjean and Landrigan, 2006; Grandjean et al., 1997; NRC, 2000; WHO, 1990). In pregnant women the transfer of inhaled Hg° to the developing foetus has also been observed (Clarkson, 1989, 2002), but in spite of it having similar mechanisms of toxicity *e.g.*, oxidative stress from the stimulation of free radical generation and reducing the amount of bioavailable selenium (Ganther, 1980; Kosta et al., 1974; Lund et al., 1993; Rana and Boora, 1992; WHO, 1991, 1990), no definitive data on its effects on children's mental development has been documented (Davidson et al., 2004; WHO, 2003).

On a biochemical level, Hg can promote lipid peroxidation by stimulating free radical generation as a catalyst in a Fenton-type reaction and through other mechanisms by interacting with antioxidant enzymes that could modify its activity (Björkman et al., 1993; Lund et al., 1993). The main antioxidant enzymes in human cells are superoxide dismutase (SOD), catalase (CAT) and the selenoenzyme glutathione peroxidase (GPx), which, among other functions, transform the superoxide radical into hydrogen peroxide and hydrogen peroxide into oxygen and water. Normal pregnancy is also associated with increased oxidative stress, resulting in the induction of antioxidant enzymes to protect embryonic development against free radical attack (Carone et al., 1993; Mistry et al., 2008; Sugino et al., 2000).

The aims of this study were to evaluate the environmental distribution and concentrations of Hg in the area of the town of Idrija, and to estimate the actual exposure of school-age children and pregnant women to Hg°, methyl Hg and inorganic Hg through inhalation of ambient air, ingestion of drinking water and locally grown seasonal vegetables, as well the intake of methyl Hg from the consumption of fish from the river Idrijca and from the fish market. Possible interactions of Hg with selenium (Se) and antioxidative enzymes were also examined in a subgroup of the studied population. This study is important in terms of identifying if the most susceptible population groups living in the contaminated area of the town of Idrija are still at risk.

2. Methods

2.1. Study design and subjects

First, we evaluated the data from environmental studies performed in the town of Idrija since the mine closed in 1995. From these data, we estimated the concentration of Hg° in air, inorganic and methyl Hg in local seasonal vegetables, drinking water and in fish from the river Idrijca. Second, we evaluated Hg absorption by determining the total Hg concentrations in urine, blood and hair of 6–11 years old school children, pregnant women and in the cord blood of newborns, with these tissues serving as biological markers of exposure to Hg (UNEP Chemicals, 2002; UNEP/WHO, 2008; WHO, 2003, 1990). Third, we determined the concentrations of Se in plasma (P-Se) in school-age children, pregnant women and, in a subgroup of pregnant women, the activity of antioxidative enzymes in erythrocytes during pregnancy and in cord blood. These act as peripheral markers of antioxidative activity associated with pro-oxidative effects of Hg (Kobal et al., 2008, 2004; Lund et al., 1993), and/or with the pregnancy itself (Agarwal et al., 2005; Sugino et al., 2000). Among these antioxidative enzymes, we focused on the activity of selenoenzyme GPx, CAT and SOD in erythrocytes.

The study included the following: 1) inhabitants of the former mercury mining area of the town of Idrija: 63 children 6–11 years of age (all together there were 289 children of the same age in the town) and 20 pregnant women aged 20–35 years (on average, 41 women gave birth per year in the study period in the town of Idrija), and 2) a control group of age-matched children from a rural uncontaminated area (Žužemberk, $n=66$) and an urban area (Ljubljana, $n=47$) and 15 age-matched pregnant women from a rural uncontaminated area.

The *study population of school-age children* was recruited from schools in each study area in the years 2008 – contact was established first with the headmaster and then with parents and their children. Written consent was obtained from a parent of each child, while oral consent was obtained from the child prior to sampling. Only healthy children, with no evidence of chronic diseases were recruited.

The *study population of pregnant women* was conducted in years between 2003 and 2008. The selection of pregnant women was based on medical examinations performed by a gynaecologist and a specialist in occupational medicine, who among other standard data also evaluated the number of dental amalgam fillings and calculated the dental amalgam score using the method of Aposhian and co-workers (Aposhian et al., 1992). The following selection criteria for pregnant women were applied: informed consent from the pregnant women to participate in the study; the study and control groups were neither currently nor previously occupationally exposed to Hg, lead, cadmium or halogenated hydrocarbons; the medical history and medical examinations did not reveal any hypertension, alcoholism, lung diseases (asthma, chronic obstructive pulmonary diseases, pulmonary fibrosis), metabolic disorders, auto-immune diseases (rheumatoid arthritis, lupus erythematodes); and hepatic and renal diseases (proteinuria) which can influence the absorption or excretion of Hg and antioxidative enzyme activity.

The National Medical Ethics Committee of Republic of Slovenia approved the study.

2.2. Biological sampling

Venous *blood* from school-age children was collected by venipuncture in 4 mL Lithium Heparin plastic tubes (Greiner-Bio One GmbH, Frickenhausen, Germany) for analysis of total Hg and plasma Se. Venous blood from pregnant women was collected during the first and last trimester of pregnancy and in cord blood of infants for analysing total Hg, Se in plasma and antioxidative enzymes CAT, CuZn-SOD and GPx in erythrocytes. Blood was collected by venipuncture in 3 mL and 7 mL BD Vacutainer® tubes (Benton Dickinson) containing the anticoagulant K3-EDTA and Lithium Heparin, respectively. The samples were transported to the laboratory within 2 h at 2–8 °C, where they were divided into aliquots and stored at −20 to −30 °C prior to analysis.

To determine *urinary Hg*, morning urine samples of school-age

children and pregnant women were collected in metal-free polypropylene tubes. The urine samples were transported to the laboratory within 2 h at 4 °C, where they were divided into aliquots and stored at −20 °C. *Hair samples* (first cm of occipital hair) of school-age children and pregnant women were also collected for analysis. Hair samples were stored in clean PVC zip-lock bags and stored in a clean room.

2.3. Questionnaire

Participating children's parents and pregnant women completed a questionnaire on dietary habits, drinking water consumption from local sources, basic residential characteristics and residential history, basic anthropometrical measures, socio-economic status, child's age and gender, number of amalgam fillings of a child, and smoking conditions inside the home; mother's smoking habits, alcohol consumption and occupational history.

2.4. Chemical and biochemical analysis

2.4.1. Total mercury in biological samples

Total Hg in biological samples was determined at the Jožef Stefan Institute (JSI) by cold vapour atomic absorption spectrometry (CVAAS) using three different analytical procedures, depending on matrix type and the study population (period of sampling). All measurements were made under strict quality control procedures and gave comparable results. In addition, the JSI laboratory participated in a series of inter-laboratory comparisons organised within the PHIME project. Three inter-comparisons used lyophilised samples of human blood from non-exposed persons, people occupationally exposed to elemental Hg, fish eaters while the fourth study used fresh blood from the general population. The obtained values were in good agreement with the assigned values (results presented by Miklavčič et al., 2013a).

Analytical procedures used are described in detail elsewhere (Akagi, 1997; Horvat et al., 1991a; Miklavčič et al., 2011a, 2013a). Estimated analytical precision of the measurements was less than 10 %, the detection limit of the procedures was below 0.1 ng/mL in blood and urine and 1 ng/g in hair. Concentrations of total Hg in the urine were normalized to urinary creatinine.

2.4.2. Selenium in blood

Selenium in plasma was determined using Zeeman graphite furnace atomic absorption spectrometry (GFAAS) with a palladium chemical modifier. The standard was Merck selenium Tritisol standard 9948. Quality control included Seronorm Trace Elements in Serum. The method is described in detail elsewhere (Kobal et al., 2004). The detection limit for plasma was 1.9 μg/L, within-run imprecision was ± 3.2 % and day to day imprecision ± 5.6 %.

2.4.3. Erythrocyte antioxidative enzyme activity in pregnant women and cord blood

Erythrocyte GPx and SOD activity was determined using a Randox test kit and measurement of GPx activity performed using a Hitachi 717 analyser (Roche, Mannheim, Germany). The methods are described in detail elsewhere (Kobal et al., 2004). Coefficient of variation (CV) of the GPx measurements was < 6.0 % and the sensitivity was 10 U/mL. For CuZn-SOD determination the CV was < 5.0 % and the sensitivity 20 U/mL. *Erythrocyte CAT activity* was assayed by a modified enzymatic UV test using a Cary UV/VIS spectrophotometer (Varian, Australia). A standard curve was prepared using catalase provided by Sigma. The method is also described in detail in Kobal et al. (2004). For CAT measurements the overall CVs were < 7.5 % and the sensitivity was 10 U/mL. All enzymatic activities in erythrocytes were expressed as units of haemoglobin (Hb).

Routine analyses (venous blood and urine) were performed using clinical biochemical methods (data not presented). Red blood cell counts were obtained using a Coulter GEN. S electronic haematology analyser (Beckman Coulter Fullerton, CA, USA).

2.4.4. Creatinine in urine

Urinary creatinine was determined by the standardized routine method (compensated Jaffé reaction) on a biochemical analyser (Dimension, Dade Behring). Repeatability was assured by using a reference material supplied by the analyser/reagent producer. Comparability and accuracy of the method was verified by the inter-laboratory comparison excercise.

2.5. Statistical analysis

Hg levels were log transformed to normalize the distribution of the data. Descriptive statistics was performed for the questionnaire data (covariates) and exposure levels. Wilcoxon sum-rank test was used to compare Hg levels between the population groups. Spearman correlation coefficients were calculated between Hg levels and the selected covariates. For school-age children multiple linear regression models were used to evaluate the correlations between Hg exposure and all covariates that were correlated to the biomarker with a significance level of $p < 0.25$. Predefined confounders were fixed into the models (age, gender of the child, education), while highly correlating variables were excluded to avoid the effect of multi-collinearity. P values < 0.05 were considered statistically significant.

3. Results

3.1. Environmental Hg distribution and concentrations

3.1.1. Concentrations of Hg° in air and total Hg in drinking water in the town of Idrija

In the early 70's Hg production reached its peak and consequently Hg air concentrations in Idrija could reach 20 μg/m^3 (Kosta et al., 1974). After the closure of the mercury mine and smelter in 1995 the air Hg concentrations decreased rapidly and after 1999 levels were between 5 ng/m^3 and 170 ng/m^3, with higher levels in parts of the town during hot summer days (Gosar et al., 1997; Grönlund et al., 2005; Kocman et al., 2011b; Kotnik et al., 2005). In certain inhabited areas near the mine entrance and the ventilation shafts (Fig. 1) the concentrations of air Hg could exceed 100 ng/m^3 (Kotnik et al., 2005), while emissions from the heavily polluted surfaces surrounding the former distillation plant could reach up to 3000 ng/m^3 during hot summer days (Kocman et al., 2011b). Total Hg concentrations in the remote non-polluted reference location (Iskrba, Southern part of Slovenia) are 0.8 ng/m^3 – 3.4 ng/m^3 with an average of 1.5 ng/m^3 as measured within the Slovenian national monitoring programe between years 2008 and 2015 (*unpublished data*).

After closure of the mine, total Hg content in drinking water decreased in comparison to the levels obtained when the mine was operational (Kobal et al., 1992; Kosta et al., 1974). Total Hg concentrations in drinking water (samples taken from water supply pipes at different locations) in Idrija were 0.6–21.5 ng/L (Kobal et al., 1992). Regular monitoring of Hg concentration in Idrija since 1992 have shown that the levels of Hg have remained constant (*unpublished data*, 2005 – archive of the City utilities monitoring).

3.1.2. Hg concentrations in seasonal vegetables from individual gardens in the town of Idrija

The total Hg concentrations in vegetables vary considerably. Parsley leaves contained the highest concentrations (520–1500 ng/

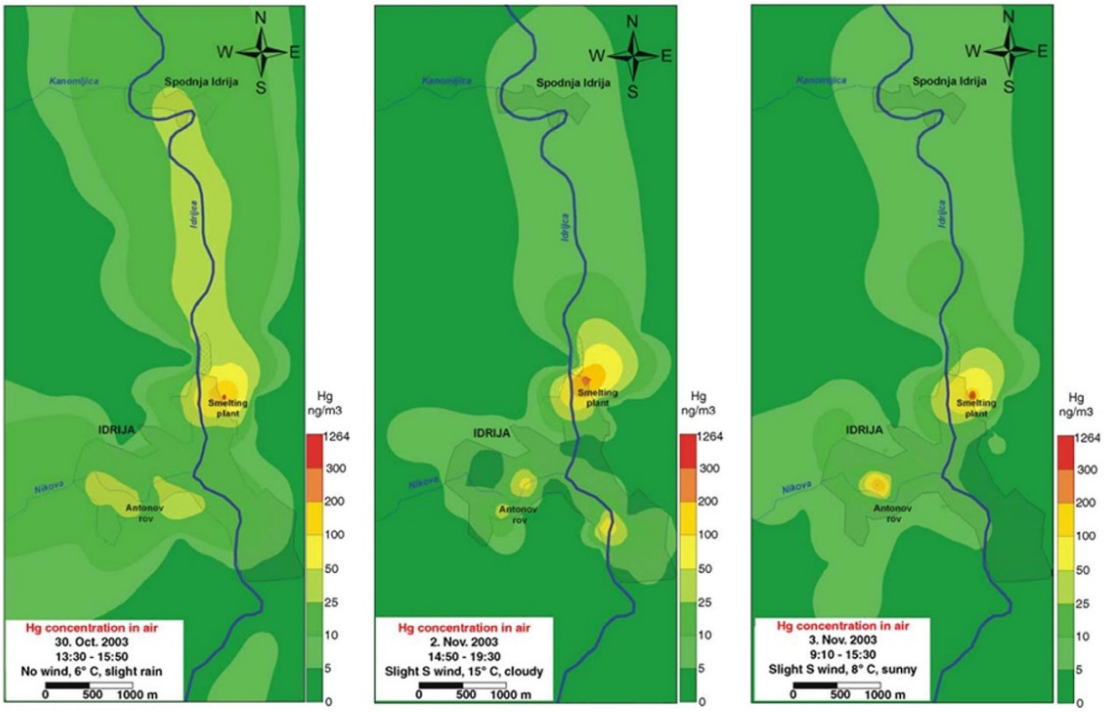


Fig. 1. Air Hg concentration maps in Idrija and its surroundings (Kotnik et al., 2005).

g) followed by parsley roots (42.8–1612 ng/g), cabbage (57–782 ng/g), chicory (22–386 ng/g), carrot (13–103 ng/g), lettuce (mean 14 ng/g), and onion (1–3.6 ng/g). In vegetables from non-contaminated gardens, corresponding levels were 1 ng/g in parsley, cabbage, chicory, and 2 ng/g in lettuce (Jereb et al., 2004). Hg levels found in these vegetables were comparable with the levels measured in 2008 and 2009 in the same types of vegetables from the same gardens (Miklavčič et al., 2013b). This indicates that the levels of Hg in vegetables have not diminished. The content of methyl Hg in vegetables was low, varying from 0.02 % to 2.3 % of the total Hg concentration (Jereb et al., 2004), but between 2008 and 2009 methyl Hg levels were up to 100 times higher (average 13.5 % of the total Hg) in certain vegetables (Miklavčič et al., 2013b).

3.1.3. *Hg concentrations in fish from the river Idrijca and from the fish market*

The total level of Hg in the river Idrijca increases several hundred-fold downstream from the mine compared to background headwater levels (Hines et al., 2006; Kocman et al., 2011a; Žižek et al., 2011, 2007). Methyl Hg levels are spatially and seasonally variable, with higher values at the most contaminated sites during summer (Žižek et al., 2007) and at impounded areas (Hines et al., 2006; Horvat et al., 2004, 2002).

Studies performed since the mine closed show that total Hg in some fish species downstream of the river Idrijca exceeded the maximum level of 1 mg/kg (wet weight) proposed by the European Commission for the muscle meat of the most contaminated fish (EC No. 78, 2005). Most fish species in the river Idrijca in the town area were below 0.5 mg/kg, the maximum level for other fishery products and muscle meat of fish (EC No. 78, 2005). Total Hg in fish muscle between various *spp.* on a fresh weight basis caught near the town were between 0.50 ($\pm$0.41) mg/kg (*Thymallus thymallus*) and 1.65 ($\pm$2.05) mg/kg (*Salmo trutta fario*), with 50 % of the Hg present as methyl Hg. Downstream of the mine at "Bača pri Modreju" the mean total Hg concentration in fish muscle was 1.35 $\pm$ 0.93 mg/kg (*Thymallus thymallus*) and 0.30 ($\pm$0.30) mg/kg (*Salmo sp.*), with 70 % present as methyl Hg (Horvat et al., 2004). In 2009, fish samples were collected again from the same locations (Miklavčič et al., 2013b) and showed similar levels of Hg as measured in past decades. This study also confirmed that fish from the most polluted site (town of Idrija) contained higher average levels of total and methyl Hg than in fish from other sampling sites, but had the lowest percentage of methyl Hg (Miklavčič et al., 2013b). The average levels of Hg in fish from the least polluted sampling site (above the town of Idrija) were comparable with the average levels of Hg in fish available on the Slovenian market. The mean total Hg content of fresh fish was 0.174 mg/kg with on average 50 % of the Hg present as methyl Hg, while the mean total Hg content of canned fish was 0.130 mg/kg (Miklavčič et al., 2011b, 2013b).

3.2. *Biological indices of Hg exposure*

3.2.1. *General characteristics of the study populations*

In total, *176 school-age children* were recruited from three areas: 65 from the town of Idrija, 47 from an urban and 69 from a rural area, with a similar distribution of age and gender across the areas. The number of amalgam fillings and frequency of fish consumption, were also similar but with a lower frequency of shellfish consumption in the rural area. Most children from the rural area ate home-grown vegetables and fruit, however the frequency of

consumption was not known (Table 1). The home-grown local vegetables consumed by children in the town of Idrija were mostly from less contaminated areas of the town; and the fish was bought on the fish market, rather than caught in the river Idrijca (data obtained from a questionnaire, not presented). Important information obtained from the questionnaire data was that most of the children were away from the town of Idrija approximately for 2 months per year in the summer time.

Twenty pregnant women were recruited from the town of Idrija and 15 control women from areas in central Slovenia, mostly rural. One of the pregnant women from the Idrija study group was excluded from data analysis because she had elevated blood and urinary Hg due to a broken Hg-containing thermometer in her house approximately one year prior to sampling. Three of the pregnant women from the control group failed to complete the study. One did not wish to participate in the study during her last trimester, one was excluded because of metabolic disorders and one was lost due to a premature delivery. Finally, 12 pregnant women from the control group and 19 pregnant women from the town of Idrija participated in the study.

During pregnancy, no adverse health effects were observed in either group of pregnant women. Equally, blood pressure were between 110/70 and 140/90 and no signs of gestosis (excessive increased body weight and urinary protein after 20 weeks of gestation in normotensive pregnant women) were observed. In both groups a decrease in Hb was observed in the 3rd trimester ($p < 0.05$), which does not differ from the level of Hb in cord blood of both groups of neonates (data not presented). Gestation before spontaneous labour varied from 38 to 40 weeks. The physical condition of the new-born (oxygenation, ventilation and degree of asphyxia) evaluated with the Apgar score at 1 and 5 min after birth (skin colour, pulse rate, respiratory effort, reflex irritability-grimace, muscle tone), was normal (9 – 10) in all but one newborn from the control group that had a lower score (score 4) and required ventilation and oxygenation.

The number of amalgam feelings and consumption of fish was comparable between the pregnant women study groups, with women having on average 3 amalgam fillings (1 – 4) and consuming fish on average 1–3 times per month (data obtained from a questionnaire, representing the last 12 months prior to sampling). The consumption of highly contaminated fish from the river Idrijca occurred only occasionally. In both study groups, the consumed fish were mostly bought from the fish market.

3.2.2. Biological indices of Hg exposure in school-age children

The levels of Hg in blood and urine did not differ significantly between children living in the former mercury mining town and those living in an urban area ($p=0.355$ and 0.179, respectively). In both areas, levels (Table 2) were significantly higher than those in the rural area, both in blood ($p < 0.001$) and in urine ($p < 0.001$ and 0.014). A distinctive difference between the Idrija and urban children was observed only in the upper 10 % of the population, with higher levels of Hg in the children from Idrija (Table 2). Similarly as with blood and urine levels, total Hg levels in the hair of the Idrija children were significantly higher than the hair levels of those living in a rural area ($p < 0.001$). Hair from urban children was not available for analysis.

Blood total Hg levels in the total studied population were correlated significantly with the frequency of fish ($r_s=0.18$, $p=0.023$) and shellfish consumption ($r_s=0.27$, $p < 0.001$) and with the number of amalgam fillings ($r_s=0.27$, $p < 0.001$). In hair, total Hg levels were correlated significantly with the frequency of fish ($r_s=0.22$, $p=0.011$) and shellfish consumption ($r_s=0.33$, $p < 0.001$) and in urine with the presence of amalgam fillings ($r_s=0.51$, $p < 0.001$). Once the levels were adjusted for a child's age, gender, educational level of the parents, and both determinants of exposure (seafood consumption and amalgam fillings) were incorporated into the multiple linear regression model ($R^2=0.19$, $p=0.004$), amalgam fillings were the only determinant having a significant influence on blood Hg levels ($p=0.010$). This was also the case in the population group-specific models (Table 3). In hair, the regression model ($R^2=0.25$, $p < 0.001$) showed only a marginally significant correlation between total Hg levels and fish ($p=0.063$) or shellfish consumption ($p=0.067$), and as demonstrated by the population-specific model this association could be attributed only to the rural group (Table 3). In urine, both general ($R^2=0.30$, $p < 0.001$) and population-specific regression models (Table 3) show a significant correlation between total Hg levels and amalgam fillings.

Table 1
General characteristic of the studied population of 6–11 years old children from three studied areas in Slovenia: former mercury mining town (Idrija), and an urban and rural area.

	Idrija	Urban	Rural
n	65	47	69
Boys (n)	35	20	36
Girls (n)	30	27	33
Age			
6–8 years	18 (28%)	14 (30%)	20 (29%)
9–11 years	46 (72%)	33 (70%)	49 (71%)
No. of amalgam fillings			
None	20 (42%)	13 (37%)	21 (31%)
1–2	10 (21%)	7 (20%)	32 (46%)
3 or more	18 (37%)	15 (43%)	16 (23%)
Consumption of sea fish			
Never or less than 1/month	17 (27%)	11 (26%)	21 (30%)
Once per month	31 (49%)	18 (43%)	32 (77%)
At least once/week	15 (24%)	13 (31%)	16 (23%)
Consumption of shellfish			
Never	27 (42%)	21 (50%)	51 (74%)
Less than once per month	22 (34%)	9 (21%)	10 (14%)
At least once per month	15 (24%)	12 (29%)	8 (12%)
Consumption of home grown food			
Yes	50 (91%)	15 (58%)	69 (100%)
No	5 (9%)	11 (42%)	0 (0%)

Furthermore, we found that urinary total Hg was correlated to chewing-gum: among the children with amalgam fillings, i.e., those who chewed daily had higher level of urine total Hg than children who did not chew daily ($p=0.033$).

Besides seafood and amalgam fillings, education level (defined as the highest in the family) also showed a significant positive correlation with blood and hair total Hg levels in children ($r_s=0.26$, $p < 0.001$ and $r_s=0.22$, $p=0.012$, respectively). This was found to be the case in the control areas ($r_s=0.29$, $p=0.053$ and $r_s=0.29$, $p=0.020$, respectively for urban and rural area), but not for the children living in Idrija ($r_s=-0.07$, $p=0.581$).

3.2.3. Biological indices of Hg exposure in pregnant women

During pregnancy total blood Hg levels were in general higher in the women living in Idrija than in the control group (Table 4). This difference was significant in the maternal and cord blood ($p=0.009$ and 0.028, respectively) but not in hair and urine ($p=0.434$ and 0.247, respectively). Total Hg concentrations in venous blood from the 3rd trimester of pregnancy in pregnant women from Idrija correlated significantly ($p < 0.05$) with the number of dental amalgam fillings (dental amalgam score).

3.3. Selenium in plasma and the activity of antioxidative enzymes

Total Hg levels determined in the blood of *school-age children* were compared with the levels of plasma selenium (P-Se).

Table 2
Total mercury levels in 6–11 years old children. n – sample number, SD – standard deviation, GM-geometric mean, 95% CI – confidential interval. P10 – 10th percentile, P25 – 25th percentile,....

Study group	n	min - max	Mean ± SD	GM (95% CI)	P10	P25	P90	P95
THg, blood (ng/mL)								
Rural	66	0.35–3.76	0.84 ± 0.58	0.72 (0.64–0.81)	0.47	0.53	161	1.82
Urban	45	0.36–3.11	1.09 ± 0.55	0.99 (0.86–1.13)	0.56	0.78	1.67	2.19
Idrija	63	0.41–4.47	1.08 ± 0.76	0.92 (0.81–1.05)	0.51	0.66	2.08	2.14
Total	174	0.35–4.47	0.97 ± 0.64	0.86 (0.79–0.92)	0.49	0.57	1.74	2.19
THg, urine (μg/g crea.)								
Rural	58	< 0.10–5.43	0.87 ± 1.13	0.47 (0.35–0.64)	0.12	0.24	1.65	4.02
Urban	43	0.20–4.15	1.11 ± 0.99	0.82 (0.64–1.03)	0.31	0.46	2.01	3.85
Idrija	64	0.15–10.3	1.74 ± 1.97	1.08 (0.84–1.37)	0.31	0.44	3.57	5.63
Total	163	< 0.10–10.3	1.28 ± 1.53	0.77 (0.66–0.91)	0.25	0.38	3.53	4.15
THg, hair (ng/g)								
Rural	67	15.0–840	181 ± 159	132 (109–161)	48.0	76.0	389	520
Idrija	64	53.0–1114	293 ± 190	241 (205–283)	107	155	516	606
Total	131	15.0–1114	236 ± 183	177 (155–204)	65.0	111	486	595

The correlation was positive and significant only in children from Idrija (Fig. 2), but not in the children from the rural ($r=0.05$, $p=0.670$) or urban areas ($r=0.02$, $p=0.874$). Otherwise, the P-Se levels did not differ significantly among the study groups ($p=0.274$).

In the *subgroup of pregnant* women from the town of Idrija ($n=11$) and in the control subgroup ($n=7$) changes in concentration of P-Se (μg/L) and antioxidative enzyme activity GPx in erythrocytes (U/g Hb) during pregnancy and in cord blood were demonstrated (Fig. 3A and B). In the exposed group, P-Se concentrations (Fig. 3A) significantly decreased ($p<0.001$) from the 1st trimester (50–76 μg/L, mean 65.9) to the 3rd trimester (36–62 μg/L, mean 50.0), and in cord blood (30–64 μg/L, mean 44.0) *versus* the 1st trimester ($p<0.001$). In the control group significant decreases ($p<0.05$) in the P-Se concentrations from the 1st trimester (60–79 μg/L, mean 69.2) to the 3rd trimester (42–61 μg/L, mean 49.4), and in cord blood (30–48 μg/L, mean 42) *versus* the 1st trimester ($p<0.001$) were observed. No significant differences in the decrease in P-Se concentrations between the 3rd trimester and cord blood within the groups, and between the

Table 3
Multiple linear regression: determinants of Hg exposure in 6–11 years old children.

	Idrija ß (95% CI)	**Urban ß (95% CI)**	**Rural ß (95% CI)**
Total Hg in blood (μg/L) Model R^2	*0.44 (p=0.552)*	*0.46 (p=0.728)*	*0.49 (p=0.095)*
Consumption of sea fish			
Never or less than 1/month	1.00	1.00	1.00
Once per month	0.87 (0.37–1.37)	1.11 (0.63–1.59)	1.04 (0.78–1.29)
At least once/week	1.08 (0.51–1.64)	1.18 (0.64–1.72)	1.20 (0.61–1.80)
Consumption of shellfish			
Never	1.00	1.00	1.00
Less than once per month	1.07 (0.63–1.51)	1.07 (0.63–1.51)	1.23 (0.85–1.61)
At least once per month	1.05 (0.58–1.54)	0.97 (0.49–1.46)	1.34 (0.87–1.82)
Amalgam fillings			
none	1.00	1.00	1.00
1–2	**1.51 (1.00–2.01)***	0.92 (0.34–1.49)	**1.25 (0.97–1.52)#**
3 or more	**1.41 (1.01–1.81)***	1.04 (0.58–1.50)	**1.31 (0.94–1.67)#**
Total Hg in hair (ng/g) Model R^2	*0.32 (p=0.642)*	–	*0.45 (p=0.097)*
Consumption of sea fish			
Never or less than 1/month	1.00	–	1.00
Once per month	0.98 (0.51–1.44)	–	1.03 (0.62–1.45)
At least once/week	1.32 (0.83–1.82)	–	1.27 (0.28–2.26)
Consumption of shellfish			
Never	1.00	–	1.00
Less than once per month	1.10 (0.67–1.52)	–	**1.53 (0.96–2.10)#**
At least once per month	1.10 (0.63–1.57)	–	**1.76 (0.94–2.56)#**
Total Hg in urine (μg/L) Model R^2	*0.59 (p=0.005)*	*0.46 (p=0.253)*	*0.45 (p=0.072)*
Amalgam fillings			
none	1.00	1.00	1.00
1–2	1.59 (0.91–2.27)#	1.21 (0.41–2.01)	**1.70 (1.04–2.36)***
3 or more	**2.25 (1.68–2.81)****	**1.74 (1.10–2.39)***	**2.36 (1.57–3.16)****

Remark: Models are adjusted for child's age, gender and educational level (highest in the family).
Margin of statistical significance:
* $p<0.05$,
# $p<0.1$,
** $p\leq 0.001$.

Table 4
Total mercury levels (THg) in pregnant women and their newborns. n – sample number, GM-geometric mean, 95% CI – confidential interval.

	n	Pregnancy (III. trimester) GM (95% CI), *range*	n	Infants GM (95% CI), *range*
Exposed group (Idrija)				
THg in urine (μg/L)	19	2.51 (1.91–4.07), *0.77–9.5*		
THg in hair (μg/g)	17	251 (192–394), *61–607*		
THg in blood (μg/L)	16	1.86 (1.14–3.51), *0.05–5.30**	8	2.61 (1.65–4.14), *1.04–5.41**
Control group				
THg in urine (μg/L)	6	*1.90 (0.93–3.88), 0.95–5.30*		
THg in hair (μg/g)	12	211 (129–344), *45 – 602*		
THg in blood (μg/L)	12	0.98 (0.55–1.72), *0.19–3.17*	6	1.17 (0.63–2.14), *0.48 – 2.13*

Margin of statistical significance:
* $p < 0.05$.

groups during pregnancy and in cord blood were found. In the exposed subgroup of pregnant women from Idrija the mean activity of GPx in erythrocytes (Fig. 3B) was lower ($p > 0.05$) in the 3rd trimester (22–116 U/g Hb, mean 55.0) *versus* the 1st trimester (47–116 U/g Hb, mean 70.8), but the mean activity was the same as in cord blood (25–65 U/g Hb, mean 50.5). No significant differences in the mean activity of GPx in erythrocytes during pregnancy and in cord blood between the two groups of pregnant women were observed.

Fig. 4 (C and D) shows changes in the activity of antioxidative enzymes CAT and SOD in erythrocytes (MU/g Hb, U/g Hb) during pregnancy and in cord blood in the group of pregnant women from Idrija and in the control group. The differences in the mean erythrocyte CAT activity levels (Fig. 4C) during pregnancy and in cord blood were not significant within and between the groups. Although not significant, the highest level of CAT activity in erythrocytes was observed in the 3rd trimester (2.51–4.84 MU/g Hb, mean 3.64) of pregnancy in the exposed group. During pregnancy SOD activity in erythrocytes (Fig. 4D) increased, reaching the highest level in cord blood in both groups. In the exposed group, the increase in SOD activity in the 3rd trimester (1238–2031 U/g Hb, mean 1604) *versus* the 1st trimester (1217–1781 U/g Hb, mean 1441) was not significant but the increase in the control group in the 3rd trimester (1000–1712 U/g Hb, mean 1451), *versus* the 1st trimester (993–1323 U/g Hb, mean 1100) was significant ($p < 0.05$). In the exposed group the increase in SOD activity in cord blood (1888–2381 U/g Hb, mean 2116) *versus* the 1st trimester

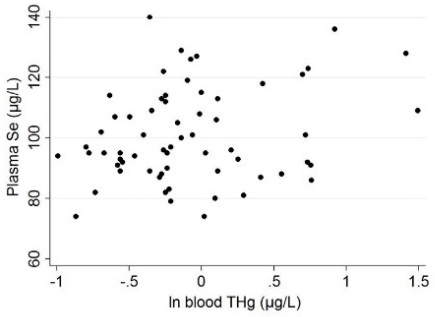


Fig. 2. Association between plasma Se and ln transformed total Hg in blood (THg) of 6–11 years old children from the former mercury mine area (r=0.25, p=0.044).

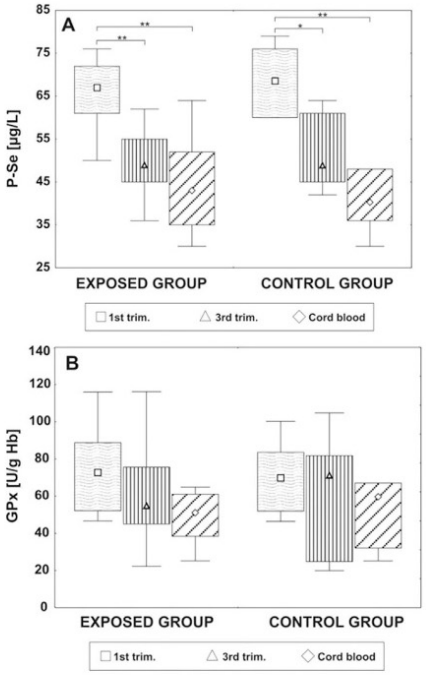


Fig. 3. Biological markers of potential Hg interaction: (A) P-Se (μg/L) concentrations, (B) GPx activity in erythrocytes (U/g Hb) during the pregnancy (1st – 3rd trimester), and in cord blood of the exposed (n=11) and control (n=7) groups. Statistically significant differences are given as *$p < 0.05$, **$p < 0.001$.

was significant ($p < 0.001$), as was the increase ($p < 0.001$) in SOD erythrocyte activity observed in cord blood (1848–2259 U/g Hb, mean 2017) in the control group. A significant increase in SOD activity was found in cord blood *versus* the 3rd trimester in both groups ($p < 0.05$). The mean level of erythrocyte SOD activity in the 1st trimester of the exposed pregnant women was significantly higher than that of the control subgroup ($p < 0.01$), but no difference between the groups was observed in the 3rd trimester and cord blood.

4. Discussion

The evaluated data on environmental Hg contamination since the shutdown of the Hg mine and smelter in 1995 suggests that inhabitants of the town of Idrija are potentially exposed to various forms of environmental Hg. Air Hg concentrations in the town have decreased considerably over the past two decades, the level of air Hg° is between 5 and 170 ng/m^3(Grönlund et al., 2005; Kocman et al., 2011b; Kotnik et al., 2005), which is under the tolerable concentration of 0.2 μg/m^3 derived from the lowest-observed-adverse-effect level (LOAEL) for long-term inhalation exposure (WHO, 2003). Vegetables from the gardens situated close to the former smelter and fish in the river Idrijca downstream from the town are still contaminated with inorganic Hg and, particularly fish, with methyl Hg (Horvat et al., 2004; Jereb et al., 2004; Miklavčič et al., 2013b). Therefore, inhabitants who frequently consume vegetables from contaminated gardens and/or fish caught in the contaminated part of the river Idrijca can be exposed to a level of inorganic and/or methyl Hg, that is above that

for the "normal" non-exposed population. Potential retention of Hg from the respective sources of Hg exposure in the town of Idrija was calculated for pregnant women (as they tend to stay at home during the pregnancy and eat locally grown food) and was based on the environmental concentrations described above (Section 3.1) and general assumptions described in the WHO methodology (WHO, 2003, 1991, 1990). The estimated retention of Hg is summarized in the Table 5.

As expected, the estimated potential retention of Hg° from inhaled air and of inorganic and methyl Hg exclusively from the consumption of vegetables in *pregnant women* from the town of Idrija is above the average daily retention for a non-exposed population (Table 5). Low concentration of inorganic Hg in drinking water in the town of Idrija does not substantially increase the exposure of inhabitants and the estimated retention is below that for the general population (Table 5). Amalgam fillings, the source of Hg° exposure that is typical for the general population, contributed equally to the Hg retention in both study groups. As estimated by WHO (WHO, 2003), the Hg retention from amalgams in 5–11 years old children is 5–11 μg/day, and 9.4 μg/day in adults. Furthermore, it was estimated that 10 amalgam fillings surfaces may, on average, increase urinary Hg level by approximately 1 μg/L (Dye et al., 2005). To obtain a more accurate estimate of exposure from amalgam fillings, a more precise measure, that takes into account the amount of time each amalgam is in the mouth, should be applied (Maserejian et al., 2008). The retention of methyl Hg from the consumption of fish from the Slovenian market is below the Reference Dose (RfD) of 0.1 μg methyl Hg/kg body weight per day (US EPA, 2001) in both study groups.

The actual exposure to Hg in *school-age children* from all three study groups was characterised by the presence of amalgam fillings and to a certain extent (particularly in the control rural area) by the consumption of seafood, bought from the fish market. The levels of total Hg in biological matrices showed comparable exposure level in Idrija and in urban area groups (Table 2). The data from the questionnaires showed that in Idrija and urban area groups there was a higher proportion of children with 3 or more amalgam fillings in comparison to the rural area; and also that more fish meals were consumed in Idrija and in the urban area (average 3 meals per month) in comparison to the rural area (average 2 meals per month). Of the fish consumed by the Idrija children, none included fish from the contaminated parts of the river Idrijca. Also, locally grown food consumed by the Idrija town children did not originate from contaminated sites surrounding the ventilation shafts and the smelting plant and children mostly ate their main meals in the school, where non-local market food was consumed. Based on this, we can assume that local food did not contribute significantly to Hg exposure in the Idrija town children. Within the project PHIME, *total Hg levels in blood of the urban children* were also compared to that of urban children of other European countries (including Croatia, Czech Republic, Poland, Slovakia, Slovenia and Sweden). Slovene children had the highest blood Hg levels. This is because Slovene children had the highest number of amalgam fillings compared to the other countries, whereas the intake of fish was close to the average intake reported in the study (Hrubá et al., 2012). In addition, children monitored within the German Environmental Survey (2003/06) showed much lower levels than Slovene children: the GM of those consuming fish meals more than three times per month was 0.29 ng/mL blood and GM of those who had more than 2 amalgam fillings was 0.21 μg/L urine (Becker et al., 2008). Despite the elevated levels, none of the studied Slovenian children exceeded the HBM I level of 5 μg/L in blood (Schulz et al., 2011), below which no biological and/or adverse health effects are expected. In hair, the levels of most children were below 1 μg/g total Hg, which corresponds to the US EPA RfD of 0.1 μg methyl Hg/ kg body weight per day and is associated with no adverse health effects in exposure to methyl Hg (Rice et al., 2003; US EPA, 2001). One child from Idrija, marginally exceeded this value (Table 2). Levels in urine showed a distinct difference between the Idrija and urban children in the upper 10 % of the studied populations, and indeed there were 5 children in the Idrija town who exceeded the HBM I level of 5 μg/g creatinine, while in the urban area none and in the rural area only one child exceeded this level. None of the children exceeded the HBM II level of 20 μg/g creatinine, which is associated with biological and/or potential adverse health effects.

Biological monitoring of Hg exposure in *pregnant women living in the contaminated area* was similar to that observed in school-age children: despite the small number of women included in our study, the results revealed differences in the amount of Hg received during pregnancy between the women living in Idrija and in the control group. The group of control women included mostly women from rural areas, which shared similar characteristics as the control group of children living in the rural area. Both rural study groups had significantly lower blood Hg levels than the exposed groups (Table 2, Table 4), but as observed in the children, also lower than the group living in the urban area (Table 2). Total Hg blood mean levels in the 3rd trimester of pregnancy in women from Idrija (Table 4) were similar as for a population that consumes 2–3 fish meals per month (Wilhelm et al., 2004), and only two women from Idrija exceeded the HBM I value of 5 μg/L blood. None of them exceeded the HBM II value of 15 μg/L (Schulz et al., 2011) that could be associated with biological and/or potential adverse health effects. Similarly, neither hair total Hg levels in pregnant women nor cord blood total Hg levels in the present

Table 5
Estimated potential average daily retention of Hg° and Hg compounds in μg/day in pregnant women living in the town of Idrija (calculated) and in the general population not occupationally exposed to Hg. Calculations were made based on the WHO methodology (WHO, 1991, 1990, 2003).

Source of exposure		Inhabitants of the Idrija town (pregnant women)	General, non-exposed population
Hg° vapour	air	0.08–1.4[a]	0.024[b]
Inorganic Hg compounds	vegetables (chicory, cabbage)	0.23 (winter)[c], 0.67 (summer)[c]	0.25[d]
	drinking water	0.0005–0.0015[e]	0.0035[f]
Methyl Hg	vegetables (chicory, cabbage)	0.032 (winter)[c], 0.09 (summer)[c]	0[g]
	fish	1.5[h]	6.5[i]

[a] air Hg° concentration range 5–170 ng/m³ in the town of Idrija; pulmonary ventilation (20 m³ per day); pulmonary absorption (80 %) (WHO, 2003, 1991, 1990);

[b] calculated from total Hg concentration in air of 2 ng/m³ (75 % as Hg°, 5 % as inorganic Hg and 20 % as methyl Hg).

[c] percentage of Hg in the vegetable as inorganic Hg (99 %) and as methyl Hg (1 %), rate of absorption of inorganic (7 %) and methyl Hg (95 %) in the digestive tract; exclusive consumption of vegetables from individual gardens in Idrija, consumption of chicory and cabbage (seasonal 500 g per week, and non-seasonal 250 g per month), the percentage of Hg in the vegetable as inorganic Hg (99 %) and as methyl Hg (1 %), rate of absorption of inorganic Hg (7 %) and methyl Hg (95 %) in the digestive tract (WHO, 2003, 1991, 1990);

[d] retention of inorganic Hg from non-fish food for the general population (WHO, 2003, 1991, 1990)

[e] daily water consumption of 2 L, rate of absorption of inorganic Hg (7 %) in the digestive tract (WHO, 2003, 1991, 1990)

[f] retention for the general population (WHO, 2003, 1991, 1990)

[g] retention of methyl Hg from non-fish food for the general population (WHO, 2003, 1991, 1990)

[h] calculated from the known concentration of methyl Hg in fish from the Slovenian market (Miklavčič et al., 2011a)

[i] calculated from the Reference Dose (RfD) of 0.1 μg methyl Hg/kg body weight per day (US EPA, 2001)

study exceeded 1 μg/g hair or 5.8 μg/L blood, the corresponding equivalents to a RfD of 0.1 μg emethyl Hg/kg body weight per day (USEPA, 2001). An earlier study of pregnant women made when the mine was operational (Horvat et al., 1991b), recorded higher cord blood total Hg values (2.25–6.11 μg/L).

Similarly as for school-age children, urinary Hg levels in 4 pregnant women in Idrija exceeded the HBM I level of 5 μg/g creatinine, which is also above the background level for an unexposed population (Clarkson and Magos, 2006). This and the elevated venous and cord blood Hg levels in pregnant women from Idrija (at a relatively lower level of total Hg in hair) suggests that total blood Hg in pregnant women and cord blood from Idrija reflect mostly intermittent exposure to Hg° (Kobal et al., 1999; Roels et al., 1987). Due to the relatively low number of amalgam fillings observed in both groups of pregnant women, a significant increase of Hg° intake from their amalgams was not expected, but the positive and significant correlation between the dental amalgam score and total Hg in venous blood of pregnant women ($p < 0.05$) confirms that dental amalgams are an important source of exposure to Hg° (WHO, 2003, 1991, 1990), which may additionally increase Hg° exposure in pregnant women from air Hg° in the town of Idrija.

4.1. Selenium in plasma and the activity of antioxidative enzymes

Selenium as an essential element is an integral part of 25 human Se-proteins including GPx and Se protein P that antagonize Hg by binding it directly and/or by catalysing redox reactions and antioxidant defence (Kryukov et al., 2003). Se protein P acts also as a transporter of Se from liver to other sites where Se is required (Reilly, 2006) and elevated exposure to Hg increases Se protein expression to sequester Hg (Chen et al., 2006; Yoneda and Suzuki, 1997). Higher accumulation of Hg in tissues of ex-miners can reduce the bioavailability of Se (Byrne et al., 1995; Falnoga et al., 2002; Kosta et al., 1975), which was demonstrated after occupational exposure to Hg°, where the P-Se levels (71.4 ± 12.4 μg/L) negatively correlated with the cumulative urinary Hg level ($r = -0.38$, $p < 0.001$) (Kobal et al., 2004). The positive correlation between blood total Hg levels and P-Se in school-age children (Fig. 2) suggests an increased Se protein expression as a consequence of moderately elevated exposure to Hg° in the town of Idrija as well as from amalgam fillings. At the same time it excludes exposure that would result in the accumulation of Hg in inhabitants of Idrija as was observed after occupational exposure. The observed decrease of P-Se concentrations from 1st to 3rd trimester and in cord blood *versus* 1st trimester ($p < 0.001$) in both observed groups of women excludes any significant interaction of Se with Hg. The mean P-Se level in pregnant women from Idrija still satisfies the Se requirement for normal GPx activity (Rea et al., 1979). The mean level of P-Se in cord blood in the exposed and control groups was below the reference value for an infant at < 1 month (50 μg/L), which can be explained by the physiological influence of the mothers diet during pregnancy (Muntau et al., 2002).

The Se enzyme GPx protects cell membranes against oxidative stress and also acts as the body's storage depot for Se, interdependently with the antioxidant vitamins (A, C and E), catalyzing the decomposition of hydrogen peroxide, lipid peroxides and hydroperoxides (Michiels et al., 1994; Rayman, 2000). In the study of Zachara and co-workers (Zachara et al., 1993) a decrease in Se in whole blood and plasma, as well as a decrease in the GPx activity in erythrocytes during normal pregnancy was observed, and the lowest Se levels and GPx activity were noted just before delivery. In other studies (Mihailović et al., 2000; Mistry et al., 2008) a highly significant decrease in serum Se concentrations and plasma GPx activity in pregnancy was also shown. In the present study, a decrease in the GPx activity in erythrocytes from the 1st trimester of pregnancy and cord blood was observed (Fig. 3B), but it was not significant. The results obtained in this and other studies (Mihailović et al., 2000; Mistry et al., 2008; Zachara et al., 1993) suggest an increased requirement for Se during pregnancy to maintain GPx antioxidative activity in blood up to delivery to ensure protection from increasing oxidative stress.

Increase in erythrocyte SOD activity in the 1st trimester of pregnancy in women from Idrija (Fig 4) ($p < 0.01$), hypothetically reflects a moderate retention of Hg^{2+} in placenta. Inorganic Hg (Hg^{2+}) results from inhaled Hg° which is then oxidised by CAT in blood (Ask et al., 2002). The SOD activity, which gradually increased during pregnancy, is likely to contribute to the maintenance of the pregnancy by preventing the accumulation of superoxide radicals, lipid peroxide and prostaglandins in decidua and endometrium (Djordević et al., 2004; Mistry et al., 2008; Sugino et al., 2000). During occupational exposure to Hg°, SOD activity in erythrocytes increases during elevated on-going exposure to Hg° (Palinkaš et al., 1995), whereas at lower levels of exposure the activity of SOD did not increase (Bårregard et al., 1995; Björkman et al., 1993; Kobal et al., 2004). This data suggests that an increase in SOD activity in erythrocytes during pregnancy is chiefly associated with increasing antioxidative capacity in the placenta to maintain a healthy pregnancy.

Many studies have shown that CAT activity does not change during normal gestation (Carone et al., 1993; Chaudhari et al., 2003; Djordević et al., 2004), which was also observed in the present study. CAT is responsible for the oxidation of Hg° to Hg^{2+} in erythrocytes and in other tissue cells (Halbach and Clarkson, 1978) and its activity in erythrocytes depends on the rate of Hg°

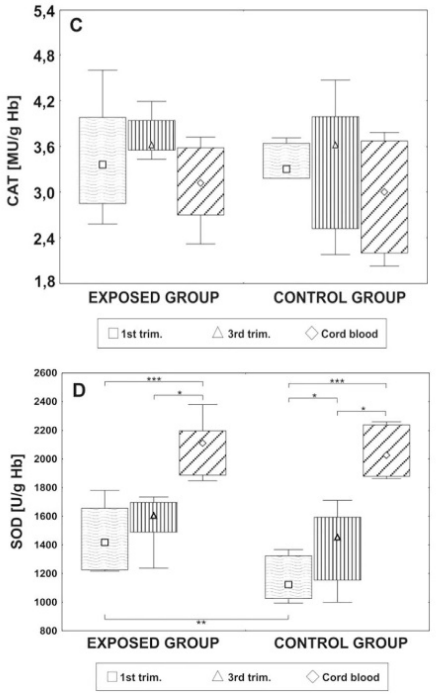


Fig. 4. Biological markers of potential Hg interaction: (C) CAT and (D) SOD activity in erythrocytes (MU/g Hb and U/g Hb) during pregnancy (1st – 3rd trimester) and in cord blood in exposed and control groups. Values are given as mean ± SD range. Statistically significant differences are given as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

absorption. This activity significantly decreases during increased Hg° absorption, while in the period after exposure it slowly increases (Kobal et al., 1999, 2008). The activity of CAT in erythrocytes observed during pregnancy and in cord blood suggests that the on-going absorption of blood Hg° in the exposed group was low.

5. Conclusions

Biological indicators of exposure to methyl and inorganic Hg reflect, predominantly, exposure to Hg° from dental amalgam and to a certain extent to air Hg° in both study groups from Idrija - school-age children and pregnant women. Although the exposure level of the two study groups living in the contaminated area was significantly higher than in the control rural groups, it is comparable to the urban group of children and is below the critical levels for biological and/or adverse health effects.

The internal doses of Hg received in children and in pregnant women living in the town of Idrija did not decrease the bioavailability of Se. Based on the observation in school-age children, a higher Se level in plasma is suggested as a consequence of moderately elevated exposure to Hg°. No association was observed between blood Hg levels in pregnant women and selected oxidative stress biomarkers. However, during the pregnancy and in newborns a decrease in Se plasma level, decrease in GPx activity, no significant changes in CAT activity and increased SOD activity in erythrocytes were observed and were similar in both study groups. It seems, therefore, that the observed changes were associated with the pregnancy *per se*. The concentrations and distribution of Hg in the environment surrounding the town of Idrija represent a potential load that could increase exposure to Hg and pose a risk for the most susceptible population groups. In view of the low number of observed pregnant women studied, we propose a further longitudinal study of a larger group of pregnant women residing in the locality of the former mercury mine, which measures more precisely local food consumption and dental amalgam exposure.

We also recommend that pregnant women and children should not consume seasonal vegetables such as cabbage, parsley leaves and carrots from domestic gardens located in the area of the former Hg smelting plant and fish from the Idrijca river because of their higher contents of inorganic and methyl Hg. The findings of the study should be considered by the competent services and particularly by the fisheries society in the Idrija municipality in order to take appropriate action to avoid/limit Hg exposure in susceptible groups of inhabitants.

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Declaration of interest

The authors report no declaration of interest.

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Chapter 4

Conclusions

According to the main subjects of the thesis, we have assessed the exposure to Hg and also other potentially neurotoxic elements (Pb, Cd, As) and the status of essential elements (Mn, Se, Cu, Zn) in the Slovenian population of childbearing age and their children. This was achieved through the national HBM program and the complementary HBM activities as presented by the papers in Chapter 3. Sections 3.1 to 3.3 cover the methodological aspect of HBM, which is crucially connected to the design of such studies, and defined the reference population to derive background exposure for the selected population. Association between prenatal exposure to Hg and neurodevelopment of children was assessed within Sections 3.4 and 3.5. The latter took into account confounders that could influence the association, arising from the non-genetic (demographic, socio-economic, dietary, and co-exposure to Pb and status of beneficial micronutrient Se), and genetic (*APOE* polymorphism) factors. The assessment of the overall association at low-levels of exposure covered prenatal Hg exposure which was mainly on account of fish consumption during pregnancy. The exposure to Hg due to the past mining activities was further assessed for the population groups residing in the town of Idrija (Section 3.6), however the sample size of the mother-child study group was too low to allow assessment of association between the exposure and neurodevelopment in this particular study population.

Findings complying with the specific aims of the thesis are the following:

- Among the biomarkers recognized as appropriate for determination of Hg and other trace element exposure in the general, occupationally non-exposed, population, urine concentrations were identified as the most critical due to inter-individual differences in diuresis and different normalization methods available. We confirmed previous findings that SG appears to be a more reliable alternative in comparison to creatinine. For the sake of comparability with other studies, the reference values in urine are provided as (1) volume expressed, (2) creatinine and (3) SG normalized.
- Moreover, using hematocrit as a confounder in the statistical models, the prediction of sources of exposure improved. This suggests that confounding or normalizing the blood elemental concentrations for hematocrit improves the precision of internal exposure assessment.
- Breast milk as a biomarker of exposure through breastfeeding in the neonatal period showed certain limitations. Elemental concentrations in milk were expressed per volume, which is appropriate for general assessment of the exposure levels, but for studying associations the concentrations in the samples should be normalized for water content. This is particularly important for the

exposome approach in the on-going and future studies, in which measurements of environmental pollutants are used for environmental wide association studies.

- The required sample size increased the most on account of variability arising from the measurements performed in different laboratories, compared to the population variability and measurement uncertainty within one laboratory. We therefore propose a single-laboratory approach as a cost-effective compromise.
- The first national HBM defined the reference population representative for the adults of childbearing age living in Slovenia, which enabled to set the national reference levels for trace elements. The established reference levels are the first available for the *postpartum* period and represent the background exposure in the selected Slovenian population.
- The Slovenian childbearing population was observed to have generally low to moderate exposure to Hg because of low to moderate consumption of fish and other seafood, and moderate contribution from amalgam fillings. The seafood consumption and consequently the internal Hg exposure were relatively higher in the coastal part of the country, where 22% of the study population of women exceeded the U.S. EPA threshold level for developmental risk (in other study areas altogether 6% of the female participants exceeded the threshold level). Hg concentrations in urine, which in the general population indicate mainly the exposure from amalgam fillings, were low, with less than 1% of the participants exceeding the health-based HBM I value.
- Prenatal Hg exposure, assessed through cord blood Hg concentrations in the population residing in the central part of Slovenia, was moderate, with 12% of the samples exceeding the corresponding equivalent of the U.S. EPA reference dose. The exposure was significantly lower than in the Croatian study population, which reported to consume fish and other seafood more frequently than ours.
- Statistical models confirmed that the majority of internal Hg concentrations in adults was associated with seafood consumption and amalgam fillings, a minor part also to Hg concentrations in soil and tap water. The prenatal exposure was also associated with the frequency of seafood consumption but not with amalgam fillings. In the school-age children, the contribution from amalgam fillings dominated over the seafood consumption.
- Exposure to other potentially toxic elements in the adult study population was generally low, with few individuals exceeding the health-based values.
- The status of essential elements was mainly within the expected levels and was similar to the levels found in other populations within Europe. No significant deficiencies or excessive levels were detected, the observed variability was mainly due to the *postpartum* / lactating status of women, and was associated with decreased Se and increased Mn, Cu and Zn levels in the blood.
- Associations between Hg prenatal exposure and cognitive, language and motor scores of the Bayley Scales of the children at 18 months of age have been established using multiple linear regression analyses. The associations were stratified according to the *APOE* genotype, differentiating between ε4 carriers and non-carriers.
- The overall assessment of association between low-level Hg exposure in pregnancy did not show any overall negative associations with cognitive and language scales of development. However, a weak but significant inverse

association between total Hg levels and developmental motor scores at 18 months was observed in Croatian and Slovenian study populations with a normal range of Bayley scores for the majority of the participating children.

- Among the non-genetic parameters, the associations were confounded by sex of the child, birth weight, mother's age at delivery, mother's educational level, smoking of the mother, breastfeeding history, mother's fish consumption during pregnancy, child's consumption of fresh fish, day care attendance at 18 months, number of children, home size, co-exposure to Pb, and status of Se.
- The role of *APOE* genetic polymorphism was recognized as a significant modifying factor of the association: low-to-moderate prenatal Hg exposure showed negative association with a cognitive score in ε4 carriers, while in non ε4 carriers, the association was insignificant.
- Confounding for co-exposure to Pb and status of Se revealed a negative association between Pb and motor function; while beneficial role of Se in language development was evident.
- The exposure of the residents of the Idrija town was similar to the residents of the urban area of Ljubljana, but higher than in the rural control areas. Among school-age children and pregnant women, there were several individuals with elevated levels of Hg in urine, indicating elevated exposure to elemental or inorganic Hg. Generally most of the residents of the town of Idrija did not exceed the values that could be associated with biological and/or potential adverse health effects.
- No association was observed between blood Hg levels in pregnant women and selected oxidative stress biomarkers. The observed changes were similar in the Idrija and control study groups and were mainly associated with the normal pregnancy status. Although higher plasma Se was observed in the school-age children living in the town of Idrija in comparison to other study areas, which could be a consequence of moderately elevated exposure to elemental Hg vapor, the internal doses of Hg received in children and in pregnant women living in the town of Idrija did not decrease the bioavailability of Se.

In view of the future research activities, the following conclusions are outlined:

- Biobanked samples obtained in the framework of the HBM studies could be exploited and used in the context of the exposomic approach, as they followed a well-defined protocol of collection, transportation and storage.
- In line with the exposomic approach, the exposure assessment in the general population should be complemented with other potentially (neuro)toxic persistent and non-persistent organic compounds. The data obtained should then be explored in view of synergistic and antagonistic effects of environmental chemicals.
- Genotyping of relevant genes involved in the toxicokinetics of Hg should be done in the population from the town of Idrija to check the distribution of alleles and compare it to the overall Slovenian population.
- We suggest to replace the collection of mixed cord blood as a biomarker of prenatal exposure with venous cord blood sampling. Mixed cord blood consists of venous and arterial blood in variable proportions which introduces uncertainty to the exposure assessment. This is particularly important in association studies, predicting health outcomes.

- Use of relevant confounders, non-genetic and genetic, has been demonstrated as very important in association studies, however, many of them have not been included yet. Among them are parameters which interact crucially with trace elements, particularly in the childbearing age (e.g. Fe and vitamin D status), PUFAs, which are important for the development of a child, and various other genetic polymorphisms which could affect toxicokinetics or toxicodynamics of environmental chemicals.
- External exposure data (concentrations of elements in soil, water, moss, etc.) was demonstrated as an important component in predicting overall exposure sources, which refines quantification of their contributions. Limitations in their use arise from differences in spatial resolutions and the coverage of various datasets for HBM and environmental data, respectively. The trend in future studies would be towards the collection of individual external exposure data using personal sensor devices.
- Standard neuropsychological assessments offer a sensitive but not a selective tool to detect potential effects of exposures. As we are dealing with low but chronic exposures throughout the lifespan, early biological responses to detect small but significant alternations in biological pathways that are still sub-clinical should be monitored on a biochemical level. However, biochemical markers of effects have yet to be developed and validated. Exposome approach using non-targeted analysis to obtain insight into alterations of biological pathways offers a promising way towards detecting the potential biomarkers.

Based on the above given conclusions, the hypothesis of the thesis has been confirmed:

The Slovenian childbearing population and their children have low to moderate exposure to Hg, as consumption of fish and other seafood is low to moderate, and contribution from dental amalgam fillings is moderate. The exposure of residents of the Idrija town is higher than in the residents of other areas, particularly to elemental or inorganic Hg, however, the majority of the residents did not exceed the values above which the health risk is elevated. However, in the most vulnerable population group, that is pregnant women and children, the exposure could still exceed the safe level, therefore, they are recommended to avoid consuming locally grown seasonal vegetables and fish from the river Idrijca because of their high contents of inorganic and methyl Hg (Kobal et al., 2017).

Low-level exposure to the organic form of Hg from seafood consumption in the prenatal period was found to be associated with cognitive development, but only in carriers of the *APOE* ε4 allele, which appears as a susceptible genotype. However, benefits of consuming seafood, which contains PUFAs and Se, and at the same time a moderate amount of Hg, can prevail over potentially negative effects of Hg. The mechanistic role of *APOE* polymorphism in Hg toxicity is yet to be investigated. One possibility is that the effect modification is on account of a lower elimination rate of Hg from the cell binding sites in ε4 carriers as they have the lowest capacity to bind and remove Hg. The other is related to its role in neurite outgrowth and synaptogenesis, but it also shows some contradiction, as in general higher developmental scores were observed among ε4 carriers in comparison to ε2 or ε3 carriers as reported by Wright et al. (2003). Nevertheless, there is evidence that the presence of *APOE* ε4 allele and Hg provoke many common effects, and therefore act synergistically (Fonseca Arrifano et al., 2018). Unlike in cases of Hg intoxication, the relationship between the two factors in cases of low-level chronic exposure might not be enough to explain the mechanistic role of APOE. This means that further epidemiologic studies, preferably using the mentioned non-targeted approach, are needed.

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Biography

The author of this thesis Janja Snoj Tratnik obtained her B.Sc. degree in Biology at the Department of Biology, Biotechnical Faculty, University of Ljubljana, Slovenia. Her graduation thesis 'Localization of cadmium and activities of selected antioxidative enzymes in *Thlaspi praecox'* was awarded the Student Prešeren Award.

After graduation, she joined the Department of Environmental Sciences, Jožef Stefan Institute as a PhD student of the Jožef Stefan International Postgraduate School, Slovenia, under the supervision of Prof. Dr. Milena Horvat. She has been involved in the environmental health studies carried out at the Department, with a focus on mercury exposure in the general population and its link to health effects at low, environmentally relevant levels. Within this scope, she has been engaged in most of the stages of the human biomonitoring (HBM) implementation, from recruitment of participants to chemical and molecular analysis in human tissues, data management and analysis of datasets arising from such studies, and also communication of results. She has gained expertise in the analysis of mercury in biological as well as environmental samples using different analytical techniques, and estimation of measurement uncertainty. She has acquired knowledge on genotyping to identify specific alleles in polymorphic genes as susceptibility markers. She has managed all databases obtained within the studies and established statistical models to analyze HBM as well as epidemiological data.

During her work, she attended various national and international conferences and workshops, mainly on the topic of mercury and other trace elements in men, HBM, toxicology, epidemiology, and gene-environment interaction. She has been actively involved in various meetings organized within the scope of on-going projects. She has published 40 original scientific papers and participated in 1 book chapter and 2 monographies.