MERCURY SPECIATION IN PRENATAL EXPOSURE

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Doctoral Dissertation
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SPECIACIJA ŽIVEGA SREBRA V PRENATALNI IZPOSTAVLJENOSTI

Doktorska disertacija

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Ljubljana, Slovenia, March 2020

To my parents

Acknowledgments

First, I would like to express my sincere gratitude to my supervisor Prof. Dr. Milena Horvat, who supported and guided my work and was an unlimited source of research inspiration. I would also like to thank my co-supervisor Asst. Prof. Ingrid Falnoga, for the selfless transfer of her valuable knowledge and for teaching me to always question previous research critically. I am sincerely grateful for all the time that both of them invested in my work.

Thanks are also addressed to the members of the committee for the evaluation of the doctoral dissertation, Asst. Prof. Aleš Lapanje, Prof. Dr. David Neubauer, and Prof. Dr. Igor Prpić, for their valuable comments and suggestions that improved this dissertation.

I am very grateful to Vesna Fajon for her immense help and valuable experience provided during mercury measurements. I sincerely appreciate the help of Dr. Janja Snoj Tratnik, who helped me at all stages of my research work. My big thanks are also due to the help and support from the current and past colleagues at the Department of Environmental Sciences.

Experimental work was performed at the Department of Environmental Sciences, Jožef Stefan Institute. This work has been financially supported by the Slovenian Research Agency through the research program P1-0143 and project Neurodys J7-9400. This work was also supported by the EU through the 6th FP FOOD-CT-2006-016253, PHIME project; and the 7th FP no.603946, HEALS and CROME-LIFE+ projects. This work would not have been possible without the pregnant women who decided to participate in birth cohort PHIME. I sincerely thank them all for agreeing to collect their valuable biological samples and for cooperating during all the stages of the project. I appreciate the personnel of the University Medical Centre Ljubljana and the University Hospital Centre Rijeka who collected biological samples and collaborated in conducting the interviews with the pregnant women. I am also grateful to the psychology students and psychologists who conducted neurodevelopmental tests on children.

I am sincerely thankful to my parents who were always by my side and made all of this possible. Their unconditional love and support, along with the safe harbor that I call home, were essential parts for my personal and professional growth. I would also like to thank my big brother Nejc, for being a great role model and showing me that dreams do come true. I am grateful for all the help and encouraging conversations that I received from him and his wife, Urška.

Special thanks are addressed to the love of my life, Rok. He was an unlimited source of love, support, positive energy, and motivation. Sparkles in his eyes turned my bad days into good and good days into better. Thank you for reminding me to always look at the big picture!

It's not where you are in life, it's who you have by your side that matters.
(unknown)

Abstract

Mercury (Hg) is one of the top ten chemicals of major public health concern. The general population is exposed to different levels of Hg through various sources; these usually include exposure to methyl-Hg (MeHg) from seafood intake and Hg^0 vapour released from dental amalgams. In contrast to irreversible dysfunctions and anomalies observed in children who were prenatally exposed to high levels of Hg, possible health effects because of prenatal low-level Hg exposure are inconsistent and controversial. Reliable exposure assessment is challenged many times by the lack of Hg speciation and unsuitability of included exposure or susceptibility biomarkers.

The objectives of this work were to show how Hg speciation in blood can contribute to associations between low-level Hg exposure and health effects, whether meconium can be a suitable biomarker of prenatal long-term Hg exposure, and to study Apolipoprotein E (ApoE) *ε4* allele as a biomarker of susceptibility. Biological samples were collected from pregnant women and their newborns from Slovenia (N = 584) and Croatia (N = 234) that were life-long exposed to Hg mainly through seafood intake and dental amalgams.

The observed results showed the importance of speciation when assessing Hg exposure and its adverse effects on the general population. Inter-individual differences in exposure to Hg^0 or Hg(II) significantly contributed to the variations in proportions of MeHg, which makes measurements of total Hg (THg) an unreliable exposure biomarker. Results showed that the proportion of THg as MeHg in maternal blood and cord blood varied between 4%-100% and 8%-100%, respectively.

Hg speciation in meconium showed that Hg(II) presented the majority (82%-100%) of measured THg in the meconium and that high Hg(II) content primarily originates from placental transport of Hg^0. Although MeHg from seafood was shown to be transported through the placental barrier, prenatal MeHg biotransformation to Hg(II) could not be confirmed nor excluded. Further studies are needed to confirm these observations.

Statistically significant differences among ApoE *ε4* carriers vs. non-carriers were observed. The results showed that mothers who were *ε4* carriers had higher levels of plasma Se (*ε4* carriers vs. non-carriers: 62.6 and 54.9 ng/mL, respectively). Moreover, children who were *ε4* carriers had marginally significant higher cord blood THg levels in comparison with non-carriers, and a Hg-associated decrease in the cognitive score of children who were *ε4* carriers was observed. Hg-associated decrease in fine motor skills of children was genotype independent. Further studies are needed to establish the physiological role(s) of ApoE.

In this dissertation the importance of Hg speciation was confirmed. We showed that Hg speciation provides clearer information about blood levels of different Hg species, which can reduce the possibility of misinterpretations when studying metabolism or associations between prenatal low-level Hg exposure and child development. Hg speciation in meconium revealed that meconium can be used as a biomarker of prenatal exposure. Higher plasma Se levels in mothers who were *ApoE ε4* carriers could be connected to beneficial effects of ApoE during pregnancy and early life. On the other hand, there is certain evidence that children who are *ε4* carriers can be more susceptible to negative effects of Hg, but further research is needed to confirm the latter.

Povzetek

Živo srebro (Hg) je ena izmed desetih kemikalij, ki najbolj ogrožajo človekovo zdravje. Splošna populacija je izpostavljena različnim koncentracijam Hg prek različnih virov, ki najpogosteje vključujejo izpostavitev metil-Hg (MeHg) prek uživanja morske hrane in izpostavitev hlapom Hg^0, ki se sproščajo iz amalgamskih zalivk. Negativni učinki na otroke, ki so bili prenatalno izpostavljeni visokim koncentracijam Hg, so dobro poznani. Po drugi strani možni negativni učinki na razvoj otrok, kot posledica prenatalne izpostavitve nizkim koncentracijam, niso jasni. Ocena izpostavitve je velikokrat neustrezna zaradi pomanjkanja podatkov o ravni izpostavitve različnim Hg specijam ter zaradi neustrezne uporabe biomarkerjev izpostavitve in občutljivosti.

Namen tega dela je ugotoviti, kako lahko speciacija Hg izboljša asociacije med nizko izpostavljenostjo Hg in njegovimi negativnimi učinki, ali je mekonij ustreznen biomarker prenatalne Hg izpostavitve, ter oceniti vlogo alela *ε4* Apolipoproteina E (ApoE) kot biomarkerja občutljivosti. Biološke vzorce smo odvzeli otrokom iz Slovenije in Hrvaške ter njihovim materam, ki so bile dolgoročno izpostavljene nizkim koncentracijam Hg zaradi uživanja morske hrane in zaradi amalgamov.

Rezultati so pokazali pomembnost Hg speciacije še posebno, kadar ocenjujemo možne negativne učinke Hg na zdravje pri splošni populaciji. Individualne razlike v izpostavitvi Hg^0 ali Hg(II) lahko doprinesejo k variabilnosti v deležu MeHg, kar posledično pomeni, da so meritve celokupnega Hg (THg) lahko nezanesljiv biomarker izpostavitve. Rezultati so pokazali, da delež THg, prisoten kot MeHg, variira med 4 %–100 % v mamini krvi ter 8 %–100 % v popkovni krvi.

Rezultati speciacije v mekoniju so pokazali, da je Hg(II) predstavljal večino (82 %–100 %) izmerjenega THg. Visok delež Hg(II) je predvsem rezultat prenosa Hg^0 prek placente. Čeprav je bil tudi MeHg iz morske hrane prenesen prek placente, biotransformacije MeHg do Hg(II) nismo potrdili.

Rezultati so pokazali tudi statistično pomembne razlike med ApoE *ε4* nosilci in ne-nosilci. Matere, ki so imele vsaj en alel *ε4,* so imele v svoji plazmi višje vrednosti selena v primerjavi z ne-nosilkami (62,6 in 54,9 ng/mL). Otroci, ki so bili *ε4* nosilci, so imeli mejno signifikantno višje vrednosti Hg v popkovni krvi. Prav tako smo opazili s Hg povezano znižanje kognitivnih sposobnosti otrok, ki so bili nosilci *ε4*. Negativna asociacija med fino motoriko in Hg je bila neodvisna od genotipa.

V tej disertaciji je dokazana pomembnost Hg speciacije, saj lahko le z jasno opredelitvijo izpostavitve različnim Hg specijam zmanjšamo možnosti neustreznega vrednotenja asociacij med prenatalno izpostavitvijo nizkim vrednostim Hg in njegovimi negativnimi učinki na razvoj otrok. Speciacija Hg v mekoniju je pokazala, da je mekonij primeren biomarker prenatalne izpostavite MeHg in Hg^0. Višje vrednosti selena pri materah, ki so nosilke *ApoE ε4* alela, bi lahko povezali s pozitivnimi učinki apolipoproteina E. Sklepamo lahko, da so otroci, ki so nosilci *ε4*, bolj dovzetni za negativne učinke Hg na nevropsihološki razvoj otrok, vendar bomo za potrditev te hipoteze izvedli še dodatne študije.

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Abbreviations

ALSPAC	...	The Avon Longitudinal Study of Parents and Children
ApoE	...	Apolipoprotein E (protein)
ApoE	...	Apolipoprotein E (gene)
CNS	...	Central nervous system
CV-AAS	...	Cold vapor atomic absorbance spectrometry
CV-AFS	...	Cold vapor atomic fluorescence spectrometry
EtHg	...	Ethyl-mercury, $C_2H_5Hg^+$
GM	...	Geometric mean
GSH	...	Glutathione
HBM	...	Human biomonitoring
Hg	...	Mercury
Hg^0	...	Elemental mercury
IHg	...	Inorganic mercury (Hg^0, Hg(II), Hg(I))
INMA	...	Environment and Childhood Project
LCPUFAs	...	Long chain polyunsaturated fatty acids
LOD	...	Limit of detection
MeHg	...	Monomethyl-mercury, CH_3Hg^+
OHg	...	Total organic Hg
PHIME	...	Public Health Impact of Long-term, Low-level Mixed Element Exposure in Susceptible Population Strata
ROS	...	Reactive oxygen species
THg	...	Total mercury; the sum of all mercury species present
SNP	...	Dingle nucleotide polymorphism
Speciation	...	Distribution of an element amongst defined chemical species in a system
Speciation analysis	...	Analytical activities of identifying and/or measuring the quantities of one or more individual chemical species in a sample
%MeHg	...	Proportion of THg as MeHg

Chapter 1

Introduction

Mercury (Hg) is identified as one of the ten chemicals of major public health concern (WHO, 2016). It is present naturally in the environment, but human activities such as mining and fossil fuel combustion have led to widespread global Hg pollution (Driscoll, Mason, Chan, Jacob, & Pirrone, 2013). Physicochemical properties enable Hg to disperse into and remain in ecosystems for generations (UNEP/AMAP, 2013). Chemically, Hg exists in various forms that can be divided in three groups: elemental Hg (Hg^0), inorganic mercury compounds, and organic mercury compounds (ATSDR, 1999).

Elemental mercury is the only metal that is liquid at room temperature and it readily forms an amalgam with other metals. Hg^0 has been used widely in the industry, to extract pure gold and silver, and for dental treatment. Inorganic Hg compounds exist in two oxidative states, Hg(I) and Hg(II), usually bound with oxygen, sulfur, or chlorine. Inorganic Hg compounds have been used in medical applications, in cosmetics, and as preservatives, but nowadays almost all of those applications have nearly disappeared (UNEP/AMAP, 2013). In biological systems, particularly aquatic, Hg combines with carbon and the formed compounds are called organic mercury compounds (Du, Ma, Igarashi, & Wang, 2019). Although there are several different organic mercury compounds (alkyl-Hg), by far the most common found in the environment is monomethyl mercury (CH_3Hg^+, MeHg). MeHg bioaccumulates and biomagnifies through the food chain and the highest concentrations of MeHg are found in large predator fish and marine mammals (NRC, 2000c).

It is known that Hg is a non-essential element for human health and as such has no known physiological role (Berlin, Zalups, & Fowler, 2015). Moreover, numerous investigations have reported the negative health effects after occupational or accidental exposures to high doses of Hg (ATSDR, 1999; Clarkson, 2002). On the other hand, the nonoccupationally exposed general population is life-long exposed to lower Hg levels in varying amounts and forms through different sources (EPA, 2015; WHO, 2016). Several researchers studied the relation between low level Hg exposure and its potential to cause negative effects, but mechanisms behind are not well understood and conclusions remain inconsistent (Karagas et al., 2012).

During pregnancy particularly organic and elemental Hg present in maternal blood or plasma can pass the placental barrier (Kajiwara, Yasutake, Adachi, & Hirayama, 1996; Yoshida, 2002), which leads to prenatal Hg exposure. Such intrauterine Hg exposure during windows of vulnerability in early life (Grandjean & Herz, 2011) could produce damage on neurodevelopmental, immune, and reproduction systems (Rice & Barone, 2000) and can have an impact on human health later in life as reviewed by Ruggieri et al. and Karagas et al. (Karagas et al., 2012; Ruggieri, Majorani, Domanico, & Alimonti, 2017). Further studies are needed in order to protect human health and to establish reliable exposure limits under which there will be no adverse effects. The goal is to recognize, summarize,

and limit the shortcomings of existing studies and provide methods of reliable exposure assessment especially at the most susceptible parts of population (e.g. during pregnancy). This will be achieved when we will recognize all of the routes of Hg exposure, its toxicokinetics and toxicodynamics mechanisms, and other influencing factors that can modify a person's risk profile on an individual level. The aim of this dissertation is to present a current approach for prenatal Hg exposure assessment and through published scientific articles provide some suggestions about possible improvements.

1.1 Routes of Hg Exposure in the General Population

The general population is exposed to Hg^0 vapour released from dental amalgam fillings and from the atmosphere, although the contribution of the latter is minor. Exposure to Hg(II) can occur through the use of some skin-lightening creams and soaps, fungicides in latex paint and as disinfect of grain seeds, although the use of Hg in such products is prohibited in many countries (UNEP/AMAP, 2013). Exposure to organic Hg (mostly as monomethyl Hg, MeHg) is usually related to consumption of freshwater and marine fish and other seafood. Human exposure to other alkyl-Hg compounds is possible and usually includes exposure to ethyl-Hg ($C_2H_5Hg^+$, EtHg), which is present in some vaccines as a preservative. In Table 1.1. major toxicokinetics and toxicodynamics characteristics are presented (adapted from Horvat, Snoj Tratnik, & Miklavčič, 2012), whereas Figure 1.1 presents those mechanisms in detail.

Table 1.1: Main routes of Hg exposure in the general population (adapted from Horvat et al., 2012).

Hg specie	Source of exposure	Route of exposure	Uptake (%)	Target organ	Half life
Hg^0 vapour	dental amalgam	inhalation	80	CNS, kidney	2 months
Hg(II)	cosmetics	dermal absorption	?	kidney	2 months
MeHg	seafood	ingestion	95	CNS	50 days
EtHg	vaccines	intramuscular injection	100	CNS	7 days

1.1.1 Toxicokinetics and toxicodynamics of Hg species

Hg^0 vapour is readily absorbed by the lungs (~80%) and diffused into the blood, while absorption through gastrointestinal tract and skin is minor (J. D. Park & Zheng, 2012). Hg^0 vapour is to some extent dissolved in plasma, but the majority is oxidised to Hg(II) by catalase (Magos, Halbach, & Clarkson, 1978). Although the oxidation process occurs in a relatively short time, it is still long enough for dissolved Hg^0 vapour to penetrate the blood-brain and placental barriers, which can lead to prenatal Hg exposure (Clarkson, Magos, & Greenwood, 1972; Yoshida, 2002). On the other hand, after ingestion only about 15% of Hg(II) is absorbed in the gastrointestinal tract. Dermal absorption of Hg(II) is possible, but available data are insufficient for an evaluation of the uptake. The Hg(II) ion in blood is bound to albumin, globulin, and haemoglobin. In contrast to Hg^0 vapour, Hg(II) crosses brain and placental barriers only to a certain extent (Ou et al., 2014). During chronic low-level exposure to Hg^0 vapour and Hg(II), the kidney tissue is the main deposit site. The main excretory pathway of Hg^0 vapor and Hg(II) are the urine and faeces, with a half-life of approximately 2 months (Horvat et al., 2012).

Once consumed, MeHg is readily absorbed by the gastrointestinal tract (~95%). MeHg is rapidly distributed to all tissues and there it is normally complexed with water-soluble sulfhydryl-containing molecules (e.g. cysteine, glutathione). In blood, the majority of MeHg was found in red blood cells (Berglund et al., 2005; Horvat et al., 2012). Similarly as Hg^0 vapour also MeHg most likely crosses cell membranes by passive diffusion, whereas the methylmercury L-cystein complex (CH_3-Hg-cysteine) is believed to be transported via amino acid transporters by mimicking L-methionine. Because of this molecular mimicry CH_3-Hg-cysteine complex can be transported across the blood-brain-barrier and placental barrier (Bridges & Zalups, 2005; Kajiwara et al., 1996; Yin et al., 2009), thus presenting prenatal exposure. Once in the bile, MeHg can be reabsorbed through the enterohepatic cycling, but the majority is demethylated to Hg(II), which is also the main MeHg excretory pathway. This less toxic Hg specie (Hg(II)) is poorly absorbed across the intestine and excreted in feces (Clarkson & Magos, 2006). The exact mechanism of MeHg demethylation is not clear, but researchers suggested the role of selenium (Khan & Wang, 2010), free radicals (Nagano, Yasutake, & Miura, 2010), and/or microbiota (Caito et al., 2018; Rothenberg et al., 2017). Further studies are needed to establish the mechanisms of MeHg biotransformation. The mean half-life of MeHg in human blood is about 50 days.

Human exposure to EtHg mainly arises from vaccination. In comparison with MeHg, half-life of EtHg is short (Table 1.1.) and once in the blood, EtHg is readily biotransformed and excreted via the gut (WHO, 2016).

As summarized above and presented in Figure 1.1, the metabolism plays crucial role in the person's internal dose and special care is required in order to accurately assess the level of exposure by including inter-individual variabilities possibly affecting Hg bioaccumulation and its biological effects. Human biomonitoring (HBM) is the most commonly used method to assess human exposure and possible health effects to different environmental contaminants. HBM is based on the use of biomarkers, which are measurements of exogenous compound(s) or its metabolite(s), measurable indicator(s) of change as a result of contaminant exposure or specific factors (genetic, non-genetic) that can modify the individuals' susceptibility (NRC, 1987).

1.2 Biomarkers of Hg Exposure

Biomarkers of exposure are measurable exogenous compounds or its metabolites within biological matrix (blood, urine, hair, etc.). In the case of Hg, the selection of appropriate biomarker of exposure is not only closely related to the toxicokinetics and toxicodynamics mechanisms, but also to the type of exposure (acute, long-term), the levels of exposure, and the time-window (prenatal, children, adults). Although the general population is exposed to different Hg species in various amounts, epidemiological studies, as an effective method for HBM, often perform measurements of total Hg (THg, organic and inorganic Hg compounds) without Hg speciation (Branco, Caito, Farina, Teixeira, & Carvalho, 2017; Horvat et al., 2012).

1.2.1 Biological samples used for Hg exposure assessment

THg measurements in urine are used to assess the long-term exposure to Hg^0 and Hg(II) and it is essential that the measured concentrations are normalized for dilution of the urine (e.g. normalization to creatinine or specific gravity) (Santonen, Aitio, Fowler, & Nordberg, 2015). Determination of Hg in whole blood provides information about recent exposure (~3 months) to organic and inorganic Hg. Assessment of MeHg exposure through measurements of THg in hair is based on binding of MeHg to sulfhydryl (-SH) groups of keratin in the

hair. It was shown that MeHg concentrations in blood are proportional to those found in hair (1:250) and that almost all Hg (>90%) in hair is in the form of MeHg. As hair grows approximately 1 cm per month, measurements of Hg in longer hair segments present long-term Hg exposure. Higher exposure to Hg^0 or Hg(II) makes measurement of hair THg for MeHg exposure assessment an unreliable biomarker (Horvat et al., 2012).

Prenatal Hg exposure is often assessed through THg measurements in maternal scalp hair or maternal blood, but neither of them is suitable for the assessment of prenatal exposure in early weeks of gestation. However, it was shown that the best biomarker of prenatal Hg exposure is cord blood (Grandjean & Budtz-Jørgensen, 2007; Grandjean, Satoh, Murata, & Eto, 2010). Concentrations in cord blood are known to be 1.5 to 2-fold higher than those found in maternal blood (Stern & Smith, 2003; US EPA, 1997). The major problem with cord blood is its problematic sampling, which needs to be done with a well trained and experienced personnel. The sample is usually not separated to venous and arterial cord blood, but is rather sampled as "mixed cord blood". Moreover, collected cord blood was shown to be frequently (reported between 10% and 20%) contaminated with maternal blood (Morin et al., 2017). As several physiological differences between cord blood and adult blood exist (e.g. the haemoglobin), with this approach individual differences can be lost and such a sample cannot be marked as representative. As foetal haemoglobin has a higher affinity for MeHg binding in comparison with the adult one and cord blood has higher haematocrit levels, maternal contamination of cord blood can provide lower exposure levels measured in cord blood (Kim et al., 2014). However, for non-essential elements that accumulate in erythrocytes, mixed cord blood is a suitable approximation of prenatal exposure, but adjustment of measured Hg levels for haemoglobin can improve the precision of exposure assessment.

Along with all these, toenail and fingernail (Sakamoto et al., 2015), neonatal scalp hair (Lindow, Knight, Batty, & Haswell, 2003; Sikorski, Paszkowski, & Szprengier-Juszkiewicz, 1986), and breast milk (Branco et al., 2017; LaKind et al., 2005; Snoj Tratnik, Falnoga, et al., 2019) are also considered appropriate biomarkers of Hg exposure, although they are not commonly used. Similarly as in the case of urine also in breast milk, the problem with reported concentrations is normalization for dilution as composition of the milk (e.g. water and fat content) varies through time to provide nutrients for the developing child (Martin, Ling, & Blackburn, 2016).

1.2.2 Levels considered safe

As prenatal life was shown to be sensitive for toxic effects of Hg (Grandjean & Herz, 2011), different thresholds of exposure considered safe are established. Although they were derived in order to prevent negative effects of prenatal Hg exposure, they use various exposure biomarkers and calculate the thresholds based on different statistical and pharmacokinetic models. Based on the observed results from the Faroe study (Grandjean et al., 1997), the National Research Council and US EPA established the only official reference dose of 0.1 µgHg/kg body weight per day as an exposure considered safe for development of neonates. This corresponds to 5.8 µgHg/L in cord blood or ~1 µgHg/g in maternal hair (NRC, 2000c). However, updated calculations taking into account the imprecision of the exposure data, maternal hair Hg limit would correspond to 0.58 µg/g (Grandjean & Budtz-Jørgensen, 2007). On the other hand, the THg threshold of 3.5 µg/L in maternal blood is considered relevant for preventing foetal neurotoxicity (Mahaffey, Clickner, & Bodurow, 2004; Mahaffey, Clickner, & Jeffries, 2009). Although many studies put a lot of effort into establishing relevant exposure limits under which there would be no observed health effects, this job is far from completed. As presented in Table 1.2., studies report various adverse

effects associated with different levels of Hg exposure, which are assessed in various exposure biomarkers.

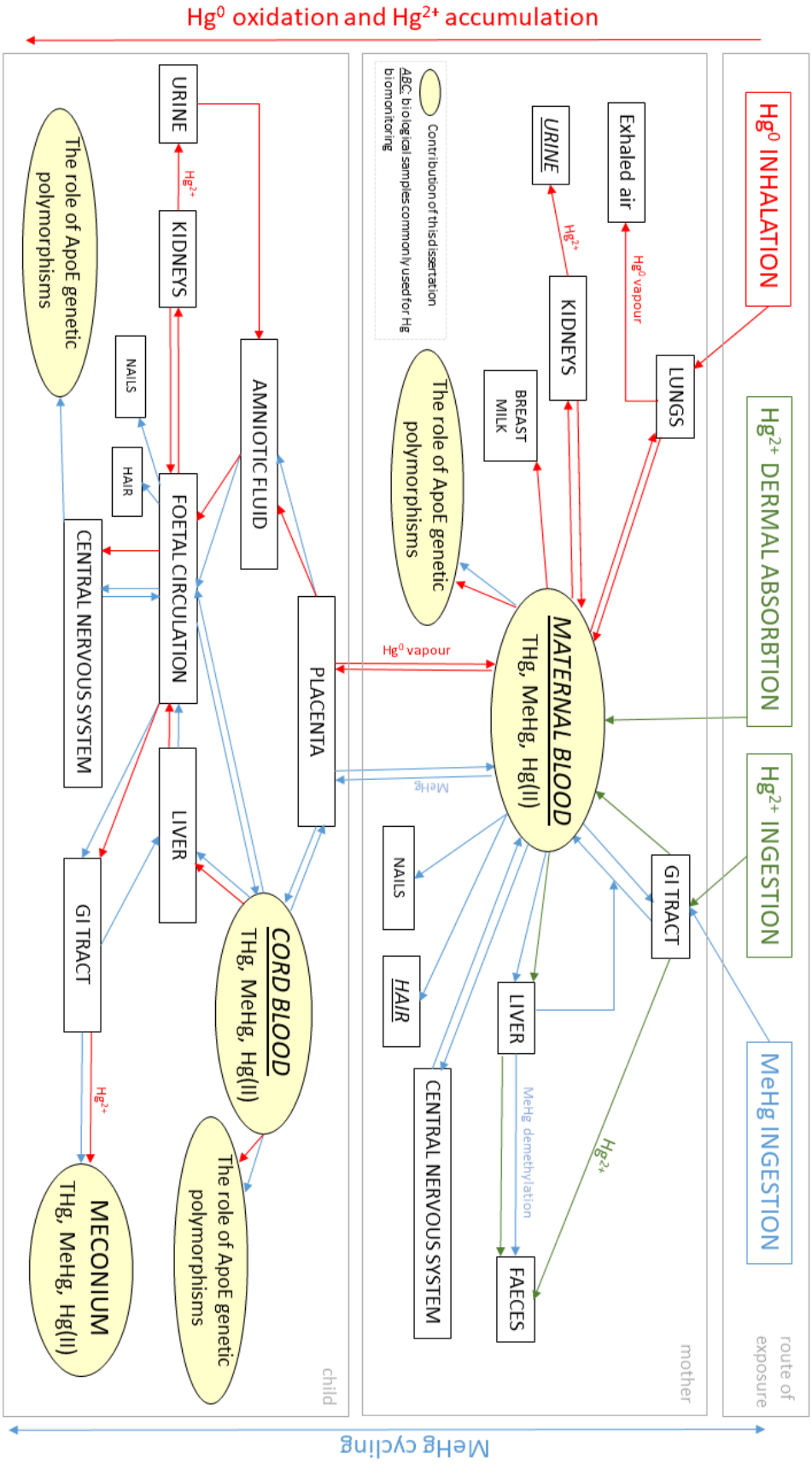

Figure 1.1: Hg toxicokinetics and toxicodynamics mechanisms with biomarkers of exposure and main contributions of this dissertation.

1.3 Examples from birth cohorts

Birth cohorts were designed to monitor individuals' exposure to selected contaminant through different life stages (prenatal, adolescence, adulthood, etc.) and associate established exposure to possible negative effects. In the case of Hg and its potential to cause neurotoxic effects, several different birth cohorts were established to study possible adverse effects after prenatal exposure to Hg. Some of the most important ones are summarised in Figure 1.2.

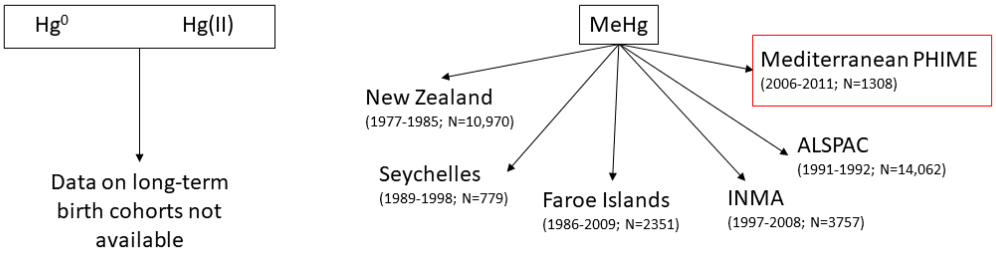

Figure 1.2: Main birth cohorts assessing prenatal Hg exposure.

MeHg neurotoxicity was extensively studied after observed evidence of irreversible dysfunctions and anomalies in children who were prenatally exposed to high levels of MeHg due to the environmental disaster in Minamata through maternal seafood consumption (Harada, 1995) and accidental poisoning with contaminated grain in Iraq (Bakir et al., 1973). As negative health effects of MeHg were observed and consumption of highly contaminated food resulted in body burdens that greatly exceeded those found in the general population, researchers focused on maternal seafood intake from less contaminated areas and in utero MeHg exposure related to neurological development of children. The first epidemiological report on adverse effects observed in children related to maternal seafood intake was documented in New Zealand (Kjellström, Kennedy, Wallis, & Mantell, 1986; Kjellström et al., 1989) and was confirmed later in the study from the Faroe Islands (Grandjean et al., 1997, 1992). The latter birth cohort along with the one conducted in Seychelles Islands (Myers et al., 1995) are the two major longitudinal cohort studies conducted to date in which children were continuously followed through adolescence to assess the relation between neuropsychological performance and current or past Hg exposure. The Faroe study observed that even low exposure to Hg during early development could cause neurobehavioral deficits later in life (Grandjean et al., 1997). However, in the Seychelles study, although exposed to comparable Hg levels as in the Faroe, adverse effects of Hg on child's neuropsychological development were initially not observed (Myers et al., 1995), but only a more recent study on the same cohort reported an association between higher exposure to MeHg and adverse symptoms (van Wijngaarden et al., 2006). Since then, several birth cohorts from different countries were designed. They all share the same aim, which is to assess the level of exposure and to evaluate the impact of such low-level exposure on growth, health, and development of the child (Figure 1.2).

Environment and Childhood Project (INMA, "INfancia y Medio Ambiente") is a study conducted in different parts of Spain, which was created to study the relation between exposure to various pollutants (including Hg) during pregnancy and in early life and its

associations with a child's growth and development. Researchers reported a Hg-associated decrease in psychomotor development among female infants (Llop et al., 2012) and a decrease in neuropsychological development (Llop et al., 2016). Additionally, they reported that prenatal Hg exposure may be associated with reduced placental and foetal growth (Murcia et al., 2016).

The Avon Longitudinal Study of Parents and Children (ALSPAC) was conducted in the county of Avon in England. In the early 90's they have recruited over 14,000 pregnant women and their families to follow the life of their newborns in order to recognize health and social issues. In this cohort researchers did not find adverse associations between maternal blood Hg levels and effects on child development (Golding et al., 2016), low birth weight or preterm birth (Taylor, Golding, & Emond, 2016). More importantly, the associations between maternal Hg levels and child development were present in beneficial direction (Golding et al., 2016).

The Mediterranean project of Public Health Impact of Long-term, Low-level Mixed Element Exposure in Susceptible Population Strata (PHIME) (Miklavčič et al., 2011; Valent et al., 2013) was conducted in Slovenia, Croatia, Italy, and Greece. The birth cohort was designed because of a renewed interest in toxic metals, due to a growing awareness that the exposure in the general population in Europe and elsewhere is at levels with a potential to cause toxic effects in susceptible individuals. In this dissertation, the research work was conducted on the samples collected within two countries (Slovenia and Croatia) included in the Mediterranean PHIME project. A recent publication including Mediterranean countries of Italy, Slovenia, Croatia, and Greece reported overall positive association between maternal Hg levels and a child's language composite score (Barbone et al., 2019). The study including only participants from Croatia reported an association between cord blood Hg levels and a decrease in fine motor skills (Prpić et al., 2017) and maternal hair Hg levels with a decreased size of a newborn's cerebellum (Cace et al., 2011).

As seen from the abovementioned examples (summarized in Table 1.2.), listed in review articles (Karagas et al., 2012; Taylor, Golding, & Emond, 2014), and emphasized by NRC (NRC, 2000a) in the chapter entitled "Comparison of studies for use in risk assessment" it is clear that further studies need to identify reasons for such inconsistencies. This is essential in order to provide clear conclusions about prenatal Hg exposure and levels considered safe to prevent possible negative health effects later in life. There are some opportunities for improvement and they will be presented next.

Table 1.2: Examples from the literature assessing prenatal low-level Hg exposure and its adverse effects (adapted from Ruggieri et al., 2017).

Birth cohort studies	Biological samples	Average exposure level	Observed associations	Reference
New Zealand	Maternal hair	>6 µg/g	Decreased psychological and scholastic performance	Kjellström et al., 1986; 1989)
Faroe Islands	Maternal hair, cord blood	4.3 µg/g 23 µg/L	Hg-related neuropsychological dysfunctions	(Grandjean et al., 1997)
the Seychelles	Maternal hair	6.9 µg/g	No direct association between exposure and neurodevelopmental outcomes	(Myers et al., 2003; van Wijngaarden et al., 2017)
INMA	Cord blood	8.4 µg/L	Decrease in psychomotor development among female infants	(Llop et al., 2012)
		8.8 µg/L	Decrease in neuropsychological development	(Llop et al., 2016)
		8.2 µg/L	Reduced placental weight and foetal growth	(Murcia et al., 2016)
ALSPAC	Maternal blood	0.17-12.8 µg/L	No adverse effects on child development between 6 and 42 months	(Golding et al., 2016)
		1.83 µg/L	No association with anthropometric variables, low birth weight or preterm birth	(Taylor et al., 2016)
Mediterranean PHIME	Maternal hair	704 ng/g	Overall positive association with language composite score	(Barbone et al., 2019)
	Cord blood	2.98 ng/g	Decrease in fine motor skills	(Prpić et al., 2017)
	Maternal hair	>1 µg/g	Decreased size of newborn's cerebellum	(Cace et al., 2011)

1.4 Opportunities for Improvement

The abovelisted studies have many strengths. To mention only a few of them, the large sample size (e.g. the study from Faroe started with ~2350 participants; INMA started with

~3700 participants; ALSPAC with ~14,000 participants), therefore the studied populations can be marked as representative. Furthermore the participants from the original birth cohorts are being followed-up to see how their exposure to Hg varies through different life stages and how past and present exposure could be associated with negative effects later in life. The researchers included many factors that could have a synergistic effect (e.g. lead, bisphenol A) on the studied relation or could act beneficially (selenium status, levels of long chain polyunsaturated fatty acids, etc.).

On the other hand, there are also some drawbacks. Limits of the three birth cohorts from New Zealand, the Seychelles, and the Faroe Islands are thoroughly discussed by NRC (NRC, 2000a), but have many shared limitations with other studies listed in Table 1.2. For example, a possible source of misinterpretations are unstandardized methods for the assessment of Hg exposure and its possible negative effects. Studies use various exposure biomarkers (maternal hair, blood, cord blood, etc.), apply different neuropsychological tests and test children at different ages. Moreover, an individual's genetic susceptibility is often not included and statistical analyses take into account different subjectively selected factors (e.g. gender, nutritional status, and sociodemographic data).

Besides that, these studies hardly investigate the importance of measurement uncertainty or inter-laboratory measurement variability, as it was shown that the sample size is greatly influenced by the latter (Snoj Tratnik, Mazej, & Horvat, 2019). The next drawback is that studies were focused mainly on Hg exposure from seafood intake, therefore mainly on MeHg, although the source of exposure is not negligible (e.g. one time consumption of whale meat vs. frequent consumption of fish). Moreover, exposure to Hg^0 is most commonly connected to occupational exposure in communities involved in gold mining (Gibb & O'Leary, 2014), although also the general population is exposed to Hg^0 vapour released from dental amalgams. Birth cohorts that would study the adverse effects in children after exposure to Hg^0 or Hg(II) are not available (Figure 1.1), although all Hg species can negatively modify physiological processes especially at the time of rapid growth (e.g. prenatal life) (Rice & Barone, 2000). One of the drawbacks is also that none of the listed and only a few non-listed (Wells et al., 2017) studies performed analytical techniques of Hg speciation. In the case of general population, Hg exposure includes exposure to Hg species from various sources (seafood intake, dental amalgams, cosmetics, etc.) at different levels (fish eating groups, the number of dental amalgams, etc.), therefore inter-individual variability should be considered. It is clear that combined influence of these factors is difficult to predict, therefore all opportunities for improvement should be considered.

1.4.1 The importance of Hg speciation at MeHg exposure

Although the majority of MeHg in blood is found in erythrocytes and Hg(II) is the major Hg specie found in plasma, measurements of THg in blood compartments are a useful approximation of exposure, but can vary considerably at an individual level (Carneiro Hornos, Grotto, & Barbosa Jr, 2014). This was shown especially in cases of low to moderate exposure to MeHg from seafood consumption (e.g. the general population), where proportions of Hg as MeHg in blood varied considerably (Sakamoto et al., 2016; Wells et al., 2016). Although individual variations in proportions of THg as MeHg in biological samples were shown in the past (Berglund et al., 2005), few studies have performed Hg speciation analysis[1] in relevant exposure biomarkers (Basu et al., 2018; Gundacker et al., 2010; Sakamoto et al., 2016; Wells et al., 2016).

THg concentration is often used as a proxy measure of MeHg exposure based on the assumption, that MeHg constitutes a substantial (~ 90%) proportion of THg in blood (Basu

[1] Speciation analysis as referred to IUPAC is "Analytical activities of identifying and/or measuring the quantities of one or more individual chemical species in a sample" (Templeton et al., 2000).

et al., 2018; Berglund et al., 2005; Hong, Kim, & Lee, 2012; Horvat et al., 2012). Moreover, Hg speciation in cord blood was marked as unnecessary, because Hg found in cord blood is mainly in its methylated form for which placenta does not constitute a barrier (Kim et al., 2014). However, this approach can result in overestimation of MeHg exposure especially in cases of low-to-moderate levels of exposure to MeHg from seafood consumption, although possible adverse effects in such low-level exposure are questionable. In such cases, inter-individual differences in exposure to Hg^0 or Hg(II) might significantly contribute to variations in MeHg proportions (Wells et al., 2017, 2016). This was shown in our paper (Trdin, Snoj Tratnik, et al., 2019) (Section 3.1), where proportion of THg as MeHg in maternal blood and cord blood varied between 4%-100% and 8%-100%, respectively. The importance of Hg speciation was further confirmed as we showed that measurements of MeHg provide clearer information about blood levels of different Hg species, which can reduce the misinterpretations when studying Hg metabolism and possible negative effects of Hg on children neurodevelopment.

Although the importance of Hg speciation was shown through published scientific papers as described above, many researchers still prefer not to perform Hg speciation. On the other hand, there are different methods available for Hg speciation. The example is a study by Berglund et al. where measurements of THg and IHg were performed, whereas OHg (total organic, presumably in the form of MeHg) was calculated (Berglund et al., 2005). Although in human blood the majority of OHg is presumably present in the MeHg form, other organic Hg compounds (such as EtHg) could also be present. In comparison with MeHg, the problem of Hg(II) measurements are interferences, which are hard to overcome (Liang, Bloom, & Horvat, 1994). This is especially important with measurements near the detection limit, as are those low-levels of Hg(II) present in human blood. The approach with Hg(II) measurements (Berglund et al., 2005) is a reliable exposure assessment for biomonitoring purposes, but in the case of establishing toxic health effects or evaluating Hg metabolism, it could act as an additional source of uncertainty. Furthermore, in the case of measurements of total organic (OHg) mercury with ion exchange techniques, obtained results can be higher, compared to other methods, especially in the case of high Hg(II) content in the sample (Horvat, 1989). There are at least two reasons behind. The first is that the isolation of OHg and IHg in this technique is non-specific and could be a result of formation neutral Hg complex, which passes through an ion exchanger. The second reason could be detection of Hg(II), which is bound to other biological complexes (e.g. the proteins) that may pass the ion exchanger, and is later detected as OHg (Horvat, 1989). However, methodologies to accurately speciate Hg in human samples are available and cost-effective, and no longer represent a barrier for improved study protocols. Direct measurements of MeHg (Liang, Horvat, & Bloom, 1994) in combination with THg measurements and Hg(II) calculations were shown to be a precise estimation of exposure to different Hg species and this approach is encouraged in epidemiological studies assessing health effects of Hg (Trdin, Snoj Tratnik, et al., 2019).

However, Hg speciation will only be cost-effective when also other factors that can have an impact on Hg levels in a human body (e.g. seafood intake, number of dental amalgams) will be collected precisely and all other sources of uncertainties (sampling, sample transport and storage, the number of freezing and thawing cycles, detailed questionnaires about possible sources of exposure) are reduced.

1.4.2 Meconium

To study Hg toxicokinetics mechanisms during pregnancy and to assess health outcomes in children, the use of biomarkers presenting the widest period of exposure is essential. As mentioned earlier, Hg measurements in whole blood present short-term exposure and

additionally, Hg in blood is in constant dynamic state of metabolism, tissue distribution, and elimination (Ostrea Jr. et al., 2002). On the other hand, it was shown that longer (~9cm) hair segments (Myers et al., 1995; NRC, 2000c), if such length is available, and meconium are ideal for investigation of foetal long-term exposure to neurotoxicants during pregnancy (Ortega Garcia, Carrizo Gallardo, Ferris i Tortajada, Garcia, & Grimalt, 2006; Ostrea Jr. et al., 2002). In the case of prenatal low-level and long-term exposure to various pollutants, meconium analysis was shown to be more sensitive compared with blood as meconium is a cumulative and repository matrix (B. Y. Park & Lee, 2014). Furthermore, meconium represents foetal tissue and is therefore a direct measure of exposure, by contrast to maternal blood or hair, which provide only indirect estimations of exposure (Ostrea Jr., Bielawski, & Posecion Jr., 2006).

Until now only a few studies have measured Hg levels in meconium (Gundacker et al., 2010; Jiang, Hsi, Fan, & Chien, 2014; Knezović, Trgo, & Sultović, 2016; Ostrea Jr. et al., 2002; Peng et al., 2015; Ramirez et al., 2003; Rothenberg, Wagner, Hamidi, Alekseyenko, & Azcarate-Peril, 2019; Unuvar et al., 2007) and only the recent one performed Hg speciation on a very limited sample size (N = 14) (Rothenberg et al., 2019). The study by Rothenberg et al. reported ~100% of measured THg being in Hg(II) form, but the origin of inorganic Hg was not identified. As all herein discussed Hg species (particularly Hg^0 and MeHg, while Hg(II) less readily) can pass the placental barrier, the toxicokinetics mechanisms behind should be evaluated in order to see whether meconium is a suitable biomarker of prenatal MeHg exposure. From this point of view, our study (Trdin, Falnoga, et al., 2019) (Section 3.2) was the first one to perform Hg speciation in meconium on a large sample size (N = 488). Our results showed that Hg(II) presented the majority (82%-100%) of measured THg in the meconium and that high Hg(II) content primarily originates from placental transport of Hg^0 and after oxidation partitioning of Hg(II) in foetal tissues, body fluids, and meconium. Moreover, MeHg levels found in meconium were associated with maternal seafood intake, therefore we assumed that MeHg is transported to the foetal side and is accumulated there. However, as MeHg demethylation to Hg(II) is a crucial step in elimination of MeHg from the adult body, based on our results prenatal MeHg biotransformation could not be confirmed nor excluded. With this study we were able to conclude that meconium is a suitable biomarker for assessing prenatal exposure to MeHg and Hg^0 under the condition that Hg speciation analysis is performed (Trdin, Falnoga, et al., 2019).

Although sampling of meconium is non-invasive and simple, methods used so far are not standardized, therefore the results obtained in different studies are not entirely comparable. Each study has its own sampling protocol – collection was done in the first few hours or even days after birth. As concentrations are believed to be correlated with the time of meconium collection (Ortega Garcia et al., 2006), this should be taken into account. Furthermore, in most cases meconium is collected from a diaper, which makes it impossible to compare the water content between the samples. Additionally, some studies lyophilized the meconium samples before the Hg measurements, which makes the comparability of results even more challenging.

It is worth mentioning that meconium was shown to be a promising new biomarker of prenatal exposure, but all of the measurements are made under the assumption that a fetus does not have independent mechanisms for metabolizing MeHg and any mechanism of elimination occurs on the maternal side or inside the placental tissue (Ruggieri et al., 2017). However, this assumption needs to be confirmed with future studies.

1.5 Biomarkers of Susceptibility

When studying possible negative health effects because of low-level Hg exposure special care must be taken to avoid misinterpretations. As mentioned above, Hg toxicity can vary considerably at an individual level because of exposure to different levels of various Hg species in different time frames. Additionally, it was shown that individuals' toxicokinetics mechanisms differ under comparable Hg exposure levels. It is clear that complex interactions between genetic and environmental factors determine a high degree of variability in individual response to a given exposure (Basu, Goodrich, & Head, 2014). Moreover, an expert committee of the U.S. National Research Council concluded that up to 25% of neurodevelopmental disorders would be the result of the interaction between exposure and individual susceptibility genes (NRC, 2000b).

Recently studies established the relation between Hg exposure and genetic polymorphisms that could help identify populations or individuals that are more vulnerable (Llop, Ballester, & Broberg, 2015). Most frequently studied were single nucleotide polymorphisms (SNPs) in genes linked to Hg toxicokinetics and neurotoxicity (Andreoli & Sprovieri, 2017; Julvez, Smith, Ring, & Grandjean, 2019). Although many studies reported associations between Hg and specific SNPs, there are many examples of inconsistencies, as reviewed by Llop et al. (Llop et al., 2015).

1.5.1 Hg and single nucleotide polymorphisms

Most of the literature studied the role of genes in the glutathione system, as glutathione (GSH) was proposed to be the main peptide involved in Hg metabolism. Inorganic and organic Hg bind to GSH possibly through the reaction with glutathione S transferase (GST) (Custodio, Harari, Gerhardsson, Skerfving, & Broberg, 2005). Stable conjugates GSH-Hg are eliminated through faeces and urine (Ballatori, 2002; Ballatori & Clarkson, 1985). For these reasons polymorphisms in *GSH*-synthesizing or *GSH*-conjugating enzymes are believed to be involved in different retention of Hg in body (Broberg, Engstorm, & Ammer, 2014). For example, study among students in Austria found statistically significant higher levels of hair Hg in students with a deletion in genes of *GST* isoform *T1* and *M1* (*GSTT1* and *GSTM1*); they concluded that the deletion is a risk factor for increased susceptibility to mercury exposure (Gundacker et al., 2007).

Other systems included in Hg transport and elimination are metallothioneins (MTs), the ATP-binding cassette transporters (ABC transporters), organic anion transporters (OATs), and L-amino acid transporters (LATs) (Bridges & Zalups, 2005). For example, study including two Mediterranean birth cohorts (PHIME and INMA) assessing SNPs in ABC transporter proteins, which are involved in active transport of various compounds across membranes, found that polymorphisms in *ABC* transporters appear to play a role in accumulation of MeHg during early development (Llop et al., 2014).

As antagonism between Hg and Se is well established (Falnoga & Tušek-Žnidarič, 2007; Khan & Wang, 2009) and Se is believed to affect the toxicokinetics of MeHg (Kim et al., 2014), polymorphisms affecting Se levels or expression of selenoproteins are among the genes of interest (Zoidis, Seremelis, Kontopoulos, & Danezis, 2018). Selenoprotein P1 (SEPP1) is a selenoprotein containing multiple selenocysteine residues and has been implicated to function as a Se transport protein and as an antioxidant (Burk & Hill, 2005; Hill & Burk, 2001). For example, a study on adult dentist professionals reported that *SEPP1* genotype was associated with urine and hair Hg levels (Goodrich et al., 2011). They assume that higher levels of Hg in urine and hair are found because the specific genotype is linked to greater *SEPP1* expression and higher ability of SEPP1 to bind mercury (Meplan et al., 2007).

On the other hand, studies also report possible interactions between Hg exposure and specific genes with potential roles in neurotoxicity (Llop et al., 2015). For example, studies address the association between Hg and brain-derived neurotrophic factor (BDNF), which is required for the survival of neurons in the brain. Similarly, Paraoxanase 1 (PON1) inhibits oxidation of lipoproteins through hydrolysis of lipid peroxides. Such oxidative damage can be induced by MeHg (Hernández et al., 2009). In the ALSPAC UK birth cohort children with *BDNF* and *PON1* minor allele showed stronger MeHg adverse effects (Julvez et al., 2013). Among other SNPs that could affect neurotoxicity of Hg (Llop et al., 2015), *Apolipoprotein E* was proposed to play a central role in neurotoxicity and neurodevelopment. As *ApoE* was included in our studies, its possible role(s) from different perspectives will be summarized next.

1.5.2 Apolipoprotein E

Apolipoprotein E (ApoE, gene *ApoE*) is plasma glycoprotein of 299 aminoacids with relative molecular mass 34-kDa, and plasma concentration 40-70 µg/mL. The most common site of synthesis is hepatic synthesis (>75%), followed by synthesis in the brain, in the macrophages, and other human tissues (lung, spleen, ovaries, and adrenal gland) (Huang & Mahley, 2014a). Although ApoE plays a central role in lipid metabolism, in the central nervous system ApoE has an important role in neurological phenomena, including neuronal plasticity, neurite outgrowth, synaptogenesis, and neuronal repair (Liu, Kuhel, Shen, Hui, & Woods, 2012; Mutter, Naumann, Sadaghiani, Schneider, & Walach, 2004). Other non-lipid related functions that have been attributed to ApoE include effects on immune response, inflammation, and oxidation. This multifunctional protein affects human physiology and pathophysiology at multiple levels (Getz & Reardon, 2009).

ApoE protein is encoded by *ApoE* gene that is located on chromosome 19q13.2 and has four exons and three introns. ApoE has three major protein isoforms ApoE2, ApoE3, which are encoded by alleles *ε2*, *ε3, ε4*, respectively. As presented in Table 1.3. (Giau, Bagyinszky, An, & Kim, 2015; Nyholt, Yu, & Visscher, 2009), the *ε2*, *ε3, ε4* system is defined by two SNPs in *ApoE* coding region of exon 4. The first SNP (rs429358) is related to the nucleotide change T to C (c.334T>C), which results in change cysteine for arginine (p.Cys112Arg). The second SNP (rs7412) is related to the nucleotide change C to T (c.472C>T), which results in change arginine for cysteine (p.Arg158Cys). According to described SNPs there are six possible *ApoE* genotypes *ε2/ε2, ε2/ε3, ε3/ε3, ε3/ε4, ε2/ε4,* and *ε4/ε4.*

Table 1.3: *Apolipoprotein E* polymorphisms and associated protein isoforms with allele frequencies (Giau et al., 2015; Nyholt et al., 2009).

Isoform	position 112 c.334T>C rs429358	position 158 c.472C>T rs7412	allele	allele frequencies (%)
ApoE2	Cys -TGC-	Cys -TGC-	*ε2*	0-14
ApoE3	Cys -TGC-	Arg -CGC-	*ε3*	49-90
ApoE4	Arg -CGC-	Arg -CGC-	*ε4*	5-37

According to SNPs presented in Table 1.3., ApoE2 has two cysteines (Cys), ApoE3 contains one Cys and one arginine (Arg), and ApoE4 contains two Arg, which differently

affect peripheral lipid and brain neuronal homeostasis (Huang & Mahley, 2014a). *ApoE ε4* is recognized as a major risk factor for Alzheimer's Disease, increasing the occurrence and lowering the age of onset (Huang & Mahley, 2014a), because ApoE4 isoform was found to be associated with a lower clearance capacity and degradation of ß-amyloid from the brain (Giau et al., 2015). It is believed that *ApoE ε4* is a global activator of innate immune function, therefore it has a survival value in populations exposed to high levels of infectious diseases, which can be detrimental when faced with diseases of advanced age (Vitek, Brown, & Colton, 2009). Moreover, *ε4* allele was shown to be associated with higher serum cholesterol levels (de Chaves & Narayanaswami, 2008; Petrovič, Zorc, & Peterlin, 2000), vitamin D (Huebbe et al., 2011), bone Ca (Egert, Rimbach, & Huebbe, 2012), and a higher progesterone level in women. These parameters can be beneficial in early life and for a higher fertility in women (Jasienska et al., 2015). *ApoE ε4* was found to be associated also with improved perinatal health and survival outcomes (Eisenberg, Kuzawa, & Hayes, 2010). The divergent effects (beneficial early in life, detrimental in post reproductive period) suggests ApoE is a candidate for antagonistic pleiotropy (Smith, Ashford, & Perffeti, 2019; Tuminello & Han, 2011). Additionally, in vitro experiments showed that ApoE can bind certain metal ions, especially divalent (e.g. Cu, Zn) (Miyata & Smith, 1996). However, because of the structural differences between isoforms there are also differences in binding capacity between them. Two SNPs in the coding region (Table 1.3) are responsible for incorporation of two different amino acids. Because Cys contains one sulfhydryl group (-SH) with high affinity to bind Hg, and Arg contains none, variants of ApoE with Cys in their structure (ApoE2 and ApoE3) were hypothesized to bind metals (including Hg) more efficiently in comparison with ApoE4.

During prenatal life, the developing child depends on nutrients provided by the mother. In this context, human placenta is essential for foetal health and development and although placenta is mainly of foetal origin, the environment provided by the mother during pregnancy is often not considered. According to Gundacker et al., 'the maternal genotype determines the placental environment, while the foetal genotype determines the capacity of the placenta to cope with this environment' (Gundacker, Neesen, Straka, Ellinger, & Dolzing, H. Hengestschläger, 2000). As ApoE variants are believed to have a different binding capacity, we studied *ApoE* polymorphisms more from a physiological point of view, how its polymorphisms could modify the concentrations of different trace elements in selected biological matrices (Section 3.3). We observed that mothers carrying at least one *ε4* allele had statistically significant higher levels of THg and MeHg in blood, hair, and cord blood; As in urine and cord blood; and Se in maternal blood and plasma (Trdin et al., 2020). However, after observed results were adjusted for covariates, the positive association between *ε4* carriers persisted only for plasma Se (*ε4* carriers vs. non-carriers: 62.6 and 54.9 ng/mL, respectively). In our study higher levels of Hg (and other elements e.g. Pb, Cu, Zn, etc.) in biological samples were not confirmed.

On the other hand, because ApoE has a major role in metabolism of cholesterol, which is crucial for neurite growth and synaptogenesis, researchers hypothesized its involvement in neurodevelopment (Wright et al., 2003). Several studies reported that the *ε4* allele is associated with neurodevelopmental advantage in children that had an unfavorable exposure or nutrient deficiency (Oriá et al., 2005; Wright et al., 2003). Recently Smith et al. reviewed the published literature and concluded that children having at least one *ε4* allele may have a positive differential impact on fitness during different life stages (Smith et al., 2019). One of the first studies that aimed to establish the relation between ApoE polymorphisms and prenatal exposure to Hg on neurodevelopment and child behavior concluded that negative associations of MeHg with neurodevelopment are positively cofounded by ApoE and that children who were *ε4* carriers may be more susceptible (Ng et al., 2013, 2015). They hypothesized that higher levels of cord blood THg in *ε4* carriers

are found because only ApoE4 has no cysteine (Table 1.2.), a thiol containing amino acid that would contribute to MeHg elimination. Therefore, *ε4* carriers might be at a greater risk of Hg-induced neurological disease development (Godfrey, Wojcik, & Krone, 2003; Ng et al., 2013). Our study (Snoj Tratnik et al., 2017) (Section 3.4) is consistent with the observation from Ng et al., although Hg exposure in our population is much lower (mean cord blood levels 2.05 ng/g vs. 12.2 µg/L). We showed that children who were *ε4* carriers had marginally significant higher THg levels in their mixed cord blood in comparison with non-carriers, and we observed a Hg-associated decrease in the cognitive score of children who were *ε4* carriers. Moreover, genotype independent Hg-associated decrease in fine motor skills was observed, which was confirmed in the same population from different points of view as mentioned above (Barbone et al., 2019; Prpić et al., 2017). However, all observed associations were found inside normal levels of neurodevelopmental scores.

1.6 Factors to Consider when Assessing Hg Exposure

It is clear that in the general population seafood intake and number of dental amalgams have the largest contribution to the levels of Hg found in human body. As described above, individuals' genetic makeup can affect toxicokinetics mechanisms under comparable levels of exposure. However, there are other individuals' characteristics and habits that may prevent or promote Hg toxicity. Various factors can modify Hg toxicokinetics mechanisms or can affect Hg accumulation. Additionally, those factors should be considered as they can have impact an on our interpretation of the observed exposure.

1.6.1 The general population

The diet represents one of the most important sources of Hg exposure. Moreover, European Food Safety Agency (EFSA) recommended that with 1-4 fish servings per week the benefits will outweigh the risks related to MeHg exposure (EFSA Scientific Committee, 2015). In this context, nutritional status has an important but complex impact on human health and individual's nutritional status can modify aspects of Hg toxicity (Walker, 2005). The Long-Chain Polyunsaturated Fatty Acids (LCPUFAs), which are most commonly ingested with seafood, have a crucial role in pre- and post-natal brain development, both during the period of growth and in the organization processes of this organ, such as cell migration and differentiation, neurogenesis, synaptogenesis and maturation of neuronal pathways (Borsonelo & Galduróz, 2008). Moreover, a cognitive decline was shown to be postponed in elderly, which had a moderate intake of PUFAs (van Gelder, Tijhuis, Kalmijn, & Kromhout, 2007).

Selenium (Se) was shown to be involved in Hg toxicity at many levels, especially as Se has a crucial role in preventing oxidative stress, which on the other hand, can be Hg-induced. Selenium is a part of selenoproteins (thioredoxin system; glutathione system, Selenoprotein P, K, T; etc.), which bind Hg with high affinity and can mediate the Hg absorption, redistribution, or biotransformation as reviewed by Spiller (Spiller, 2018). Moreover, the formation of stable insoluble Hg-Se complexes was also reported as protective effects of Se (Falnoga & Tušek-Žnidarič, 2007; Khan & Wang, 2009).

The microbiome is increasingly recognized as a critical component in human development, health, and disease. Its relevance toxicokinetics and toxicodynamics involve challenges to current concepts related to absorption, metabolism, gene-environment interactions, and pathways of response. Moreover, the gut microbiome possesses many, if not all, of the genes present in the human genome that encode enzymes that influence the

toxicity of Hg (Silbergeld, 2017). For those reasons, the role of microbiome should be studied and included in future studies.

Dietary correlates assessed within US NHANES (National health and Nutrition Examination Survey data) showed that wine was the only food category significantly associated with MeHg concentration among non-seafood consumers; additional research on wine as a potential source of MeHg exposure is recommended (Wells, 2019).

Studies reported that a persons' body mass index could be associated with Hg levels in a human body (Rothenberg, Korrick, & Fayad, 2015). Although the exact mechanism is not known, the negative association between BMI and blood Hg concentrations could be a result of BMI-related differences in MeHg uptake, metabolism or excretion. It could be that changes in GSH or other hepatic enzymes alter the enterohepatic cycling of MeHg, thereby affecting blood Hg concentrations (Cave et al., 2010). On the other hand, stromal vascular cells in adipose tissue may be a reservoir of MeHg; as stromal cells increase in size with increasing BMI, they could affect the MeHg distribution in the human body (Rothenberg et al., 2015).

It was shown that Hg levels are positively associated with age (Caldwell, Mortensen, Jones, Caudill, & Osterloh, 2009), although the mechanisms behind are not totally clear. It is unlikely that this is related only to diet; it could be that the reason behind is Hg bioaccumulation. However, observed associations are not always consistent (Kozikowska et al., 2013; Vahter et al., 2000). Moreover, it was reported that Hg is present in cigarettes (Suzuki, Shishido, & Urushiyama, 1976) and some studies reported that smoking status could affect Hg levels found in blood (Bernhard, Rossmann, & Wick, 2005).

1.6.2 Pregnancy and newborns

During pregnancy, several physiological and anatomical changes occur in woman's body that are essential for the normal development of the child. They are related to changes in the cardiovascular system, body water metabolism, respiratory and endocrine systems, and metabolism of glucose, lipids, and proteins (Soma-Pillay, Nelson-Piercy, Tolppanen, & Mebazaa, 2016). Some of them need to be taken into account in order to assess the level of Hg exposure accurately.

Haematological changes are related to the increase in the plasma volume. The 50 % plasma increase is not followed by an increase in red blood cell mass, therefore the concentration of haemoglobin and haematocrit is decreased (Soma-Pillay et al., 2016). As the majority of MeHg is found in red blood cells, the MeHg concentration measured in whole blood was reported to decrease during pregnancy (Berglund et al., 2005). Normalization for haemoglobin or haematocrit would limit this shortcoming.

Parity is the number of times women gave birth to a foetus with a gestational age of at least 24 weeks or more regardless of whether the babies were born alive or stillborn. A negative association between parity and cord blood Hg was reported (Vahter et al., 2000) and the same was observed in our study (Trdin, Snoj Tratnik, et al., 2019). This could be related to the fact that with parity the placental function is increased (Prior, Mullins, Bennet, & Kumar, 2014), which could include higher ability of the placenta to detoxify (accumulate or eliminate) potentially toxic metals, such as Hg.

Gender-related differences in Hg toxicokinetics and toxicodynamics, the influence of specific hormones (e.g. estrogens and thyroid hormones) on child development, and neurotoxicity was observed from different points of view (Gochfeld, 2007; Llop, Lopez-Espinosa, Rebagliato, & Ballester, 2013; Tatsuta et al., 2017).

Chapter 2

Aims and Hypothesis

The effects of chronic prenatal exposure to low-levels of Hg on child development remain controversial with inconsistent conclusions. In this dissertation, we evaluated how Hg speciation in maternal blood and cord blood contribute to associations between exposure and health effects, assessed whether meconium can be used as a biomarker of prenatal long-term Hg exposure, and studied Apolipoprotein E genotype as a susceptibility biomarker.

The general population is life-long exposed to low levels of different Hg species in varying amounts and from various sources. Most commonly epidemiological studies use THg measurements in blood to assess MeHg exposure, which is based on the assumption that the majority of measured THg is in the form of MeHg. This approach can lead to overestimation of MeHg exposure and consequently to misinterpretations when studying the effects of MeHg on child development. In such cases, inter-individual differences in exposure to inorganic Hg from dental amalgams and other sources might significantly contribute to variations in MeHg proportions. We expected that Hg speciation is especially important for the assessment of Hg related toxicity at low levels of exposure. Detailed information on Hg exposure is essential at an individual level, when studying Hg toxicokinetics, and possible negative effects on child development.

To study Hg toxicokinetics mechanisms during pregnancy and to assess health outcomes in children, the use of biomarkers presenting the widest period of exposure is essential. Meconium represents a foetal excretory product and is therefore a direct measure of long-term exposure, by contrast to maternal blood or scalp hair, which provide only indirect estimations of exposure. Our aim was to speciate Hg in meconium samples in order to evaluate the adequacy of meconium as a biomarker. Furthermore, based on the conducted speciation analysis in meconium, we expect that toxicokinetics or metabolic mechanisms occur during prenatal Hg exposure. Our study is the first one to perform Hg speciation in meconium on such a large sample size which enabled us to study the maternal characteristics and habits that can contribute to prenatal Hg exposure.

ApoE isoforms were hypothesized to behave differently in relation to metal kinetics (especially Hg and Pb). In order to identify susceptible parts of population, ApoE genetic polymorphisms were included to see how *ApoE* genotypes can modify the concentrations of different elements in maternal blood. We studied the relation between maternal *ApoE* genotypes and concentrations of selected trace elements (Hg, Se, Pb, Cd, As, Zn) in maternal blood, as environment provided by the mother during pregnancy is often not included in exposure and effect assessment. Moreover, *ApoE* genotypes were hypothesized to be differently involved in prenatal neurodevelopment. For these reasons, maternal and/or newborn's *ApoE* genotypes were also studied in relation to neurodevelopment and Hg levels in mixed cord blood.

2.1 Research Goals and Hypothesis

Goals of the dissertation are:

i. To define the exposure to different Hg species (MeHg, Hg(II)) in a population of mothers and their newborns from Slovenia and Croatia.
ii. To compare associations between Hg species in maternal blood and mixed cord blood with children's neurodevelopmental outcomes.
iii. To study meconium as a biomarker of prenatal long-term Hg exposure and discuss possible mechanisms of Hg biotransformations during pregnancy.
iv. To evaluate how specific ApoE genetic polymorphisms are associated with levels of selected elements (Hg, Se, Pb, Cd, As, Zn).
v. To assess the relation between prenatal Hg exposure, ApoE genetic polymorphisms, and child's neurodevelopment.

Hypotheses of the doctoral dissertation are:

i. The majority of blood total Hg will be present in organic form (MeHg), particularly in Croatian participants, because they come from the coastal part (Rijeka with surroundings) and according to the questionnaires eat fish more frequently than Slovenians.
ii. Total Hg measurements alone are currently the most commonly used approach to evaluate exposure to Hg. We expect that blood methyl-Hg measurements in combination with THg levels will provide clearer information about exposure to different Hg species, which is especially important at an individual level when studying associations between prenatal Hg exposure and child development.
iii. Among foetal bioindicators, meconium is covering 2^{nd} and 3^{rd} trimester of the pregnancy, while blood samples obtained at delivery are covering only the last trimester. Therefore, Hg determination and Hg speciation of meconium samples will help to estimate long-term foetal exposure to Hg and explain Hg biotransformation processes during pregnancy (maternal and foetal). Possible sources of Hg in meconium are different and are presumably connected to some demethylation processes of MeHg and/or oxidation of Hg^0, both resulting in continuous accumulation of Hg(II).
iv. According to literature suggestions, the presence of ApoE $\varepsilon 4$ allele will be associated with higher levels of Hg in maternal and mixed cord blood, which could have a synergistic modifying effect on a child's neurodevelopment.

Chapter 3

Scientific Publications

Publications included in this dissertation are listed in such a manner that their consecutive appearances follow the general dissertation content. The candidate's contributions to an individual scientific article are described before each article. In the dissertation, publications appear in the following order:

Trdin, A., Snoj Tratnik, J., Mazej, D., Fajon, V., Krsnik, M., Osredkar, J., Prpić, I., Špirić, Z., Petrović, O., Marc, J., Neubauer, D., Kodrič, J., Kobal, A.B., Barbone, F., Falnoga, I., Horvat, M. (2019). Mercury speciation in prenatal exposure in Slovenian and Croatian population – PHIME study. Environmental research, 177. https://doi.org/10.1016/j.envres.2019.108627

Trdin, A., Falnoga, I., Fajon, V., Živković, I., Snoj Tratnik, J., Prpić, I., Špirić, Z., Horvat, M. (2019). Mercury speciation in meconium and associated factors. Environmental research, 179. https://doi.org/10.1016/j.envres.2019.108724

Trdin, A., Snoj Tratnik, J., Stajnko, A., Mazej, D., Sešek Briški, A., Kastelec, D., Prpić, I., Petrović, O., Špirić, Z., Marc, J., Horvat, M., Falnoga, I. (2020). Trace elements and APOE polymorphisms in pregnant women and their newborns. Accepted for publication: Environment International.

Snoj Tratnik, J., Falnoga, I., Trdin, A., Mazej, D., Fajon, V., Miklavčič, A., Kobal, A.B., Osredkar, J., Sešek Briški, A., Krsnik, M., Neubauer, D., Kodrič, J., Stropnik S., Gosar, D., Lešnik Musek, P., Marc, J., Jurkovič Mlakar, S., Petrović, O., Vlašić-Cicvarić, I., Prpić, I., Milardović, A., Radić-Nišević, J., Vuković, D., Fišić, E., Špirić, Z., Horvat, M. (2017). Prenatal mercury exposure, neurodevelopment and apolipoprotein E genetic polymorphism. Environmental research, 152, 375-385. https://doi.org/10.1016/j.envres.2016.08.035

3.1 Mercury Speciation in Prenatal Exposure in Slovenian and Croatian Population – PHIME Study

Trdin, A., Snoj Tratnik, J., Mazej, D., Fajon, V., Krsnik, M., Osredkar, J., Prpić, I., Špirić, Z., Petrović, O., Marc, J., Neubauer, D., Kodrič, J., Kobal, A.B., Barbone, F., Falnoga, I., Horvat, M. (2019). *Environmental Research*, 177. https://doi.org/10.1016/j.envres.2019.108627

Possible negative effects after prenatal exposure to low levels of Hg are not fully understood. Moreover, different studies have controversial conclusions. Most of the published epidemiologic studies assess Hg exposure through measurements of blood THg rather than performing Hg speciation, which can increase the possibility of misinterpretations. In this paper, the authors evaluated the importance of Hg speciation in prenatal low-level exposure. Analyses were conducted on samples drawn from a Mediterranean population included in an epidemiological study PHIME to associate low-level prenatal Hg exposure and neurodevelopmental performance of newborns at 18 months of age. The studied population were pregnant women with their newborns from Slovenia (N = 584) and Croatia (N = 234), who were chronically exposed to low-to-moderate levels of Hg through seafood consumption and/or amalgam fillings.

Maternal blood and scalp hair were sampled during the 3rd trimester of pregnancy, while mixed cord blood was sampled at delivery. Socio-demographic data, personal characteristics and habits were collected from questionnaires. The exposure to Hg was assessed through measurements of THg and MeHg in maternal hair, blood, and mixed cord blood. Levels of Hg(II) were calculated by subtracting MeHg from THg. Neurodevelopmental performance of newborns was assessed by using standardized Bayley-III and Apolipoprotein E polymorphisms were used from the existing PHIME cohort dataset.

The authors presented levels of exposure to different Hg species. Median THg concentration in maternal blood (collected only in Croatian population, N = 225) was 2.04 (0.55-20.5) ng/g. Cord blood median THg concentrations were 1.55 (0.16-14.1) ng/g and 2.94 (0.33-32.3) ng/g for participants from Slovenia (N = 435) and Croatia (N = 210), respectively. It was shown that the proportion of THg as MeHg in maternal and cord blood varied between 4% and 100% (CV = 32%) and between 8% and 100% (CV = 20%), respectively. With lower THg concentrations, the variability of MeHg proportion increased. The authors presented maternal *ApoE* genotype dependent negative association between cord blood MeHg levels and cognitive performance of children (inside normal levels). Decreases in fine motor skills (inside normal levels) were genotype independent and were associated with Hg(II) and MeHg. Multivariate statistical analysis showed that Hg speciation provides additional and more precise information on exposure and effect assessment. Based on the presented results the authors concluded that direct measurements of MeHg are especially important at an individual level and provide detailed information when other influencing factors are collected/measured precisely and all sources of uncertainties (such as sampling, sample transport and storage, and detailed questionnaires) are reduced.

The candidate's contribution to this paper included direct measurements of MeHg in the samples of maternal blood and cord blood, ApoE genotyping for mothers and children, and statistical analysis. She drew figures and tables, and prepared the manuscript in cooperation with co-authors.

Environmental Research 177 (2019) 108627

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Mercury speciation in prenatal exposure in Slovenian and Croatian population – PHIME study

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ARTICLE INFO

Keywords:
Methylmercury
Speciation
Prenatal exposure
Neurodevelopment
Apolipoprotein E (ApoE)

ABSTRACT:

In recent years, several studies have addressed the issue of prenatal exposure to methylmercury (MeHg); however, few have actually analysed MeHg blood concentrations. Our study population included mothers and their new-borns from Slovenia (central region; N = 584) and Croatia (coastal region; N = 234). We have measurements of total Hg (THg) and MeHg in maternal hair, maternal peripheral blood, and cord blood. Cord blood Hg concentrations were low to moderate (median THg = 1.84 ng/g and MeHg = 1.69 ng/g). The proportion of THg as MeHg (%MeHg) in maternal and cord blood varied between 4% and 100% (coefficient of variation, CV = 32%) and between 8% and 100% (CV = 20%), respectively. Our data shows that variability of %MeHg was higher at lower blood THg levels. Concentrations of MeHg in maternal blood and cord blood were highly correlated (R_s = 0.943), in the case of inorganic Hg correlation was significant but weaker (R_s = 0.198). MeHg levels in maternal blood and cord blood were positively associated with seafood intake, maternal age, and negatively associated with pre-pregnancy BMI. Additionally, MeHg in maternal blood was positively associated with plasma selenium levels, and cord blood MeHg was negatively associated with parity. The results of multiple linear regression models showed that speciation analysis provides more defined estimation of prenatal exposure in association modelling. Associations between Hg exposure and cognitive performance of children (assessed using Bayley Scales of Infant and Toddler development) adjusted for maternal or child Apolipoprotein E genotypes showed higher model R^2 and lower p-values when adjusted for MeHg compared to THg. This study demonstrates that Hg speciation improves the association between exposure and possible negative health effects.

1. Introduction

Mercury (Hg) is one of the top ten chemicals of major public health concern. It disperses into and remains in ecosystems for generations, causing severe ill health and intellectual impairment in exposed populations (Berlin et al., 2015; UNEP/AMAP, 2013). With the signing of the UNEP Minamata Convention in 2013 and its ratification in 2017, the world's governments are committed to reduce Hg emissions from the energy and other industrial sectors (UNEP/AMAP, 2013).

The general population is exposed to organic mercury (mostly as monomethyl Hg; CH_3Hg^+, MeHg) mainly through seafood consumption and to Hg^0 through dental amalgam fillings (Horvat et al., 2012). The central nervous system is the primary target site where toxic effects of MeHg are manifested (ATSDR, 1999; Karagas et al., 2012; WHO, 2007). Studies often focus on neurotoxicological effects after prenatal MeHg exposure, the time window in which the developing nervous system of the foetus is especially vulnerable (Ha et al., 2017; Sheehan et al., 2014). As shown in the past prenatal exposure to high doses of

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https://doi.org/10.1016/j.envres.2019.108627
Received 8 March 2019; Received in revised form 30 July 2019; Accepted 4 August 2019
Available online 05 August 2019
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MeHg results in different irreversible dysfunctions and anomalies (Bakir et al., 1980; Elhassani, 1982; Harada, 1995). However, the effects of chronic prenatal exposure to low levels of MeHg on child development remain controversial with inconsistent conclusions, as researchers recently reported negative associations between Hg and child development (Prpić et al., 2017; Vejrup et al., 2018) whereas others observed positive associations (Taylor et al., 2016). However, all agree that prenatal Hg exposure needs further research.

Few studies have addressed long-term low-level prenatal exposure to MeHg and measured MeHg in relevant exposure biomarkers (Basu et al., 2018; Gundacker et al., 2010; Sakamoto et al., 2016; Wells et al., 2016). Otherwise, total Hg (THg) concentration is often used as a proxy measure of MeHg exposure based on the assumption, that MeHg constitutes a substantial (~90%) proportion of THg in blood (Basu et al., 2018; Berglund et al., 2005; Hong et al., 2012; Horvat et al., 2012). However, this approach can result in overestimation of MeHg exposure especially in cases of low-to-moderate levels of exposure to MeHg from seafood consumption. In such cases, inter-individual differences in exposure to inorganic mercury (IHg; Hg(II) and Hg^0; from dental amalgam fillings and/or other sources) might significantly contribute to variations in MeHg proportions.

This study aimed to speciate Hg through measurements of MeHg in blood samples of Mediterranean population (Slovenia and Croatia, both included in an epidemiological study PHIME) which is life-long exposed to low-to-moderate levels of Hg through seafood consumption and/or amalgam fillings. We used existed data set of personal and lifestyle characteristics and THg measurements in maternal hair, maternal blood, and cord blood. Total Hg measurements were reported previously (Miklavčič et al., 2013, 2011), as well as associations between prenatal Hg exposure, as indicated through total Hg in cord blood, and neurodevelopment assessed by Bayley Scales of Infant and Toddler Development, third edition (Bayley III) (Barbone et al., 2019; Prpić et al., 2017; Snoj Tratnik et al., 2017). In this paper, we performed Hg speciation in maternal blood and cord blood and re-evaluated the associations based on MeHg measurements.

2. Materials and methods

2.1. Study population, sampling, and data collected

Our study population included mothers and their new-borns from Slovenia (the city of Ljubljana and its surroundings up to 50 km) and from the Adriatic coastal region of Croatia (the city of Rijeka and its county Primorsko-goranska). They were recruited in 2007–2009 as part of the birth cohort study PHIME (Public Health Impact of Long-term Low-level Mixed Element Exposure in Susceptible population Strata; EU 6th Framework Programme). The study was conducted in accordance with the Declaration of Helsinki; the Republic of Slovenia National Medical Ethics Committee approved the protocol (No. 98/05/06) for the Slovenian participants and the Ethics Committee of University Hospital Centre Rijeka approved the protocol (No. 2170-29-02/1-07-1) for the Croatian participants. All subjects gave their informed consent.

Valent et al. (Valent et al., 2013a, 2013b) and Miklavčič et al. (2013) described the study design, recruitment, sampling and questionnaires in detail. Briefly, recruitment and sampling took place in (1) Slovenia at the Maternity Hospital of the University Medical Centre of Ljubljana and in (2) Croatia at the University Hospital Rijeka. For logistics reasons, the timing of sample collection differs and, in some cases, biological material was not collected (Table 1). Hair samples were stored at room temperature in a zip-lock plastic bag and then analysed without any cleaning. All other biological samples (maternal blood and cord blood) were stored in a freezer below −24 °C.

Our study only included healthy pregnant women with no reported serious illness or dysfunction of the child or twin gestation (N = 818). Pregnant women were sampled in the 3rd trimester. The participating pregnant women filled out a short and a long questionnaire. At the time of recruitment, the short questionnaire provided an assessment of the individual's characteristics such as demographics, smoking status, intake of alcoholic beverages, and frequency of consumption of specific food (e.g. vegetables; milk; meat; and fresh, frozen, or tinned fish). After delivery, the long questionnaire provided more detailed information about the sociodemographic and health status of the whole family, smoking habits, food frequency (especially seafood consumption), number of dental amalgams, etc.

Questions regarding smoking were present in both the short and the long questionnaires. Women reported smoking before or during pregnancy. If the answers between the long and the short questionnaires differed (yes/no), we took the positive one as the more reliable one. To include smokers regardless of the period of smoking, we combined both smoking during or before pregnancy in the group of 'ever-smokers'.

Seafood intake during pregnancy was estimated from a long questionnaire completed by mothers one month after delivery. This questionnaire included seven questions on seafood that addressed the frequency (never, 1x/month, 1–3x/month, 1x/week, 2–4x/week, 5–6x/week, 1x/day, 2–3x/day, more than 3x/day) of consumption of 150-g servings of fish, crustaceans, and molluscs (boiled, grilled, fried, or baked) and tuna, mackerel, or sardines in oil. To estimate daily seafood intake, we used the same method as that described in detail by Valent et al. and Miklavčič et al. (Miklavčič et al., 2013; Valent et al., 2013b). For each seafood item, conversion from categories of consumption into continuous intake of seafood servings per day was done by assigning to each category a consumption level equal to the median value for that category (e.g., 2–4 times/week was converted to 3 times/week), and this was recalculated into servings per day of each seafood type. Overall seafood intake was calculated by summing up the estimated daily intake of all seafood types.

To assess the number of dental amalgams, women were divided into four classes (< 3 amalgams, 3–5 amalgams, 6–9 amalgams, 10 + amalgams). Information on dental amalgam fillings was incomplete, especially for the Croatian part of the population; therefore, data about amalgam fillings was not included in the statistical analysis.

Women also reported how many births they gave to a foetus with a gestational age of at least 24 weeks or more regardless of whether the babies were born alive or stillborn. According to their answer, we divided them into two groups: nulliparous and parous women.

2.2. Mercury measurements methods

All analyses of THg and MeHg in biological samples were performed at the Jožef Stefan Institute, Ljubljana, Slovenia. Total mercury in maternal hair, blood, and cord blood was determined using a Direct Mercury Analyzer (DMA; Milestone, USA). The method is described in detail elsewhere (EPA Method 7473, 1998; Miklavčič et al., 2011). All measurements were performed under strict quality control procedures and gave comparable results. To check the accuracy of the method, we used the following reference materials (RMs): NIES CRM no. 13, human

Table 1
Sampling protocol summary: sample phases, biological samples, and data collected.

Country (Number of recruited women)	Short questionnaire, maternal hair	Maternal blood	Mixed cord blood	Long questionnaire
Slovenia (N = 584)	3rd trimester	–	At delivery	approx. 1 month after delivery
Croatia (N = 234)	3rd trimester	3rd trimester	At delivery	approx. 1 month after delivery

Table 2
Average, range, and number of collected basic demographic data from Slovenian and Croatian participants with p-values for comparisons between both populations.

Demographic data	Slovenia	Croatia	All	p-value
Maternal age (years)	30.5 (18–45), N = 580	30.1 (19–44), N = 221	30.4 (18–45), N = 818	0.285[a]
Body mass index (BMI; kg/m^2)	23.8 (17.1–44.5), N = 582	23.0 (16.8–41.1), N = 232	23.6 (16.8–44.5), N = 814	**0.012**[a]
Parity (% of nulliparous)	52, N = 562	45, N = 233	50, N = 795	0.080[b]
Seafood intake (meals per day)	0.28 (0–4.5), N = 372	0.45 (0–2.21), N = 200	0.34 (0–4.5), N = 375	**0.000**[c]
Ever-smoking (% of smokers)	31, N = 584	42, N = 234	35, N = 818	**0.002**[b]
Gestational age (EGA; weeks)	39.5 (28–42), N = 371	39.4 (34–41), N = 169	39.4 (28–42), N = 541	0.302[c]
Birth weight (kg)	3.4 (2.0–4.9), N = 371	3.5 (2.4–4.8), N = 196	3.5 (2.0–4.9), N = 568	**0.037**[c]
Child sex (% boys/girls)	49/51, N = 373	50/50, N = 208	49/51, N = 582	0.728[b]

Bolded are statistically significant differences between both populations. Statistical tests used to test the difference between Slovenian and Croatian population:
[a] ANOVA.
[b] χ^2.
[c] Mann Whitney U.

hair; IAEA-086, methylmercury, total mercury and other trace elements in human hair; and Seronorm, trace elements in whole blood L-1. For additional information, see Miklavčič et al. (Miklavčič et al., 2013, 2011).

MeHg in hair was measured only in those samples in which THg concentrations in hair exceeded 1 μg/g, Horvat et al. gave a more detailed description of this method (Horvat et al., 1988; Horvat and Byrne, 1990), and results were previously published by Miklavčič et al. (Miklavčič et al., 2013, 2011). Methylmercury in maternal blood and cord blood was measured in all available samples. For this purpose, ~0.2 g of a sample was weighed into glass tubes. Then, 10 mL of 4 M HNO_3 was added and the mixture was heated at 72 °C for 24 h (Hammerschmidt et al., 2013). After cooling, the digested samples were diluted to 30 mL with MQ water. We took an aliquot of digested sample, added MQ water, and after adjusting the pH using citrate buffer, MeHg was ethylated using an ethylation reagent (1% $NaBEt_4$ in 1% KOH) (Liang et al., 1994). For measurements, we used an automatic system based on cold-vapor atomic fluorescence detection (CV AFS, Tekran 2700 instrument; Tekran Instruments Corporation, Canada). To check the accuracy of MeHg measurements in maternal blood and cord blood, we analysed lyophilized whole human blood PT-WB1 obtained from non-exposed population as an RM. MeHg in PT-WB1 was determined in the PHIME project through interlaboratory comparisons. The assigned value (5.8 ± 0.5 μg/kg) was in good agreement with the determined value (5.69 ± 0.57 μg/kg, k = 2; n = 50).

Inorganic mercury levels were calculated by subtracting MeHg from THg. In cases where the percentage of Hg as MeHg 100%, we used LOD/2 for the IHg level.

The estimated measured uncertainty for Hg determination in blood was 14% (k = 2) for THg measurements (Miklavčič et al., 2011), 10.2% (k = 2) for MeHg measurements, and 17.3% (k = 2) for IHg. The LODs calculated as three times the standard deviation of the blank sample were 0.02 ng/g for THg and MeHg in blood samples. The LOQs calculated as ten times the standard deviation of the blank sample were 0.07 ng/g for THg and MeHg in blood samples.

2.3. ApoE genotyping

Maternal DNA was isolated from their peripheral blood leukocytes using High Pure PCR Template Preparation Kit (Roche, Switzerland). Genotyping for Apolipoprotein E (ApoE; rs429358 and rs7412) was performed using TaqMan® pre-designed SNP genotyping small scale (Applied Biosystems, CA, USA) on LightCycler® 480 II (Roche, Switzerland). We determined ApoE genotype for 398 women, 59 of which were ε4 carriers. ApoE genotypes were in Hardy-Weinberg equilibrium. Details are presented elsewhere (Trdin, 2015).

2.4. Neurodevelopmental assessment

Children neurodevelopment was assessed using Bayley Scales of Infant and Toddler development third edition (Bayley-III). Children (N = 444) were tested at the age between 15 and 22 months. Bayley standard scores were derived from raw scores according to standardization sample for child's age at test administration in one-month interval, which is relevant for ages 5 months 16 days–36 months 15 days (Bayley, 2006). Additional information about the Bayley III test and outcomes are reported elsewhere (Barbone et al., 2019; Prpić et al., 2017; Snoj Tratnik et al., 2017).

2.5. Statistical analysis

All data obtained were analysed using STATA 12 software. To check the distribution of measured Hg species in samples, the Shapiro-Wilk test was used. Mercury concentrations in all measured samples were not normally distributed. Therefore, median values were used to describe the original data and Spearman correlations between continuous variables. For comparison between populations, we used the Mann Whitney *U* test, χ^2, and ANOVA. Before performing multiple linear regression analysis, the THg and MeHg concentrations in maternal blood and cord blood were log transformed. The regression models were used to evaluate associations between THg and MeHg concentrations and other possible influencing factors (Table 5). Cofounders and covariates were selected based on knowledge of physiology about possible influencing factors and correlations from our results or from literature and then included to provide the highest prediction value (R^2) of the models. Model 1 MB was adjusted for seafood consumption, smoking status, pre-pregnancy BMI, parity, estimated gestational age, child's sex, ln blood Pb, and ln plasma Se. Model 2 CB was adjusted for seafood consumption, smoking status, pre-pregnancy BMI, parity, estimated gestational age, child's sex, birth weight, ln cord blood Pb, and ln cord blood plasma Se. Re-evaluation models, assessing ApoE genotypes, cord blood Hg levels, and Bayley III neurodevelopmental outcomes (Table 6) were designed during our first study (Snoj Tratnik et al., 2017). Models were adjusted for ApoE genotype, cord blood Hg levels, country and particular socio-demographic characteristics (mother's age at delivery, child's gender, birth weight, educational level of mother, smoking during pregnancy), ln cord blood plasma Se, and ln cord blood Pb. The same as in our previous study (Snoj Tratnik et al., 2017), models were not adjusted for child's age. Models A, C, and E are adjusted for ln THg [ng/g] concentrations. Models B, D, and F are adjusted for ln MeHg [ng/g] concentrations. Models A and B include child's genotype. Models C and D include maternal genotype. Models E and F include child's and maternal genotype where ε4 carriers are mother-child pairs and at least one of them is an ε4 carrier. Afterwards we re-ran the regression models

Table 3
Median concentrations [ng/g] of total Hg (THg), methyl Hg (MeHg) expressed as ng Hg/g, inorganic Hg (IHg), and % MeHg [%] in maternal hair (MH), maternal blood (MB), and cord blood (CB).

	THg (ng/g) median range N				MeHg (ng/g) median range N			% MeHg (%) median range N			IHg (ng/g) median range N		
	Slo	Cro	All	All*	Slo	Cro	All	Slo	Cro	All	Slo	Cro	All
MH	298 15–2439 571	604 16–8710 234	346* 15–8710 805	1510* 1010–8710 75	1268 576–2439 26	1651 928–8710 49	1475* 576–8710 75	100 55–100 26	99 72–100 49	100 55–100 75	0.1 0.1–474 26	16.3 0.1–1302 49	0.1 0.1–1302 75
MB	/	2.04 0.55–20.5 225	/	/	/	1.73 0.03–19.6 225	/	/	85.9 4.3–100 225	/	/	0.36 0.01–12.6 225	/
CB	1.55 0.16–14.1 435	2.94 0.33–32.3 210	1.84* 0.16–32.3 645	/	1.43 0.08–13.3 426	2.84 0.16–31.9 210	1.69* 0.08–31.9 636	98.6 20–100 423	97.9 7.8–100 210	98.4 7.8–100 633	0.02 0.01–2.09 423	0.08 0.01–4.76 210	0.04* 0.01–4.76 633

Slo-Slovenian population. Cro-Croatian population. All-both populations combined. *statistically significant ($p < 0.05$) difference between Slo and Cro population.
[a] THg levels in hair of participants which have measurements of THg and MeHg levels in hair.

using sub-sets, dividing participants into two groups according to the presence or absence of ε4 in children and/or mothers (genotypes ε4 vs. ε2 And ε3). The level of significance (p) was set at 0.05 and p values < 0.1 as marginally significant.

3. Results and discussion

To estimate prenatal exposure to Hg in our population, we sampled pregnant women and their new-borns from central Slovenia (N = 584) and the coastal region of Croatia (N = 234). Maternal hair and peripheral whole blood were sampled during the 3rd trimester (Table 1), at which time the foetuses' brains are in the critical phase of development and are particularly vulnerable to neurotoxic chemicals such as Hg (Grandjean and Herz, 2011). The age of pregnant women included in this study was 30.4 years, and their average body mass index (BMI) before pregnancy was 23.6. Of the participating women, 50% have never given birth (nulliparous women). Approximately 3% of women self-reported never eating any kind of fish or other seafood. Based on their answers from the short and the long questionnaires, we identified 35% women as 'ever-smokers'. The average gestational age of newborns was 39.4 weeks, average birth weight was 3.5 kg, and 49.3% of them were boys. Collected demographic data are presented in Table 2.

At delivery, we sampled cord blood, which was shown to be a suitable biomarker of prenatal Hg exposure (Grandjean et al., 2010; Grandjean and Budtz-Jørgensen, 2007). Total Hg concentrations were measured in all collected samples (N = 1685). MeHg was measured in available samples of maternal blood (N = 225) and cord blood (N = 636). MeHg in hair was measured in cases when hair THg exceeded 1 μg/g (N = 75). The median values, range, and observed THg, MeHg, (% MeHg), and IHg concentrations are listed in Table 3.

The median value of THg in maternal hair was 346 ng/g. Methylmercury in hair presented ~100% of THg. In maternal blood, which was available only for the Croatian population, the median THg value was 2.04 ng/g. Methylmercury on average presented 85.9% of THg in maternal peripheral blood. The median value of IHg concentrations in maternal blood was 0.36 ng/g. The median value of THg in cord blood was 1.84 ng/g. Methylmercury presented 98.4% of THg in cord blood on average. The median value of IHg concentrations in cord blood was 0.04 ng/g. The maximum level of IHg in maternal and cord blood was observed in the same participant. As shown in Table 3, participants from Slovenia had lower hair and cord blood Hg concentrations than participants from Croatia ($p < 0.001$). This is related to higher fish consumption in Croatian than in Slovenian study population, and was confirmed by the questionnaire data (Table 2), and reported previously by Miklavčič et al. (Miklavčič et al., 2013, 2011). As observed from Table 2, women from Croatia also had higher percentage of smokers. Although there was statistically significant difference between Slovenian and Croatian women in pre-pregnancy BMI and birth weight of their children, average BMI and birth weight (Table 2) was comparable between the two countries. As we did not have all the demographic data for all the participants, we pooled the data from both countries into one database for further statistical analysis.

In our population, ~10% of women exceeded the threshold of 1 μg/g for total mercury in hair, which was established by NRC (National Research Council, 2000). Based on the updated hair Hg limit of 0.58 μg/g (Bellanger et al., 2013; Grandjean and Budtz-Jørgensen, 2007) a substantial proportion of our population (29%) is above this limit. Given the US EPA threshold of 5.8 μg/L in cord blood, which appeared to be safe for the development of neonates (Rice, 2004; US EPA, 1997), 11% of women exceeded this value. 23% of women had blood THg ≥ 3.5 μg/L, which is the threshold considered relevant for preventing foetal neurotoxicity by Mahaffey et al. (Mahaffey et al., 2009, 2004). Considering the health-based value established by the German Human Biomonitoring Commission, ~12% of women exceeded limit value (HBM-I) for THg defined at 5 μg/L in maternal blood, and two women exceeded the action level (HBM-II) of 15 μg/L (Schulz et al., 2011). Based on the above comparisons with "safe" values, we can conclude, that in general our population was exposed to low-to-moderate levels of Hg (Table 3).

We observed that with lower THg blood concentrations, the average % of MeHg decreases (Fig. 1a and b), which is consistent with literature (Berglund et al., 2005; Wells et al., 2017). The coefficient of variation (CV) for % of MeHg in maternal blood was 32.3%. With maternal MeHg levels lower than the median level of 1.73 ng/g, MeHg presented only 62.4% (range: 4%–100%, CV: 42%) of THg on average; if we included only those lower than 0.72 ng/g (25th percentile), MeHg presented only 44.1% (range: 4%–72%, CV: 41%) of THg. Trends in cord blood were similar; however, the decrease in % of MeHg was less obvious. The CV for % of MeHg in cord blood was 20.2%. For cord blood MeHg levels lower than the median value of 1.69 ng/g, MeHg presented 92.4% (range: 8%–100%, CV: 27%) of THg on average; if we included only those lower than 0.76 ng/g (25th percentile), MeHg presented 79.2% (range: 8%–100%, CV: 35%) of THg. This could be related to variations in only the MeHg concentrations and the population's exposure to IHg remains at a comparable level. The decreased proportion of MeHg at lower levels of THg exposure can be the result of some other influencing factors (e.g. genetic polymorphisms, gut microbiome). These results showed that at comparable THg levels, the individual proportion of MeHg can vary considerably. One woman presented a special case (in Fig. 1a: THg ~ 13 ng/g, %MeHg ~ 5%), but based on our data, we could not identify the reason for such low %MeHg for this individual.

Table 4
Spearman's correlations (N) between total Hg (THg), methyl Hg (MeHg), and inorganic Hg (IHg) in maternal hair, maternal blood, cord blood, and maternal personal characteristics (daily seafood intake, BMI, age, parity).

		MB				CB		Seafood intake	BMI	Age	Parity
		THg	MeHg	IHg	THg	MeHg	IHg				
MH	THg	0.892*** (225)	0.887*** (225)	−0.255*** (225)	0.896*** (622)	0.903*** (611)	−0.060 (611)	0.509*** (554)	−0.221*** (798)	0.140*** (784)	−0.082* (776)
MB	THg		0.960*** (225)	−0.183* (225)	0.929*** (208)	0.913*** (208)	−0.304*** (208)	0.440*** (198)	−0.199** (223)	0.174* (212)	−0.128 (224)
	MeHg			−0.352*** (225)	0.929*** (208)	0.943*** (208)	−0.338*** (208)	0.480*** (198)	−0.217** (223)	0.199** (212)	−0.143* (224)
	IHg				−0.291*** (208)	−0.312*** (208)	0.198** (208)	−0.181* (198)	0.144* (223)	−0.085 (212)	0.087 (224)
CB	THg					0.972*** (633)	0.052 (633)	0.550*** (533)	−0.210*** (630)	0.129** (623)	−0.058 (624)
	MeHg						−0.109* (633)	0.562*** (527)	−0.194*** (619)	0.142*** (612)	−0.057 (613)
	IHg							−0.009 (527)	−0.046 (619)	−0.026 (612)	0.045 (613)
Seafood intake									−0.126** (565)	0.035 (567)	−0.005 (563)
BMI										0.087* (794)	0.099* (786)
Age											0.305*** (773)

MH-maternal hair; MB-maternal blood; CB-cord blood; *p < 0.05; **p < 0.005; ***p < 0.0001.

However, this woman and her child were not included in further multiple linear regression modelling. On the other hand, seafood intake was correlated with % of MeHg in maternal blood ($R_s = 0.354$, $p < 0.001$, $N = 198$) and cord blood ($R_s = 0.126$, $p < 0.05$, $N = 526$).

To better understand all possible sources of Hg exposure, especially in this low-level concentration range, we examined Spearman's correlations (Rs) between Hg concentrations in different matrices and maternal characteristics (Table 4). Furthermore, by using multiple linear regression we adjusted the THg and MeHg concentrations in maternal blood and cord blood for possible covariates and cofounders (Table 5). The regression models were not significant for IHg; therefore, these results are not included.

As observed from the correlations in Table 4 and additionally confirmed by the multiple linear regression in Table 5, the major source of Hg exposure in our population was seafood consumption, mainly presenting exposure to MeHg. A further examination of the correlations from Table 4 showed that daily seafood consumption correlated the strongest with cord blood MeHg but also with THg in maternal hair. Measurement of Hg in hair is a commonly used method to asses MeHg exposure in the general population, because the Hg concentration in the hair reflects the blood Hg concentration during hair formation (Ha et al., 2017; Sakamoto et al., 2018). Further, hair collection is non-invasive, storage is simple and analytical determination is relatively precise (Miklavčič et al., 2011). Correlation between THg in hair and cord blood MeHg was stronger as compared to the correlation between THg in the cord blood and THg in the hair.

The correlation between Hg in maternal blood and cord blood was high and significant, but Spearman's correlations coefficient was somewhat higher in the case of MeHg (Table 4). By contrast, the IHg correlation between maternal blood and cord blood was significant but much lower. Donohue et al. observed positive correlation between MeHg and IHg concentration in maternal blood (Donohue et al., 2018), but we observed a significant negative correlation between MeHg and IHg concentrations in maternal blood and cord blood. This can be explained by either lower numbers of amalgams or lower demethylation potential of MeHg (Dock et al., 1994) in women with higher MeHg levels. Unfortunately, due to incomplete data on the number of amalgams we cannot explain these correlations.

Associations that were observed from multiple regression (Table 5) after adjusting for potential covariates and confounders include a negative association between pre-pregnancy BMI and MeHg concentrations in maternal blood and cord blood. Associations between Hg and BMI were observed before in a study performed on the general (non-pregnant) population (Rothenberg et al., 2015). In present study, total Hg in maternal blood did not show an association with BMI, whereas MeHg did.

In the case of cord blood associations were found for BMI and there was no marked difference between them (Table 5). Furthermore, Kozikowska et al. (2013) reported that Hg decreases with age, which is opposite to the results reported by Vahter et al. (2000). Consistently with the latter, we found a positive association between maternal age and Hg concentrations in maternal blood and cord blood. In the case of cord blood, the difference between associations with maternal age and THg or MeHg was minor. In contrast, maternal blood THg was marginally associated with the age, but blood MeHg association was significant. Table 5 shows also negative associations between parity and Hg in cord blood, which is consistent with literature (Vahter et al., 2000). The effect of parity on Hg cord blood level was stronger in the case of MeHg than THg. Additionally, maternal blood Hg was associated with maternal plasma Se levels; however, the association with maternal blood THg was only marginally statistically significant, while with MeHg it was significant. Cord blood MeHg was marginally associated also with cord blood Pb levels, and cord blood THg levels were marginally associated with cord blood plasma Se. From the results of multiple linear regression above, we can conclude that MeHg concentrations better predicted factors influencing Hg concentrations in

Table 5
Multiple linear regression for THg and MeHg in maternal blood (MB) and cord blood (CB).

MODEL 1 MB	ln THg MB β; p (95% CI) p < 0.001, R^2 = 0.2465; N = 150	ln MeHg MB β; p (95% CI) p < 0.001, R^2 = 0.3029; N = 150
Seafood intake (meals per day)	**0.80; 0.000 (0.47; 1.13)**	**1.27; 0.000 (0.80; 1.74)**
Smoking (smokers vs. non-smokers)	−0.02; 0.853 (−0.23; 0.19)	−0.03; 0.831 (−0.34; 0.28)
Pre-pregnancy BMI[a] (kg/m^2)	−0.02; 0.100 (−0.04; 0.004)	**−0.04; 0.020 (−0.07; −0.001)**
Mother's age (years)	***0.02; 0.090 (-0.003; 0.04)***	**0.04; 0.028 (0.004; 0.07)**
Parity (nulliparous vs. parous)	−0.17; 0.129 (−0.40; 0.05)	−0.23; 0.159 (−0.56; 0.09)
EGA[b] at sampling (weeks)	−0.005; 0.890 (−0.09; 0.07)	−0.04; 0.522 (−0.15; 0.08)
Child's sex (girls vs. boys)	−0.04; 0.719 (−0.25; 0.19)	−0.005; 0.976 (−0.31; 0.30)
Maternal blood ln Pb (ng/g)	0.04; 0.680 (−0.16: 0.25)	−0.01; 0.959 (−0.31; 0.29)
Maternal plasma ln Se (g/L)	***0.46; 0.081 (-0.06; 0.97)***	**0.92; 0.016 (0.17; 1.66)**
MODEL 2 CB	**ln THg CB β; p (95% CI) p < 0.001, R^2 = 0.3030; N = 388**	**ln MeHg CB β; p (95% CI) p < 0.001, R^2 = 0.3016; N = 388**
Seafood intake (meals per day)	**1.54; 0.000 (1.25; 1.82)**	**1.71; 0.000 (1.39; 2.04)**
Smoking (smokers vs. non-smokers)	0.02; 0.791 (−0.14; 0.19)	0.04; 0.683 (−0.15; 0.22)
Pre-pregnancy BMI[a] (kg/m^2)	**−0.04; 0.000 (−0.06; −0.03)**	**−0.04; 0.000 (−0.06; −0.02)**
Mother's age (years)	**0.02; 0.014 (0.005; 0.04)**	**0.03; 0.007 (0.01; 0.05)**
Parity (nulliparous vs. parous)	***−0.14; 0.097 (-0.31; 0.03)***	**−0.19; 0.045 (−0.38; −0.004)**
EGA[b] (weeks)	−0.03; 0.299 (−0.10; 0.03)	−0.03; 0.382 (−0.11; 0.04)
Child's sex (girls vs. boys)	0.0001; 0.770 (−0.001; 0.001)	−0.0002; 0.573 (−0.001; 0.001)
Birth weight (kg)	0.0001; 0.572 (−0.0001; 0.0003)	0.0001; 0.612 (−0.0001; 0.0003)
Cord blood ln Pb (ng/g)	0.13; 0.119 (−0.03; 0.30)	***0.17; 0.069 (-0.013; 0.36)***
Cord plasma ln Se (g/L)	***0.32; 0.094 (-0.05, 0.69)***	0.29; 0.174 (−0.13; 0.71)

Concentrations of maternal blood and cord blood THg, MeHg, Pb and Se were log transformed (ln) before statistical analysis. Bolded are statistically significant associations; bolded in italic are marginally significant associations.

[a] BMI-body mass index.

[b] EGA-estimated gestational age; bolded are statistically significant associations; bolded in italic are marginally significant associations.

maternal blood and cord blood, particularly the influence of seafood intake and explanatory level (R^2) of the model for maternal blood. Our results are consistent with recent studies in which speciation of Hg was shown to improve the interpretation of association between exposure biomarkers and health outcome (Wells et al., 2016, 2017).

In line with the objective of our previously published work (Snoj Tratnik et al., 2017), namely, to identify susceptible subgroups of the presented population, Apolipoprotein E (ApoE) was studied as a potential genetic factor that could influence Hg exposure and/or effects of exposure. The ε4 allele of ApoE is recognized as an important risk factor for Alzheimer's disease (Buttini et al., 1999), but researchers have also reported the positive role of ApoE in neurodevelopment (Tuminello and Han, 2011; Wright et al., 2003). Recently, Ng et al. reported that the effects of MeHg on neurodevelopment were modified by ApoE in children who were ε4 carriers (Ng et al., 2015, 2013). Similarly, in our study (Snoj Tratnik et al., 2017), we found that children who were ε4 carriers had higher cord blood THg levels in comparison with ε4 non-carriers (geometric mean: 2.37 ng/g vs 1.98; p = 0.079), and we observed a Hg-associated decrease in the cognitive scores (within the normal range of scores) of children who were ε4 carriers. However, both mentioned studies did not include information about contribution of different Hg species to these associations. To clarify these associations, in present study we performed additional measurements of MeHg in cord blood for the whole population, analysed maternal ApoE genotypes, and re-evaluated multiple linear regression models designed by Snoj Tratnik et al. (Snoj Tratnik et al., 2017). It is worth mentioning, that in our first evaluation some participants were excluded from statistical analysis because of suspected high IHg exposure.

Modified multiple linear regression models were used for participants for whom information about THg and MeHg in their cord blood was available. In case of maternal blood the samples were available only for Croatian population; therefore, these data were not included (as confounder) because of the major participant downsize and consequently misleading results. We included the influence of maternal ApoE genotype (alone or in combination with child genotype) because the environment provided by the mother during prenatal development is probably as important as is that in which children grew until 18 months of age (Rappaport, 2011), when the Bayley III tests were assessed. With this approach, we tested whether measurements of MeHg in cord blood (in comparison with cord blood THg) would provide additional information about studied associations. The models (A, B, C, D, E, F) are presented in Table 6. Model A is adjusted for cord blood ln THg and child's ApoE genotype. Model B is the same as model A except that it is adjusted for cord blood ln MeHg. Model C is adjusted for cord blood ln THg and maternal ApoE genotype. Model D is adjusted for cord blood ln MeHg and maternal genotype. Models E and F are adjusted for maternal and child's ApoE genotype (where at least one in the mother-child pair is an ε4 carrier) and cord blood ln THg (Model E) or cord blood ln MeHg (Model F). All were adjusted also for variables that could potentially influence the outcome including country and particular socio-demographic characteristics (mother's age at delivery, child's gender, birth weight, educational level of mother, smoking during pregnancy), cord blood plasma Se, and cord blood Pb as described in Methods.

In general, most of the models were statistically non-significant and with low explanatory values ($R^2 < 0.2$). Anyway, some of them were significant (marked as bolded in Table 6), and inside non-significant a few variables had statistically significant modifying effects. Models adjusted for the child's ApoE genotype (Table 6, Models A and Models B) showed statistically negative association between cognitive score and Hg cord blood levels in children who were ε4 carriers. In the group of ε4 carriers the β-estimate of the change in cognitive score is comparable for THg (β = −5.55) and for MeHg (β = −5.33). As both models are not significant, the significance of selected variables have to be interpreted very carefully.

Models C and models D which are adjusted for maternal ApoE genotype confirmed similar associations as those described above. In the group of children, whose mothers were ε4 carriers, a negative associations were observed between child's cognitive score and Hg levels, where β-estimates of change were higher in the case of cord blood THg (Model C) in comparison with MeHg (Model D); −7.09 and −6.06,

Table 6
Bayley-III score associations with ln cord blood (CB) Hg using multiple linear regression models adjusted for ApoE genotype. β-estimate of change in neurodevelopment score. CI 95%–95% confidence interval.[a]

		β (CI 95%), p-value dependent variable model R^2 model p-value; N				
Models **Child ApoE**		**Cognitive**	**Language**	**Motor**	**Fine motor**	**Gross motor**
ln CB THg (Models[A])		−1.15 (−3.20, 0.90), 0.272 0.108, < 0.001; 290	−0.43 (−2.75, 1.88), 0.717 0.027, 0.657; 288	−1.02 (−2.63, 0.58), 0.211 0.033, 0.489; 290	**−0.32 (−0.65, −0.01), 0.050** **0.065, 0.008; 290**	−0.05 (−0.39, 0.26), 0.748 0.048, 0.126; 287
	ε2, ε3 carriers	−0.10 (−2.36, 2.16), 0.929 0.109, < 0.001; 240	0.233 (−2.43, 2.90), 0.863 0.030, 0.637; 239	−0.61 (−2.44, 1.22), 0.511 0.037, 0.449; 240	−0.27 (−0.64, 0.10), 0.151 0.057, 0.031; 240	0.01 (−0.35, 0.36), 0.977 0.051, 0.141; 238
	ε4 carriers	−5.55 (−10.7, −0.41), 0.035 0.165, 0.334; 50	−2.58 (−6.85, 1.69), 0.229 0.330, 0.049; 49	−2.55 (−5.99, 0.90), 0.143 0.221, 0.286; 50	−0.51 (−1.16, 0.13), 0.117 0.208, 0.018; 50	−0.25 (−0.88, 0.39), 0.441 0.255, 0.127; 49
ln CB MeHg (Models[B])		−1.35 (−3.15, 0.46), 0.144 0.110, < 0.001; 290	−0.22 (−2.27, 1.82), 0.834 0.027, 0.666; 288	−0.73 (−2.16, 0.69), 0.312 0.031, 0.540; 290	***−0.24 (−0.53, 0.04), 0.098*** ***0.061, 0.013; 290***	−0.04 (−0.31, 0.23), 0.767 0.048, 0.126; 287
	ε2, ε3 carriers	−0.38 (−2.38, 1.61), 0.705 0.109, < 0.001; 240	0.24 (−2.11, 2.58), 0.843 0.030, 0.636; 239	−0.37 (−1.99, 1.26), 0.658 0.036, 0.472; 240	−0.18 (−0.51, 0.14), 0.272 0.054, 0.043; 240	0.002 (−0.31, 0.31), 0.990 0.050, 0.141; 238
	ε4 carriers	−5.33 (−9.78, −0.88), 0.020 0.183, 0.253; 50	−2.01 (−5.87, 1.85), 0.298 0.324, 0.056; 49	−2.33 (−5.50, 0.88), 0.150 0.220, 0.292; 50	−0.42 (−0.99, 0.15), 0.147 0.255, 0.039; 50	−0.21 (−0.78, 0.37), 0.475 0.253, 0.130; 49
Maternal ApoE		**Cognitive**	**Language**	**Motor**	**Fine motor**	**Gross motor**
ln CB THg (Models[C])		−1.03 (−3.24, 1.18), 0.362 0.097, 0.002; 242	0.38 (−1.94, 2.70), 0.748 0.030, 0.724; 241	−0.36 (−2.00, 1.29), 0.669 0.013, 0.978; 244	***−0.29 (−0.63, 0.05), 0.090*** ***0.065, 0.026; 244***	0.18 (−0.14, 0.50), 0.279 0.044, 0.299; 243
	ε2, ε3 carriers	−0.28 (−2.73, 2.18), 0.824 0.086, 0.012; 206	1.00 (−1.43, 3.42), 0.419 0.050, 0.334; 205	−0.08 (−1.89, 1.74), 0.934 0.017, 0.949; 207	−0.25 (−0.63, 0.12), 0.187 0.076, 0.014; 207	0.20 (−0.14, 0.4, 0.249 0.062, 0.116; 206
	ε4 carriers	**−7.09 (−12.3, −1.88), 0.009** **0.407, 0.027; 36**	1.64 (−6.80, 10.1), 0.693 0.497, 0.017; 36	−2.51 (−7.38, 2.35), 0.298 0.165, 0.793; 37	−0.75 (−1.67, 0.17), 0.106 0.145, 0.546; 37	0.92 (−0.29, 2.12), 0.129 0.299, 0.204; 37
	Interaction ε4 x THg	−1.60 (−3.42, 0.21), 0.081 0.321, 0.109; 36	0.51 (−2.30, 3.32), 0.711 0.491, 0.020; 36	−1.03 (−2.59, 0.53), 186 0.191, 0.695; 37	−0.18(-0.49, 0.12), 0.230 0.107, 0.729; 37	0.08 (−0.32, 0.48), 0.682 0.249, 0.356; 37
ln CB MeHg (Models[D])		−1.10 (−3.04, 0.84), 0.266 0.098, 0.002; 242	0.64 (−1.42, 2.71), 0.539 0.031, 0.679; 241	−0.22 (−1.67, 1.22), 0.762 0.013, 0.981; 244	−0.23 (−0.53, 0.07), 0.130 0.062, 0.032; 244	0.17 (−0.11, 0.46), 0.236 0.045, 0.288; 243
	ε2, ε3 carriers	−0.39 (−2.57, 1.79), 0.723 0.087, 0.011; 206	1.05 (−1.12, 3.22), 0.341 0.051, 0.314; 205	−0.06 (−1.67, 1.56), 0.944 0.017, 0.949; 207	−0.21 (−0.55, 0.12), 0.204 0.076, 0.015; 207	0.18 (−0.12, 0.49), 0.244 0.063, 0.115; 206
	ε4 carriers	**−6.06 (−10.3, −1.87), 0.006** **0.422, 0.020; 36**	3.05 (−3.84, 9.95), 0.371 0.510, 0.014; 36	−1.49 (−5.82, 2.84), 0.487 0.146, 0.854; 37	−0.41 (−1.18, 0.35), 0.278 0.102, 0.753; 37	0.92 (−0.07, 1.90), 0.067 0.325, 0.145; 37
	Interaction ε4 x MeHg	***−1.89 (-3.79, 0.02), 0.052*** ***0.326, 0.088; 36***	0.97 (−2.04, 3.98), 0.513 0.497, 0.018; 36	−0.88 (−2.61, 0.85), 0.305 0.170, 0.775; 37	−0.11 (−0.44, 0.23), 0.508 0.076, 0.866; 37	0.09 (−0.35, 0.52), 0.683 0.249, 0.356; 37
Combined ApoE		**Cognitive**	**Language**	**Motor**	**Fine motor**	**Gross motor**
ln CB THg (Models[E])		−0.09 (−2.49, 2.31), 0.943 0.120, < 0.001; 220	0.71 (−1.88, 3.30), 0.588 0.022, 0.915; 219	0.02 (−1.82, 1.86), 0.983 0.013, 0.987; 220	−0.22 (−0.60, 0.16), 0.258 0.073, 0.023; 220	0.21 (−0.15, 0.56), 0.258 0.050, 0.290; 219
	ε2, ε3 carriers	1.34 (−1.68, 4.35), 0.383 0.125, 0.003; 164	2.08 (−1.09, 5.26), 0.196 0.039, 0.708; 163	0.55 (−1.81, 2.92), 0.646 0.012, 0.957; 164	−0.17 (−0.65, 0.30), 0.468 0.073, 0.062; 164	0.30 (−0.15, 0.74), 0.188 0.076, 0.133; 163
	ε4 carriers	−3.51 (−7.39, −0.36), 0.074 0.168, 0.234; 56	−1.50 (−5.58, 2.59), 0.464 0.349, 0.012; 56	−1.14 (−3.84, 1.56), 0.399 0.199, 0.279; 56	−0.40 (−1.00, 0.20), 0.188 0.175, 0.133; 56	0.13 (−0.43, 0.70), 0.630 0.222, 0.131; 56
ln CB MeHg (Models[F])		−0.24 (−2.39, 1.91), 0.824 0.120, < 0.001; 220	0.82 (−1.52, 3.15), 0.491 0.022, 0.904; 219	0.07 (−1.58, 1.72), 0.932 0.013, 0.987; 220	−0.15 (−0.49, 0.19), 0.381 0.071, 0.028; 220	0.17 (−0.16, 0.49), 0.308 0.048, 0.308; 219
	ε2, ε3 carriers	1.07 (−1.64, 3.78), 0.436 0.125, 0.004; 164	1.85 (−1.02, 4.71), 0.204 0.039, 0.714; 163	0.50 (−1.62, 2.63), 0.640 0.020, 0.957; 164	−0.12 (−0.55, 0.30), 0.568 0.072, 0.067; 164	0.24 (−0.17, 0.64), 0.249 0.074, 0.150; 163
	ε4 carriers	−3.48 (−6.92, −0.04), 0.048 0.181, 0.186; 56	−0.87 (−4.58, 2.84), 0.639 0.345, 0.014; 56	−0.94 (−3.45, 1.57), 0.457 0.196, 0.291; 56	−0.31 (−0.85, 0.24), 0.264 0.167, 0.158; 56	0.15 (−0.36, 0.66), 0.547 0.224, 0.125; 56

[a] Models were designed in our previous work (Snoj Tratnik et al., 2017). Models are adjusted for ApoE genotype, ln cord blood Hg levels, country and particular socio-demographic characteristics (mother's age at delivery, child's gender, birth weight, educational level of mother, smoking during pregnancy), ln cord blood plasma Se, and ln cord blood Pb. Models A, C, and E are adjusted for ln THg [ng/g] concentrations. Models B, D, and F are adjusted for ln MeHg [ng/g] concentrations. Models A and B include child's genotype. Models C and D include maternal genotype. Models E and F include child's and maternal genotype where ε4 carriers are mother-child pairs and at least one of them is an ε4 carrier. Interaction ε4xTHg(MeHg) model was adjusted the same as that in the group of ε4 mothers, but instead of cord blood THg/MeHg levels and maternal ApoE ε4 genotype we included maternal ApoE ε4xTHg(MeHg). Bolded are statistically significant associations; bolded in italic are marginally significant associations.

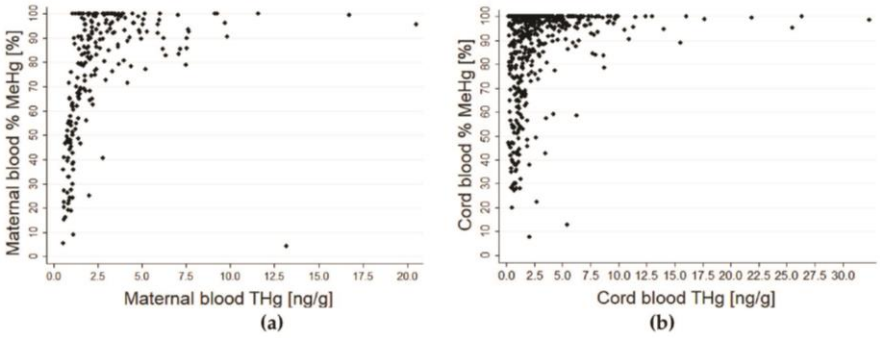

Fig. 1. Correlations between THg and % MeHg in (a) maternal blood (N = 225) and (b) cord blood (N = 633).

respectively. However, association with MeHg showed higher model R^2 (0.422 vs. 0.407) and narrower 95% CI in comparison with THg models. Both models were significant as a whole and had relatively high prediction value (R^2). So we also tested the interaction between maternal presence of ApoE ε4 and cord blood Hg levels (ApoExHg). We observed marginally significant interaction between ApoE ε4 and THg ($p = 0.081$) on child's cognitive score and the model was not significant ($p = 0.109$). However, interaction between ApoE ε4 and MeHg was significant ($p = 0.052$) and the model was marginally significant ($p = 0.088$), suggesting synergistic modifying effect of MeHg and maternal ApoE ε4 allele on decrease in child's cognitive score.

Models E and models F, adjusted for combination of the child's and maternal genotype (where at least one in the mother-child pair is an ε4 carrier), cord blood MeHg levels were significantly associated with decrease in cognitive score in the group of ε4 carriers ($p = 0.048$), compared with marginally significant association with THg ($p = 0.074$). In this case, β-estimates of change were comparable between models, but associations with MeHg had slightly higher model R^2 (0.181 vs. 0.168). However, models are again not significant.

We observed genotype independent Hg-associated modifying effect in the child's fine motor function (decrease inside normal levels). The difference between THg and MeHg suggests that inorganic Hg might as well be associated with the Hg-associated decrease in child's fine motor score, as observed from the small but significant estimates of change. Models with THg (Model A) had lower p-value in comparison with MeHg (Model B; 0.050 and 0.098, respectively).

According to observed statistically significant, or marginally, significant results (bold and bold italic, Table 6) we suppose that with inclusion of MeHg values and maternal genotypes we improved our previously reported models. The main improvement/enhancement was in the case of interaction between maternal ApoE ε4 and MeHg (in comparison with cord blood THg) levels on decrease in child's cognitive score. Interactions between child's fine motor scores and Hg were genotype independent and Hg speciation showed that probably IHg and MeHg contributed to this negative association. Associations between THg or MeHg and other Bayley-III scores (language, motor, and gross motor), generally showed no statistically significant difference regardless of which genotype was included. We can conclude that Hg speciation provides profound information on exposure and is of great importance when studying Hg metabolism and its possible negative effects.

4. Conclusions

The studied population was exposed to low-to-moderate levels of Hg, mainly to MeHg, through seafood intake. In average, MeHg presented 86% of THg in maternal blood and 98% of THg in cord blood. However, we observed substantial individual variations; the proportion of MeHg in maternal blood was 4%–100% (CV = 32%) and that in cord blood was 8%–100% (CV = 20%). Our data shows that at lower blood THg concentrations the variability of % MeHg was higher. By comparing results of multiple linear regression adjusted for ApoE genotypes, we observed better prediction values when studying associations between the child's cognitive score and cord blood MeHg levels in comparison with cord blood THg levels.

Although our study suffers from incomplete data in questionnaires, we confirmed that Hg speciation significantly improves interpretation of association between exposure and health outcomes. Therefore, we strongly recommend that Hg speciation is included in future studies. Today, methodologies to accurately speciate Hg in human samples are available and cost-effective, and no-longer represent barriers for improved study protocols.

Author contributions

A.T. wrote the paper and performed all MeHg measurements. J.S.T. performed post-partum sampling for the Slovenian population, THg measurements, statistical analysis, and helped with data interpretation. D.M. performed post-partum sampling for the Slovenian population. V.F. performed THg measurements. M.K. was responsible for the recruitment of the study population and sampling. J.O. contributed to the study design and protocols. I.P., Z.Š., and O.P. contributed to the study design and sampling in Croatia. J.M. was responsible for DNA isolations and ApoE genotyping. D.N. and JK performed and supervised neurodevelopmental tests on children. I.F., A.B.K. and F.B. contributed to the study design, protocols, and I.F. contributed to the interpretation of the data. M.H. contributed to the study design and protocols, supervised all Hg measurements, and interpreted the data.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by the EU through the 6th FP, FOOD-CT-2006-016253, PHIME project and 7th FP no. 603946, HEALS project, CROME-LIFE + project and a programme funded by the Slovenian Research Agency (ARRS) P1-0143 and project NEURODYS J7-9400.

The authors are grateful to all pregnant women and their children who participated in this study; personnel of the "Jožef Stefan" Institute, University Medical Centre Ljubljana, Slovenia, and University Hospital

Centre Rijeka, Croatia, who collected biological samples and collaborated in conducting the interviews with the pregnant women; and psychology students and psychologists of the University Medical Centre Ljubljana and University Hospital Centre Rijeka who conducted neurodevelopmental tests on children.

References

ATSDR, 1999. Toxicological Profile for Mercury. U.S. Department of Health and Human Services [WWW Document]. https://www.atsdr.cdc.gov/toxprofiles/tp46.pdf.

Bakir, F., Rustam, H., Tikriti, S., Al-Damluji, S.F., Shihristani, H., 1980. Clinical and epidemiological aspects of methylmercury poisoning. Postgrad. Med. J. 56, 1–10.

Barbone, F., Rosolen, V., Mariuz, M., Parpinel, M., Casetta, A., Sammartano, F., Ronfani, L., Vecchi, L., Bin, M., Castriotta, L., Valent, F., Latesha, D.A., Mazej, D., Snoj Tratnik, J., Miklavčič, A., So, K., Špirič, Z., Krsnik, M., Neubauer, D., Kodrič, J., Prpič, I., Petrovič, O., Vlašič, I., Horvat, M., 2019. Prenatal mercury exposure and child neurodevelopment outcomes at 18 months: results from the Mediterranean PHIME cohort. Int. J. Hyg Environ. Health 222, 9–21. https://doi.org/10.1016/j.ijheh.2018.07.011.

Basu, N., Horvat, M., Evers, D.C., Zastenskaya, I., Weihe, P., Tempowski, J., 2018. A state-of-the-science review of mercury biomarkers in human populations worldwide between 2000 and 2018. Environ. Health Perspect. 126, 1–14. https://doi.org/10.1289/EHP3904.

Bayley, N., 2006. Bayley Scales of Infant and Toddler Development, third ed. Technical manual, San Antonio, Tx (Harcourt).

Bellanger, M., Pichery, C., Aerts, D., Berglund, M., Castaño, A., Čejchanova, M., Crettaz, P., Davidson, F., Esteban, M., Fischer, M.E., Gurzau, A.E., Halzlova, K., Katsonouri, A., Knudsen, L.E., Kolossa-Gehring, M., Koppen, G., Ligocka, D., Miklavčič, A., Fátima Reis, M., Rudnai, P., Snoj Tratnik, J., Weihe, P., Budtz-Jørgensen, E., Grandjean, P., 2013. Economic benefits of methylmercury exposure control in Europe: monetary value of neurotoxicity prevention. Environ. Health 12. https://doi.org/10.1186/1476-069X-12-3.

Berglund, M., Lind, B., Björnberg, K.A., Palm, B., Einarsson, Ö., Vahter, M., 2005. Inter-individual variations of human mercury exposure biomarkers: a cross-sectional assessment. Environ. Health 4. https://doi.org/10.1186/1476-069X-4-20.

Berlin, M., Zalups, R.K., Fowler, B.A., 2015. Mercury. In: Nordberg, G.F., Fowler, B.A., Nordberg, M. (Eds.), Handbook on the Toxicology of Metals. Volume II, Specific Metals. Academic Press, London, UK, pp. 1013–1075.

Buttini, M., Orth, M., Bellosta, S., Akeefe, H., Pitas, R.E., Wyss-Coray, T., Mucke, L., Mahley, R.W., 1999. Expression of human apolipoprotein E3 or E4 in the brains of ApoE -/- Mice: isoform-specific effects on neurodegeneration. J. Neurosci. 19, 4867–4880. https://doi.org/10.1523/JNEUROSCI.19-12-04867.1999.

Dock, L., Rissanen, R.L., Vahter, M., 1994. Demethylation and placental transfer of methyl mercury in the pregnant hamster. Toxicology 94, 131–142. https://doi.org/10.1016/0300-483X(94)90033-7.

Donohue, A., Wagner, C.L., Burch, J.B., Rothenberg, S.E., 2018. Blood total mercury and methylmercury among pregnant mothers in Charleston, South Carolina, USA. J. Expo. Sci. Environ. Epidemiol. 28, 494–504. https://doi.org/10.1038/s41370-018-0033-1.

Elhassani, S.M., 1982. The many faces of methylmercury poisoning. J. Toxicol. Clin. Toxicol. 19, 875–906. https://doi.org/10.3109/15563658208992523.

EPA Method 7473, 1998. Mercury in solids and solutions by thermal decomposition, amalgamation, and atomic absorption spectrophotometry. [WWW Document]. URL. https://www.epa.gov/homeland-security-research/epa-method-7473-sw-846-mercury-solids-and-solutions-thermal-decomposition.

Grandjean, P., Budtz-Jørgensen, E., 2007. Total imprecision of exposure biomarkers: implications for calculating exposure limits. Am. J. Ind. Med. 50, 712–719. https://doi.org/10.1002/ajim.20474.Total.

Grandjean, P., Herz, K.T., 2011. Brain development and methylmercury: underestimation of neurotoxicity. Mt. Sinai J. Med. 78, 107–118. https://doi.org/10.1002/msj.20228.BRAIN.

Grandjean, P., Satoh, H., Murata, K., Eto, K., 2010. Adverse effects of methylmercury: environmental health research implications. Environ. Health Perspect. 118, 1137–1145. https://doi.org/10.1289/ehp.0901757.

Gundacker, C., Fröhlich, S., Graf-Rohrmeister, K., Eibenberger, B., Jessenig, V., Gicic, D., Prinz, S., Wittmann, K.J., Zeisler, H., Vallant, B., Pollak, A., Husslein, P., 2010. Perinatal lead and mercury exposure in Austria. Sci. Total Environ. 408, 5744–5749. https://doi.org/10.1016/j.scitotenv.2010.07.079.

Ha, E., Basu, N., Bose-O'Reilly, S., Dórea, J.G., McSorley, E., Sakamoto, M., Chan, H.M., 2017. Current progress on understanding the impact of mercury on human health. Environ. Res. 152, 419–433. https://doi.org/10.1016/j.envres.2016.06.042.

Hammerschmidt, C.R., Finiguerra, M.B., Weller, R.L., Fitzgerald, W.F., 2013. Methylmercury accumulation in plankton on the continental margin of the Northwest Atlantic Ocean. Environ. Sci. Technol. 47, 3671–3677.

Harada, M., 1995. Minamata Disease: methylmercury poisoning in Japan caused by environmental pollution. Crit. Rev. Toxicol. 25, 1–24. https://doi.org/10.3109/10408449509089885.

Hong, Y.-S., Kim, Y.-M., Lee, K.-E., 2012. Methylmercury exposure and health effects. J. Prev. Med. Pub. Health 45, 353–363. https://doi.org/10.3961/jpmph.2012.45.6.353.

Horvat, M., Byrne, A.R., 1990. A modified method for the determination of methylmercury by gas chromatography. Talanta 37, 207–212. https://doi.org/10.1016/0039-9140(90)80024-A.

Horvat, M., May, K., Stoeppler, M., Byrne, A.R., 1988. Comparative studies of methylmercury determination in biological and environmental samples. Appl. Organomet. Chem. 2, 515–524. https://doi.org/10.1002/aoc.590020604.

Horvat, M., Snoj Tratnik, J., Miklavčič, A., 2012. Mercury: biomarkers of exposure and human biomonitoring. In: Knudsen, L.E., Merlo, D.F. (Eds.), Biomarkers and Human Biomonitoring. Volume 1: Ongoing Programs and Exposures. The Royal Society of Chemistry, Cambridge, UK, pp. 381–417.

Karagas, M.R., Choi, A.L., Oken, E., Horvat, M., Schoeny, R., Kamai, E., Cowell, W., Grandjean, P., Korrick, S., 2012. Evidence on the human health effects of low-level methylmercury exposure. Environ. Health Perspect. 120, 799–806. https://doi.org/10.1289/ehp.1104494.

Kozikowska, I., Binkowski, J.Ł., Szczepanska, K., Sławska, H., Miszczuk, K., Sliwinska, M., Łaciak, T., Stawarz, R., 2013. Mercury concentrations in human placenta, umbilical cord, cord blood and amniotic fluid and their relations with body parameters of newborns. Environ. Pollut. 182, 256–262. https://doi.org/10.1016/j.envpol.2013.07.030.

Liang, L., Bloom, N.S., Horvat, M., 1994. Simultaneous determination of mercury speciation in biological materials by GC/CVAFS after ethylation and room-temperature precollection. Clin. Chem. 40, 602–607.

Mahaffey, K.R., Clickner, R.P., Bodurow, C.C., 2004. Blood organic mercury and dietary mercury intake: national health and nutrition examination survey, 1999 and 2000. Environ. Health Perspect. 112, 562–570. https://doi.org/10.1289/ehp.6587.

Mahaffey, K.R., Clickner, R.P., Jeffries, R.A., 2009. Adult women's blood mercury concentrations cary regionally in the United States : association with patterns of fish consumption. Environ. Health Perspect. 117, 47–53. https://doi.org/10.1289/ehp.11674.

Miklavčič, A., Casetta, A., Snoj Tratnik, J., Mazej, D., Krsnik, M., Mariuz, M., Sofianou, K., Špirić, Z., Barbone, F., Horvat, M., 2013. Mercury, arsenic and selenium exposure levels in relation to fish consumption in the Mediterranean area. Environ. Res. 120, 7–17. https://doi.org/10.1016/j.envres.2012.08.010.

Miklavčič, A., Cuderman, P., Mazej, D., Snoj Tratnik, J., Krsnik, M., Planinšek, P., Osredkar, J., Horvat, M., 2011. Biomarkers of low-level mercury exposure through fish consumption in pregnant and lactating Slovenian women. Environ. Res. 111, 1201–1207. https://doi.org/10.1016/j.envres.2011.07.006.

National Research Council, 2000. Toxicological Effects of Methylmercury. National Academy Press, Washington.

Ng, S., Lin, C.-C., Hwang, Y.-H., Hsieh, W.-S., Liao, H.-F., Chen, P.-C., 2013. Mercury, APOE, and children's neurodevelopment. NeuroToxicology 37, 85–92. https://doi.org/10.1016/j.neuro.2013.03.012.

Ng, S., Lin, C.-C., Jeng, S.-F., Hwang, Y.-H., Hsieh, W.-S., Chen, P.-C., 2015. Mercury, APOE, and child behavior. Chemosphere 120, 123–130. https://doi.org/10.1016/j.chemosphere.2014.06.003.

Prpić, I., Milardović, A., Vlašić-Cicvarić, I., Špirić, Z., Radić Ništević, J., Vukelić, P., Snoj Tratnik, J., Mazej, D., Horvat, M., 2017. Prenatal exposure to low-level methylmercury alters the child's fine motor skills at the age of 18 months. Environ. Res. 152, 369–374. https://doi.org/10.1016/j.envres.2016.10.011.

Rappaport, S.M., 2011. Implications of the exposome for exposure science. J. Expo. Sci. Environ. Epidemiol. 21, 5–9. https://doi.org/10.1038/jes.2010.50.

Rice, D.C., 2004. The US EPA reference dose for methylmercury: sources of uncertainty. Environ. Res. 95, 406–413. https://doi.org/10.1016/j.envres.2003.08.013.

Rothenberg, S.E., Korrick, S.A., Fayad, R., 2015. The influence of obesity on blood mercury levels for U.S. non-pregnant adults and children: NHANES 2007-2010. Environ. Res. 138, 173–180. https://doi.org/10.1016/j.envres.2015.01.018. (THE).

Sakamoto, M., Murata, K., Domingo, J.L., Yamamoto, M., Oliveira, R.B., Kawakami, S., Nakamura, M., 2016. Implications of mercury concentrations in umbilical cord tissue in relation to maternal hair segments as biomarkers for prenatal exposure to methylmercury. Environ. Res. 149, 282–287. https://doi.org/10.1016/j.envres.2016.04.023.

Sakamoto, M., Tatsuta, N., Izumo, K., Phan, P.T., Vu, L.D., Yamamoto, M., Nakamura, M., Nakai, K., Murata, K., 2018. Health impacts and biomarkers of prenatal exposure to methylmercury: lessons from Minamata, Japan. Toxics 6. https://doi.org/10.3390/toxics6030045.

Schulz, C., Wilhelm, M., Heudorf, U., Kolossa-Gehring, M., 2011. Update of the reference and HBM values derived by the German human biomonitoring commission. Int. J. Hyg Environ. Health 215, 26–35. https://doi.org/10.1016/j.ijheh.2011.06.007.

Sheehan, M.C., Burke, T.A., Navas-Acien, A., Breysse, P.N., McGready, J., Fox, M.A., 2014. Global methylmercury exposure from seafood consumption and risk of developmental neurotoxicity: a systematic review. Bull. World Health Organ. 92, 254–269. https://dx.doi.org/10.2471/BLT.12.116152.

Snoj Tratnik, J., Falnoga, I., Trdin, A., Mazej, D., Fajon, V., Miklavčič, A., Kobal, A.B., Sešek Briški, A., Krsnik, M., Neubauer, D., Kodrič, J., Stropnik, S., Gosar, D., Lešnik Musek, P., Marc, J., Jurkovič Mlakar, S., Petrović, O., Vlašič Cicvarić, I., Prpič, I., Milardović, A., Radić Ništević, J., Vuković, D., Fišić, E., Špirić, Z., Horvat, M., 2017. Prenatal mercury exposure, neurodevelopment and apolipoprotein E genetic polymorphism. Environ. Res. 152, 375–385. https://doi.org/10.1016/j.envres.2016.08.035.

Taylor, C.M., Golding, J., Emond, A.M., 2016. Blood mercury levels and fish consumption in pregnancy: risks and benefits for birth outcomes in a prospective observational birth cohort. Int. J. Hyg Environ. Health 219, 513–520. https://doi.org/10.1016/j.ijheh.2016.05.004.

Trdin, A., 2015. Connection between Mutation in the Gene for Apolipoprotein E with Concentrations of Mercury in the Mothers and Newborns (Master's Thesis).

Tuminello, E.R., Han, S.D., 2011. The apolipoprotein E antagonistic pleiotropy hypothesis: review and recommendations. Int. J. Alzheimer's Dis. https://doi.org/10.4061/2011/726197. 2011.

UNEP/AMAP, 2013. Technical Background Report for the Global Mercury Assessment

2013. Arctic Monitoring and Assessment Programme. Norway/UNEP Chemicals Branch, Geneva, Switzerland Oslo.
US EPA, 1997. Mercury Study. Report to Congress, vol. I Executive Summary.
Vahter, M., Akesson, A., Lind, B., Bjors, U., Schutz, A., Berglund, M., 2000. Longitudinal study of methylmercury and inorganic mercury in blood and urine of pregnant and lactating women, as well as in umbilical cord blood. Environ. Res. 84, 186–194. https://doi.org/10.1006/enrs.2000.4098.
Valent, F., Horvat, M., Sofianou-Katsoulis, A., Spiric, Z., Mazej, D., Little, D., Prasouli, A., Mariuz, M., Tamburlini, G., Nakou, S., Barbone, F., 2013a. Neurodevelopmental effects of low-level prenatal mercury exposure from maternal fish consumption in a mediterranean cohort: study rationale and design. J. Epidemiol. 23, 146–152. https://doi.org/10.2188/jea.JE20120030.
Valent, F., Mariuz, M., Bin, M., Little, D., Mazej, D., Tognin, V., Tratnik, J., McAfee, A.J., Mulhern, M.S., Parpinel, M., Carrozzi, M., Horvat, M., Tamburlini, G., Barbone, F., 2013b. Associations of prenatal mercury exposure from maternal fish consumption and polyunsaturated fatty acids with child neurodevelopment: a prospective cohort study in Italy. J. Epidemiol. 23, 360–370. https://doi.org/10.2188/jea.JE20120168.
Vejrup, K., Eek, R., Lise, A., Katrine, H., Henriette, I., Alexander, J., Lundh, T., Margrete, H., Magnus, P., Haugen, M., 2018. Prenatal mercury exposure, maternal seafood consumption and associations with child language at five years. Environ. Int. 110, 71–79. https://doi.org/10.1016/j.envint.2017.10.008.
Wells, E.M., Herbstman, J.B., Lin, Y.H., Hibbeln, J., Halden, R.U., Witter, F.R., Goldman, L.R., 2017. Methyl mercury, but not inorganic mercury, associated with higher blood pressure during pregnancy. Environ. Res. 154, 247–252. https://doi.org/10.1016/j.envres.2017.01.013. (Methyl).
Wells, E.M., Herbstman, J.B., Lin, Y.H., Jarrett, J., Verdon, C.P., Ward, C., Caldwell, K.L., Hibbeln, J.R., Witter, F.R., Halden, R.U., Goldman, L.R., 2016. Cord blood methylmercury and fetal growth outcomes in Baltimore newborns: potential confounding and effect modification by omega-3 fatty acids, selenium, and sex. Environ. Health Perspect. 124, 373–379. https://doi.org/10.1289/ehp.1408596.
WHO, 2007. Health risks of heavy metals from long-range transboundary air polution. Joint WHO Convention Task Force on Health Aspects of Air Pollution. pp. 85–127.
Wright, R.O., Hu, H., Silverman, E.K., Tsaih, S.W., Schwartz, J., Bellinger, D., Palazuelos, E., Weiss, S.T., Hernandez-Avila, M., 2003. Apolipoprotein E genotype predicts 24-month Bayley scales infant development score. Pediatr. Res. 54, 819–825. https://doi.org/10.1203/01.PDR.0000090927.53818. (DE).

3.2 Mercury Speciation in Meconium and Associated Factors

Trdin, A., Falnoga, I., Fajon, V., Živković, I., Snoj Tratnik, J., Prpić, I., Špirić, Z., Horvat, M. (2019). *Environmental Research, 179*. https://doi.org/10.1016/j.envres.2019.108724

In the case of prenatal low-level and long-term exposure to pollutants, meconium analysis was shown to be more sensitive compared with blood, as meconium is a cumulative and repository matrix. Moreover, meconium is a foetal excretory product and therefore presents a direct measure of long-term prenatal exposure in comparison with maternal blood or hair, which are indirect measures of exposure. Few epidemiological studies have used meconium as a biomarker of prenatal Hg exposure. Additionally, Hg toxicokinetics and metabolic mechanisms during prenatal life are not fully understood. With Hg speciation in meconium, the authors aimed to assess possible Hg exposure sources and prenatal toxicokinetics or biotransformation mechanisms. Analyses were conducted on the same population as blood Hg speciation was performed previously (described in Section 3.1).

Maternal blood and hair were sampled during the 3rd trimester and meconium was sampled in the first few days after birth. Sociodemographic data, personal characteristics and habits, and other possible Hg exposure sources (maternal seafood intake, number of dental amalgams) were assessed with questionnaires. Maternal blood was collected only in Croatian women, whereas maternal hair and meconium were available for both populations. Statistical analysis included both populations, except for linear regressions modelling, where only the Slovenian population was included, because only in this case the subset database consisted of complete information about possible sources of Hg exposure (seafood intake, dental amalgams).

The authors presented the levels of different Hg species in meconium. It was shown that this is the first study that performed Hg speciation on a large sample size (N = 488). Median THg concentration in meconium was 11.1 (0.41-375.2) ng/g and Hg(II) presented from 82%-100% of measured THg. MeHg from maternal blood was correlated with MeHg in meconium, whereas Hg(II) from maternal blood was not significantly correlated with Hg(II) in meconium. The authors observed a significant correlation between Hg levels in meconium and Hg levels in maternal hair, with the highest correlation between hair THg and meconium MeHg. Maternal seafood intake was significantly correlated with meconium MeHg and Hg(II), but this was not confirmed with linear regression. Maternal seafood intake was positively associated only with meconium MeHg levels, but not with Hg(II). The number of dental amalgams were positively associated with meconium Hg(II).

Although MeHg demethylation is a crucial step in the excretion of MeHg from an adult body, based on the presented results, the authors could neither confirm nor exclude the hypothesis of intestinal MeHg demethylation during prenatal life. They assume it is more likely that MeHg transported through placental barrier is distributed into foetal tissues and only a small part is excreted in meconium, without any biotransformation. They suggested that high Hg(II) content in the meconium results from the transport of Hg^0 vapour from maternal dental amalgam fillings through the placental barrier. The authors concluded that meconium is a suitable biomarker of prenatal MeHg and Hg^0 exposure, under the condition that Hg speciation analysis in performed.

The candidate's contribution to this paper included measurements of THg and MeHg in the samples of meconium, and statistical analysis. She summarized the figures and tables, and prepared the manuscript in collaboration with other co-authors.

Environmental Research 179 (2019) 108724

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Mercury speciation in meconium and associated factors

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ARTICLE INFO

Keywords:
Meconium
Mercury speciation
Prenatal exposure
Biomarker of exposure

ABSTRACT

Meconium is formed early in gestation and it is normally not excreted until after birth. Thus it may provide a longer and cumulative record of exposure to mercury (Hg). The present study aims to speciate Hg in meconium samples (N = 488) from Slovenian and Croatian new-borns prenatally exposed to low levels of methyl-Hg (MeHg) from maternal seafood intake and to Hg^0 from maternal dental amalgam fillings. We had complete data of total Hg (THg) and MeHg in meconium and THg in maternal hair (MH), while THg and MeHg in maternal blood (MB) were available only for Croatian mothers. Personal data namely maternal seafood intake, age, pre-pregnancy BMI, parity, smoking, estimated gestational age at birth, sex, and birth weight were available for the majority of participants, except the number of dental amalgams which was in most cases missing for Croatian mothers. The median THg concentration in meconium was 11.1 (range: 0.41–375.2) ng/g and inorganic Hg (Hg (II)) presented 98.8% (range: 82%–100%, CV: 2%) of THg. We observed significant correlation between meconium and MH Hg levels, with the highest correlation between hair THg and meconium MeHg. Correlation analysis including MB (available only for Croatian population) showed a significant positive correlation between THg in meconium and THg in MB (R_s = 0.642). Additionally, MeHg from MB was correlated with MeHg in meconium (R_s = 0.898), while the correlation between Hg(II) in MB and meconium was positive, but not significant. Maternal seafood intake was significantly correlated with meconium MeHg (R_s = 0.498) and Hg(II) (R_s = 0.201). Multiple linear regression (performed on the Slovenian population, N = 143) confirmed a positive association between meconium MeHg and seafood intake. Furthermore, meconium Hg(II) was positively associated with the number of maternal dental amalgam fillings, but linear regression models did not confirm correlation between seafood intake and meconium Hg(II) levels. We assume that Hg^0 released from maternal dental amalgam fillings and MeHg from seafood intake were both transported through the placental barrier and portioned between different foetal compartments including meconium. Weak correlation between maternal seafood intake and Hg(II) levels in meconium suggests that there is certain evidence of MeHg demethylation. However, because this correlation was not confirmed by the multiple regression, MeHg demethylation during prenatal life cannot be neither confirmed nor excluded. Further investigations at higher level of exposure are needed to confirm this observations. We can conclude that meconium is a suitable biomarker for MeHg and Hg^0 exposure during pregnancy. However, comparability of the results reported in meconium in different studies is hindered by a lack of standardized sampling protocols, storage, and analysis.

1. Introduction

Meconium is formed early in gestation, presumably from the 13th week of gestation, and it is normally not excreted until after birth, usually in the first 72 h in amount of 20–70 g (Bearer, 2003; Concheiro and Huestis, 2018). It is a dark viscous material that mostly consists of eliminated mucosa epithelia, water, bile, bile acids, epithelium cells, and other lipids swallowed with the amniotic fluid (Concheiro and Huestis, 2018). The total weight of meconium increases exponentially with gestational age; about 1 g of meconium accumulated in the foetal gut during 23–26 weeks of gestation and about 5 g after 27–32 weeks. According to recent data ~80% of meconium accumulates after 38 weeks of pregnancy (Bakdash et al., 2010; Concheiro and Huestis, 2018). Accordingly, meconium analysis provides an overview of the

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https://doi.org/10.1016/j.envres.2019.108724
Received 11 June 2019; Received in revised form 21 August 2019; Accepted 4 September 2019
Available online 20 September 2019
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exposure primarily during the last trimester of pregnancy. In the case of prenatal low-level and long-term exposure to various pollutants, meconium analysis was shown to be more sensitive compared with blood as meconium is cumulative and repository matrix (Park and Lee, 2014). Furthermore, meconium represents foetal tissue and is therefore a direct measure of exposure, by contrast to maternal blood (MB) or hair (MH), which provide only indirect estimations of exposure (Ostrea et al., 2006).

Few studies have reported mercury (Hg) levels in meconium (Gundacker et al., 2010; Jiang et al., 2014; Knezović et al., 2016; Ostrea et al., 2002; Peng et al., 2015; Ramirez et al., 2003; Rothenberg et al., 2019; Unuvar et al., 2007). Nonetheless, Hg concentrations in meconium are known to generally be higher than those in MB (Ramirez et al., 2000). Except the recent one (Rothenberg et al., 2019) none of the studies so far performed Hg speciation. On a small sample size (N = 14) Rothenberg et al. reported 99%–100% of meconium Hg being in inorganic (Hg(II)) form (Rothenberg et al., 2019). Based on listed studies we cannot conclude what are Hg kinetics and biotransformation mechanisms in meconium during prenatal life. The general population is usually exposed to low levels of different Hg species, including organic Hg from seafood intake (mostly as monomethyl-Hg, CH_3Hg^+, MeHg), elemental Hg (Hg^0) vapour from dental amalgam fillings, and, potentially, inorganic Hg (Hg(II)) from cosmetics and food. Placental transport of Hg^0 vapour released from dental amalgam and inhaled from the atmosphere has already been confirmed before (Clarkson et al., 1972; Yoshida, 2002). Further, MeHg readily penetrates placental and blood-brain barriers (Kajiwara et al., 1996) where it can enter foetal circulation. However, the exact MeHg dynamics (including individual variations) remain unclear (Caito et al., 2018). At the same time, it was long believed that Hg(II) is accumulated in the placenta and therefore limits the amount of Hg reaching the foetus (Al-Saleh et al., 2011). However, researchers showed that in rats, although having slightly different Hg kinetics than humans, Hg(II) can also pass the placental barrier in small amounts (Oliveira et al., 2015). Hg transport from MB to foetal tissues is further supported by some studies that reported positive correlations between meconium total Hg (THg) levels with fish consumption during pregnancy (Jiang et al., 2010; Knezović et al., 2016) or with dental amalgams (Gundacker et al., 2010); however, all studies do not concur on this point (Unuvar et al., 2007). Because meconium is not usually used in studies assessing prenatal Hg exposure, little is known about the possible kinetic mechanisms of different Hg species in meconium.

The present study aims to speciate Hg in meconium samples (N = 488) from Slovenian and Croatian new-borns prenatally exposed to low levels of MeHg from maternal seafood intake and to Hg^0 from maternal dental amalgam fillings. We used existing datasets which contained information on participants' lifestyle and personal characteristics and THg and MeHg concentrations in MH and MB (Miklavčič et al., 2013, 2011; Trdin et al., 2019). In this study, we performed Hg speciation in meconium and evaluated the associations between Hg species in meconium with Hg concentrations in MH and MB as well as with specific participants' characteristics and habits that could contribute to Hg levels in meconium.

2. Materials and methods

2.1. Study population and sampling

Our study population included healthy pregnant women and their new-borns from Slovenia (the city of Ljubljana and its surroundings up to 50 km) and from the Adriatic coastal region of Croatia (the city of Rijeka and its county Primorsko-goranska). They were recruited in 2007–2009 as a part of the PHIME (Public Health Impact of Long-term Low-level Mixed Element Exposure in Susceptible population Strata; EU 6th Framework Programme) birth cohort study. The study was conducted in accordance with the Declaration of Helsinki; the Republic of Slovenia National Medical Ethics Committee approved the protocol (No. 98/05/06) for the Slovenian participants and the Ethics Committee of University Hospital Centre Rijeka approved the protocol (No. 2170-29-02/1-07-1) for the Croatian participants. All subjects gave their informed consent.

Valent et al. (2013b) and Miklavčič et al. (2013) described the study design, recruitment, sampling, and questionnaires in detail. MH samples were stored at room temperature in a zip-lock plastic bag and then analysed without any cleaning. MB and meconium were stored in a freezer at temperature below −24 °C. Unfortunately, not all women who gave their informed consent answered precisely to all questions from questionnaires and not all of them were sampled for listed matrices. Therefore, the number of included participants in statistical analysis varies throughout the text; precise number of included participants have been provided in the tables and figures.

Pregnant women were recruited and sampled in the 3rd trimester (28–41 weeks of gestation). MB was available only for the Croatian part of the population, but maternal hair was available for both populations. In most cases meconium was sampled on the day of birth. However, there were cases where meconium was sampled in the first few days (≤3 days) after birth. Unfortunately, the information when meconium was sampled was not available for all of the collected samples. However, in all cases meconium was sampled (5–10 g) from the diaper and was frozen (below −24 °C) until analysis were carried out.

2.2. Data collected with questionnaires

During 3rd trimester women filled out short questionnaire for assessment of their individual characteristics. Approximately one month after delivery, women filled out the long questionnaire which provided more detailed information about the socio-demographic and health status of the whole family, smoking habits, food intake frequency, number of dental amalgams, etc. Women reported smoking before or during pregnancy, and we generated the new variable of 'ever-smokers' to include all smokers regardless of the period of smoking. Seafood intake during pregnancy was estimated from the long questionnaire. This questionnaire included seven questions on seafood that addressed the frequency of consumption of 150-g servings of fish, crustaceans, and molluscs and tuna, mackerel, or sardines in oil. We converted and combined all listed categories to estimate daily seafood intake (Miklavčič et al., 2013; Trdin et al., 2019; Valent et al., 2013a). To assess the number of dental amalgams, women were divided into four classes (< 3 amalgams, 3–5 amalgams, 6–9 amalgams, 10 + amalgams). Information on dental amalgam fillings was incomplete, especially for the Croatian part of the population. Women also reported the number of births with gestational age of at least 24 weeks regardless of whether the babies were born alive or stillborn. According to their answer, we divided them into two groups: nulliparous and parous women.

2.3. Mercury measurements methods

All analyses of THg and MeHg in biological samples were performed at the Jožef Stefan Institute, Ljubljana, Slovenia. Total mercury in MH, MB, and meconium of Croatian population was determined using a Direct Mercury Analyser (DMA; Milestone, USA). The method is described in detail elsewhere (EPA Method 7473, 1998; Miklavčič et al., 2011). Meconium samples of the Slovenian population were digested with the addition of 2 mL of a mixture of 65% HNO_3 + 70% $HClO_4$ (1:1, v/v) and 5 mL of 96% H_2SO_4. Samples were heated to 220 °C for 20 min. For determining THg in the digested samples, a semi-automated Mercury Analyser based on cold vapour atomic absorption spectrometry (CVAAS, Model Hg-201; Sanso Seisakusho Co. Ltd., Japan) was used. The method is described in detail elsewhere (Akagi, 1997). All measurements were performed under strict quality control procedures and gave comparable results (Miklavčič et al., 2011; Trdin et al., 2019).

Table 1
Basic demographic characteristics of studied population.

Participant's characteristics	Slovenia (N = 287)	Croatia (N = 201)	All (N = 488)	p-value
Seafood intake (meals per day)	0.30 (0–4.5), N = 225	0.44 (0–2.21), N = 183	0.37 (0–4.5), N = 408	0.000[b]
Dental amalgam (% of < 3 amalgams/3–5 amalgams/6–9 amalgams/≥10 + amalgams)	7/34/40/19, N = 212	–	–	–
Ever smoking (% of smokers)	35, N = 98	42, N = 85	38, N = 183	0.002[c]
Pre-pregnancy BMI (kg/m^2)	24.3 (17.1–44.5), N = 277	22.9 (16.8–40.7), N = 200	23.7 (18.8–44.5), N = 477	0.001[b]
Maternal age (years)	30.6 (20–45), N = 278	30.0 (19–43), N = 190	30.4 (19–45), N = 468	0.285[d]
Parity (% of nulliparous)	49, N = 132	44, N = 88	47, N = 220	0.080[c]
Child's sex (% of boys/girls)	53/47	50/50	52/48	0.728[c]
Gestational age (weeks)	39.5 (28–42), N = 224	39.3 (34–41), N = 154	39.4 (28–42), N = 378	0.302[b]
Birth weight (kg)	3.4 (2.0–4.8), N = 224	3.5 (2.4–4.8), N = 180	3.5 (2.0–4.8), N = 404	0.037[a]

[a] Mann Whitney U.
[b] Kruskal-Wallis.
[c] χ^2.
[d] ANOVA.

To check the accuracy of the meconium measurement method, we used the NIST SRM 2781 Domestic sludge. The assigned value (3.68 ± 0.14 μg/g) was in good agreement with the determined value (3.62 ± 0.08 μg/g, k = 2; n = 12).

Unfortunately, in some cases there was not enough sample to perform both measurements for THg and MeHg. Therefore, MeHg in meconium was measured in samples that were available. For this purpose, the samples were first homogenised and then ~0.2 g was weighed into glass tubes. Then, 10 mL of 4 M HNO_3 was added and the mixture was heated at 72 °C for 24 h (Hammerschmidt et al., 2013). After cooling, the digested samples were diluted to 30 mL with MQ water. An aliquot of the digested sample was transferred to measuring glass vials, and neutralised with 0.7 mL of 13 M KOH and the pH was adjusted to 4.6 with the addition of 1 mL of citrate buffer. Then, 50 μL of ethylating reagent (1% $NaBEt_4$ in 1% KOH) (Liang et al., 1994) was added to the measuring vials. The vials were capped and placed into the Tekran 2700 automated system with an auto sampler. Ethylated MeHg as ethylmethylmercury was purged onto a Tenax trap and thermally desorbed (180 °C) onto an isothermal gas chromatography (GC) column. Hg species were converted to Hg^0 by pyrolysis at 600 °C and measured by cold vapour atomic fluorescence detector (CVAFS, Tekran Instruments Corporation, Canada). To check the accuracy of MeHg measurements, we analysed lyophilised whole human blood PT-WB1 obtained from a non-exposed population as a reference material. MeHg in PT-WB1 was determined in the PHIME project through interlaboratory comparisons. The assigned value (5.8 ± 0.5 μg/kg) was in good agreement with the determined value (5.76 ± 0.25 μg/kg, k = 2; n = 12).

Total Hg and MeHg concentrations were determined on a wet weight basis because we observed some MeHg degradation during lyophilisation. The average H_2O content calculated on a subsample (N = 10) was 75.8 ± 2%. Inorganic mercury levels (presumably the majority was in the form of Hg(II)) were calculated by subtracting MeHg from THg.

The estimated measured uncertainty for Hg determination in meconium was 9.8% (k = 2) for THg measurements, 11.4% (k = 2) for MeHg measurements, and 15.0% (k = 2) for Hg(II) levels. The limits of detection (LODs) calculated as three times the standard deviation of the blank sample were 0.11 ng/g for THg and 0.02 ng/g for MeHg measurements. The limits of quantifications (LOQs) calculated as ten times the standard deviation of the blank sample were 0.36 ng/g for THg and 0.05 ng/g for MeHg.

2.4. Statistical analysis

Principal component analysis (PCA) was performed to summarise the main patterns of variations between Hg in meconium, seafood intake, and number of dental amalgams as active variables and personal characteristics and habits as supplementary variables (Spearman rank correlation with Varimax rotation; N = 100). PCA was performed using the XLSTAT statistical package.

Further statistical analysis were performed using STATA 12 software. The distribution of measured Hg species in samples was checked using the Shapiro-Wilk test. Hg concentrations in all measured samples were not normally distributed. Therefore, median values were used to describe the original data. For comparison between populations, we used the Mann Whitney U; chi-square (χ^2); ANOVA; and Kruskal-Wallis tests, depending on the variable used.

In the next step, Spearman correlations between meconium Hg species and Hg in MB was performed only for Croatian population, while other correlations (meconium Hg levels and MH Hg, dental amalgams, seafood intake, BMI, age, gestational age, birth weight) included both populations. To confirm observed Spearman's correlations we performed multiple linear regression on Slovenian population, as they had complete information on dental amalgams and seafood intake. Before performing multiple linear regression analysis, the Hg

Table 2
Results of total Hg (THg), methyl-Hg (MeHg as Hg), and inorganic Hg (Hg(II)) in ng/g wet weight; and percentage of Hg(II) (%) in the samples of meconium from our study; and Hg concentrations in meconium as reported in the selected literature. Median values, range, and number observed are shown below.

		Our study			Literature					
Meconium Hg		Slovenia	Croatia	All	Philippines, Manila[a]	USA[b]	Austria[c]	Philippines, Tagum[d]	Taiwan[e]	Turkey[f]
THg [ng/g]	Median	11.5	10.1	11.1	3.17	3.9	4.0	48.6	82.6	9.4 (μg/g)
	Range	0.41–375.2	1.18–359.8	0.41–375.2	0.43–71.6	0.64–11.0	0.4–128	20–200		0–66.5
	N	287	201	488	426	14	36	36	545	143
	CV (%)	170	165	169						
MeHg	Median	0.07	0.19	0.10		0.0078				
[ng/g]	Range	0.005–1.39	0.005–3.13	0.005–3.13		< MDL-0.078				
	N	204	145	349		17				
	CV (%)	139	124	147						
Hg(II)	Median	10.6	9.98	10.4		3.9				
[ng/g]	Range	0.40–375.2	1.63–359.7	0.40–375.2		0.64–11.0				
	N	204	145	349		14				
	CV (%)	195	179	189						
%Hg(II)	Median	99.2	98.1	98.8		100				
[%]	Range	81.9–100	88.2–100	81.9–100		99–100				
	N	204	145	349		14				
	CV (%)	2.2	1.9	2.1						

All-combined Slovenian and Croatian population; CV-coefficient of variation.
[a] Ostrea et al., 2002, in ng/ml.
[b] Rothenberg et al. (2019).
[c] Gundacker et al. (2010).
[d] Ramirez et al. (2000).
[e] Jiang et al. (2014).
[f] Unuvar et al. (2007), in μg/g.

concentrations in meconium were log-transformed. Models were designed to study associations between meconium Hg species and possible covariates. Models were adjusted for selected personal characteristics and habits (seafood intake, number of dental amalgam fillings, smoking status, pre-pregnancy body mass index (BMI), maternal age, parity, gestational age, child's sex, and birth weight) and were included to provide the highest R^2. They were designed to cover and include all possible variables that could have an impact on meconium Hg levels. The level of significance (p) was set at 0.05.

3. Results and discussion

Our studied population included pregnant women and their newborns from central Slovenia (N = 287 pairs) and coastal Croatia (N = 201 pairs). The average age of mothers at delivery was 30.4 years, their pre-pregnancy BMI was 23.7 kg/m^2, and for 47% of them having their first pregnancy. Approximately 3% of women self-reported never eating any kind of seafood. We identified 38% of women as 'ever-smokers'. New-borns' average gestational age was 39 weeks and their average birth weight was 3.5 kg, and 52% of them were boys. Table 1 lists all collected demographic data, including p value to determine the difference between both populations.

In this issue we have presented the Hg speciation results in MB and cord blood from the same population (Trdin et al., 2019). Table 2 lists the results of Hg measurements in meconium. Median THg concentrations in meconium (reported in wet weight) are on average higher than those reported previously in MB and cord blood of the same participants, namely 2.04 and 1.84 ng/g, respectively (Miklavčič et al., 2013). Median THg concentrations measured in meconium from our study are higher compared to those found in Manila, the Philippines, USA, and Austria, and lower than those reported in Tagum, the Philippines, Taiwan, and Turkey (Table 2). The study from Tagum, Philippines (Ramirez et al., 2000) reported much higher levels of meconium Hg in comparison with study from Manila, Philippines (Ostrea et al., 2002). It could be that results differ because the Tagum study included participants from artisanal gold mining and processing community. In contrast, study from Manila included randomly selected newborns from different hospitals, which were included to present socioeconomic cross-section of hospitals in Manila.

On the other hand, our data showed wider range of exposure with higher maximum values (Table 2). Only one study from Istanbul reported much higher Hg levels with average value of 9.4 μg/g (Unuvar et al., 2007). We did not observe a statistically significant difference between Slovenian and Croatian population in meconium THg levels ($p = 0.601$) or Hg(II) levels ($p = 0.437$), however, there was a statistically significant difference between populations in meconium MeHg ($p < 0.001$) and % Hg(II) ($p < 0.001$) levels. The MeHg level in the Croatian population is higher because pregnant women from Croatia ate seafood more frequently than did Slovenian women (Table 1). Levels of meconium THg were significantly correlated with meconium MeHg ($R_s = 0.345$, $p < 0.001$) and Hg(II) ($R_s = 0.999$, $p < 0.001$), and also correlation between meconium MeHg and Hg(II) was significant ($R_s = 0.329$, $p < 0.001$).

As observed from Table 2, Hg(II) presented the majority (82%–100%) of measured THg in meconium. To the best of our knowledge, this is the first study to perform Hg speciation in meconium with such a large sample size. Median values of meconium Hg are ~2-fold higher compared to study from USA. Additionally, our results showed wider range of exposure to all Hg species (Table 2). The latter is presumably related to the fact that they performed Hg speciation on a very limited sample size (N = 14) and median THg concentrations found in blood of participating women were also lower, compared with ours (0.48 vs. 2.04 ng/g) (Rothenberg et al., 2019; Trdin et al., 2019).

Intestinal MeHg demethylation and biotransformation to inorganic Hg are clearly important steps in MeHg excretion from the adult body (Caito et al., 2018; Rowland et al., 1977), but in some cases higher proportions of MeHg in faeces indicated that MeHg can also be trapped in faeces until defecation (Rand et al., 2016). In comparison with MeHg, Hg(II) in faeces has lower (re)absorption capacity (~95% vs. ~7%) and the major part of excreted Hg(II) is retained in faeces until defecation (Horvat et al., 2012). According to literature data, we hypothesised that MeHg demethylation makes the highest contribution to Hg(II) content not only in faeces (Ou et al., 2014) but also in meconium (Rothenberg et al., 2019), although intestinal demethylation mechanisms are not fully characterised (Rothenberg et al., 2017). For comparison, Hg(II) content in human stool samples varies from 68% to >

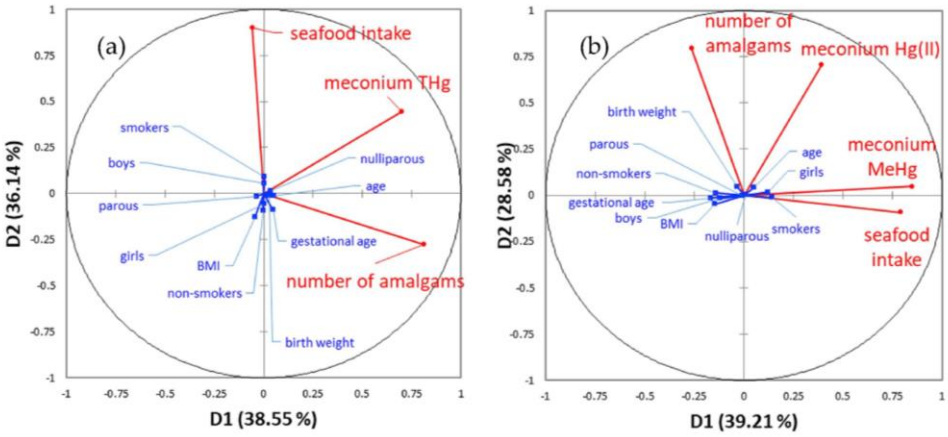

Fig. 1. PCA between active variables (marked in red), namely, **(a)** seafood intake, number of dental amalgams, and meconium THg levels; as well as **(b)** seafood intake, number of dental amalgams, meconium Hg(II), and meconium MeHg; and supplementary variables (marked in blue), namely, maternal smoking status, maternal age, pre-pregnancy BMI, parity, gestational age, sex, and birth weight for the Slovenian population. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

95% of THg (Rand et al., 2016). In addition to MeHg demethylation, high Hg(II) content also results from Hg^0 oxidation released from dental amalgam fillings and inhaled from the atmosphere which is oxidised by catalase in blood and other tissues (Clarkson, 1997) and from accumulation of Hg(II) from food, cosmetics and other sources (Ou et al., 2014). According to all possible sources of Hg(II) it is difficult to find a reliable estimation of contribution for each one.

PCA was performed to test the most probable origin of meconium Hg, the factors that could contribute to high Hg(II) content in meconium, and how our data is correlated. Fig. 1a shows results of PCA between main (active) variables (marked in red), namely, meconium THg, maternal seafood intake, and number of dental amalgams, and covariate (supplementary) variables (marked in blue), namely, maternal smoking status, maternal age, pre-pregnancy BMI, parity, gestational age, sex, birth weight). The first PCA covered 74.69% of variability (N = 100). In the second PCA (Fig. 1b) we included Hg speciation in meconium, and therefore the active variables include meconium Hg(II) and MeHg, maternal seafood intake, and number of dental amalgams and the supplementary variables are the same as in Fig. 1a. The second PCA covered 67.79% of variability (N = 100). Based on the results in Fig. 1a, we can assume that meconium THg levels are correlated with both, maternal seafood intake and number of dental amalgams. Furthermore, Fig. 1b shows that MeHg in meconium was highly related with maternal seafood intake. By contrast, Hg(II) in meconium was more related to number of dental amalgam fillings than to seafood intake. However, the correlation between meconium Hg(II) and amalgams is not as strong as that between meconium MeHg and seafood intake. The reason can be that not all Hg(II) is accumulated in meconium, a part is circulating between foetal swallowing of amniotic fluid and foetal urine excretion (Gilbert and Brace, 1989; Stigter et al., 2011).

Beside the PCA we further examined the Spearman correlation coefficients, which are presented in Table 3. Results of Spearman's correlation analysis are consistent with correlations observed from PCA. We found a significant correlation between seafood intake and Hg species in meconium, with the highest correlation in case of meconium MeHg levels (R_s = 0.498). Maternal seafood intake was also significantly correlated with meconium Hg(II) levels (R_s = 0.201). Furthermore, number of dental amalgam fillings were weakly correlated with THg (R_s = 0.175) and Hg(II) (R_s = 0.145) in meconium but not with MeHg (negative, nonsignificant correlation). Correlations between Hg species in meconium and maternal blood were additionally performed as rough estimations, because we sampled MB at only one point during pregnancy, but meconium starts to form at the beginning of the 2nd trimester and MB was available only for Croatian population. However, all Hg that can be passed to the foetal side originates from maternal blood (plasma). The correlation between MeHg in MB and meconium was high and significant (R_s = 0.898); however Hg(II) levels in MB and meconium were not significantly correlated. These results are consistent with the recently published study (Rothenberg et al., 2019). Moreover, Table 3 showed correlation between meconium and MH Hg levels, as the latter, like meconium, indicates, a long-term exposure (Horvat et al., 2012). The highest correlation was observed between THg in hair and MeHg in meconium (R_s = 0.829). Despite the discrepancy regarding the time of sampling as described above, the observed correlations obviously reflect constant dietary habits of the mothers during the pregnancy period.

To confirm the correlations shown in Table 3, we performed multiple linear regression analyses (Table 4). In linear regression modelling we did not adjust for MB. The major reason for exclusion of blood is to avoid misinterpretations, as blood was taken only once in the 3rd trimester; further, we did not have the exact time of the collection of meconium after birth, and in literature it was shown that Hg concentrations measured in meconium depend on the time of its collection (Ortega Garcia et al., 2006). Furthermore, blood was available only for the Croatian population, which also had a lot of missing values for dental amalgams. Therefore, we decided to include only the Slovenian population in the regression modelling (N = 143) and to include only those variables (e.g. seafood intake, dental amalgam), which present exposure to Hg and presumably did not change throughout the pregnancy or could modify Hg kinetics mechanisms (BMI, age, smoking status, parity, etc.).

As shown in Table 4, meconium THg and Hg(II) levels were positively associated with the number of dental amalgams. Furthermore, meconium MeHg levels were associated with maternal seafood intake. The meconium ratio between Hg(II) and MeHg levels suggested that this ratio is negatively associated with maternal seafood intake and positively associated with the number of dental amalgams. Girls had statistically higher levels of MeHg in meconium (0.24 vs. 0.20 ng/g); however, this difference is probably not physiologically important. By

Table 3
Spearman's correlations (Rs) between Hg species in meconium and Hg levels in maternal hair (MH), maternal blood (MB), and personal characteristics and habits.

	MH	MB[$]				Seafood intake	Dental amalgam	BMI	Age	Gestational age	Birth weight
	THg	THg	MeHg	Hg(II)	%MeHg						
THg in meconium	0.314*** (N = 471)	0.642*** (N = 198)	0.573*** (N = 198)	0.075 (N = 198)	0.267*** (N = 198)	0.184*** (N = 408)	0.175** (N = 255)	−0.108* (N = 477)	0.112* (N = 468)	−0.015 (N = 378)	−0.001 (N = 404)
MeHg in meconium	0.829*** (N = 337)	0.857*** (N = 142)	0.898*** (N = 142)	−0.287** (N = 142)	0.646*** (N = 142)	0.498*** (N = 289)	−0.094 (N = 181)	−0.233*** (N = 341)	0.144** (N = 336)	−0.183** (N = 268)	−0.042 (N = 286)
Hg(II) in meconium	0.297*** (N = 337)	0.614*** (N = 142)	0.539*** (N = 142)	0.112 (N = 142)	0.192* (N = 142)	0.201*** (N = 289)	0.145* (N = 181)	−0.130* (N = 341)	0.084 (N = 336)	−0.050 (N = 268)	−0.007 (N = 286)

MH-maternal hair; MB-maternal blood; *p < 0.05; **p < 0.005; ***p < 0.0001; [$]available for Croatian mothers only.

combining the results from Tables 3 and 4, we can suggest, that both MeHg from seafood (Kajiwara et al., 1996) and Hg^0 vapour from dental amalgams (Clarkson et al., 1972) were transported through the placental barrier and accumulated in meconium.

Regression modelling showed that meconium MeHg was highly associated with maternal seafood intake; however, statistically significant correlation (Table 3) between seafood intake and meconium Hg (II) was not confirmed in linear regression modeling (Table 4). Therefore, MeHg demethylation process could not be entirely confirmed nor excluded. As multiple regression (Table 4) was performed only on Slovenian population, where seafood intake is lower compared with Croatian participants (Table 1), it is possible, that at higher levels of exposure the results of statistical analysis would be different. However, results from Rothenberg et al. (2019) which observed no correlation between fish servings and meconium Hg(II) during late gestation could be consistent with our study. On the other hand, the lack of an association in the study of Rothenberg et al. could be explained by their limited sample size (N = 14) or with low seafood intake. Although MeHg demethylation in the environment and biota is well established (Du et al., 2019), and gut demethylation of MeHg in adults was confirmed as an essential step in reducing its bioavailability (Li et al., 2018; Rand et al., 2016), based on our data, we cannot confirm nor exclude the hypothesis of intestinal MeHg demethylation during prenatal life. If MeHg demethylation occurs we expect that seafood intake, as a major source of MeHg in MB, would be positively associated with Hg(II) in meconium. This agrees with an in vitro study indicating that human neonate and infant cannot demethylate MeHg in the gut (Rowland et al., 1983). Furthermore, MeHg demethylation was shown to be initiated postnatally (Byczkowski and Lipscomb, 2001). It is assumed that with increased maternal seafood intake the MeHg concentration in maternal plasma increases, resulting in increased placental transport of MeHg (Kajiwara et al., 1996; Kuhnert et al., 1981). As we found relatively low MeHg concentrations in meconium, we hypothesise that the major part of MeHg is distributed throughout foetal tissues and partially returned in maternal blood system, and that only a small part is excreted to the meconium. Once inside the meconium, MeHg is probably there accumulated without any biotransformation, given that intestinal functions are not initiated during the prenatal period and that the processes of bile excretion, gut absorption, and gut demethylation are usually assumed to be inactive for physiologically based pharmacokinetic modelling (Ou et al., 2018). Clearly, further studies are needed, to confirm this observations and to establish mechanisms at higher levels of exposure.

If we focus on the statistical model with meconium Hg(II) levels (Table 4), the results showed weak, but significant association between Hg(II) in meconium and the mother's number of dental amalgam fillings. Similarly, Rothenberg et al. reported significant positive correlation between meconium THg and maternal amalgam fillings (Rothenberg et al., 2019). Consistently, Gundacker et al. reported positive association between both THg in meconium and Hg(II) levels in placenta and the number of dental amalgam fillings (Gundacker et al., 2010). Two mechanisms may be responsible for such association. The first is the Hg^0 transport through the placental barrier (Clarkson et al., 1972; Yoshida, 2002) and oxidation of Hg^0 to Hg(II) on the foetal side (Takahashi et al., 2003) and the second is Hg^0 oxidation in MB (Magos et al., 1978) and Hg(II) transport through the placenta (Oliveira et al., 2015; Ou et al., 2014).

Hg^0 vapour is readily absorbed by the lungs and diffused into the blood (Park and Zheng, 2012). Hg^0 is, to some extent, dissolved in blood; a minor part is bound to haemoglobin; and the majority is oxidised to Hg(II) by catalase (Magos et al., 1978). Although the oxidation process occurs in a relatively short time, it is still long enough for lipid-soluble Hg^0 to penetrate the blood-brain and placental barriers (Park and Zheng, 2012). The exact mechanism and location of Hg^0 oxidation is currently unknown; however, it was shown that as in maternal tissues foetal tissues could also convert Hg^0 to Hg(II). An animal study

Table 4
Results of multiple regression models (N = 143) for Slovenian population. Levels of ln Hg species in meconium adjusted for seafood intake, number of dental amalgams and some other personal characteristics and habits.

MODELS	ln THg β; p (95% CI) model: p = 0.05, R^2 = 0.119	ln MeHg β; p (95% CI) model: p < 0.001, R^2 = 0.263	ln Hg(II) β; p (95% CI) model: p < 0.05, R^2 = 0.119	ln Hg(II)/MeHg β; p (95% CI) model: p < 0.001, R^2 = 0.222
Seafood intake (meals per day)	0.22; 0.602 (−0.62; 1.07)	**2.15; 0.000 (0.29; 3.01)**	0.19; 0.659 (−0.67; 1.05)	**−1.83; 0.000 (−2.75; −0.92)**
Number of dental amalgams	**0.31; 0.002 (0.12; 0.50)**	0.07; 0.489 (−0.13; 0.26)	**0.32; 0.002 (0.12; 0.51)**	**0.24; 0.011 (0.06; 0.43)**
Smoking (non-smokers vs. smokers)	−0.33; 0.075 (−0.68; 0.03)	−0.10; 0.588 (−0.46; 0.26)	−0.33; 0.074 (−0.69; 0.03)	−0.22; 0.263 (−0.62; 0.17)
Pre-pregnancy BMI[a] (kg/m^2)	−0.02; 0.211 (−0.06; 0.01)	−0.02; 0.359 (−0.05; 0.02)	−0.02; 0.211 (−0.09; 0.01)	−0.001; 0.976 (−0.04; 0.04)
Mother's age (years)	−0.01; 0.648 (−0.05; 0.03)	0.02; 0.323 (−0.02; 0.06)	−0.01; 0.643 (−0.05; 0.03)	−0.03; 0.207 (−0.08; 0.02)
Parity (nulliparous vs. parous)	0.02; 0.907 (−0.32; 0.36)	−0.23; 0.192 (−0.57; 0.12)	0.02; 0.885 (−0.32; 0.37)	0.29; 0.131 (−0.09; 0.67)
Estimated gestational age (weeks)	−0.06; 0.211 (−0.16; 0.04)	−0.08; 0.104 (−0.18; 0.02)	−0.06; 0.219 (−0.16; 0.04)	0.03; 0.591 (−0.08; 0.14)
Child's sex (boys vs. girls)	0.23; 0.201 (−0.59; 0.12)	**0.48; 0.009 (0.12; 0.84)**	−0.24; 0.186 (−0.60; 0.12)	**−0.64; 0.001 (−1.03; −0.25)**
Birth weight (g)	0.0001; 0.864 (−0.0004; 0.001)	7.8×10^{-6}; 0.971 (−0.0004; 0.0004)	0.00003; 0.887 (−0.0004; 0.0005)	0.0001; 0.727 (−0.001; 0.00004)

Bolded are significant associations.
[a] -body mass index.

Table 5
Average contents (with range and number observed) of inorganic Hg (%Hg(II)) content in different biological matrices as reported in literature and herein. For MB and meconium are presented median values, and for placenta and amniotic fluid arithmetic means.

	MB[a]	placenta[b]			amniotic fluid[c]	meconium[d]
		central	amnion	chorion		
% Hg(II)	14.1	79	65	75	75	98.8
	0–95.7	43–95	38–90	52–94	65–88	81.9–100
	225	17	17	17	57	349

[a] Trdin et al. (2019).
[b] Horvat et al., 1991.
[c] Suzuki et al. (1977).
[d] Present study.

indicated that the distribution of Hg^0 and oxidation to Hg(II) is determined by tissue-specific factors such as perfusion rate and the concentration of Hg-binding ligands (Morgan et al., 2002). Literature on Hg speciation in human placenta and amniotic fluid is limited. To compare levels of Hg species in different biological matrices we took data from Hg speciation in maternal blood from the same population (Trdin et al., 2019) and data on Hg speciation in placental tissue performed on Slovenian population (central Slovenia) (Horvat et al., 1991). Unfortunately, Suzuki et al. did not report on Hg levels in maternal blood, but to the best of our knowledge, this is the only study which performed Hg speciation in amniotic fluid (Suzuki et al., 1977). However, Table 5 shows that the Hg(II) content is relatively low only in MB. By contrast, in placenta and amniotic fluid, Hg(II) presents more than half of measured THg, and in meconium, the majority of THg is in Hg(II) form. From these data, we assumed that Hg^0 oxidation occurs in the placenta or amniotic fluid (Bogdanović Pristov et al., 2009). During gestation, the placenta increases in mass and perfusion to provide nutrients and gases to the foetus, and consequently, the amount of Hg^0 reaching the foetus is increased. At this time the formation of reactive oxygen species is an unavoidable consequence of aerobic metabolism and accordingly, the activity of enzymes required for antioxidant defence (superoxide dismutase, catalase, and peroxidase) increases (Watson et al., 1998). Therefore, we can assume that although the influx of Hg^0 is increased, the activity of catalase, a major factor required for Hg^0 oxidation, is also increased. This would lead to rapid oxidation of Hg^0 to Hg(II) and the majority of Hg(II) is probably complexed in placenta (Kuhnert et al., 1981). The placenta has key factors, glutathione (Mover and Ar, 1997), metallothioneins (Itoh et al., 1996), and selenium (Khan and Wang, 2009) that all have high affinity for Hg(II), and they may limit the amount of Hg(II) reaching the foetus. As shown recently, some of Hg(II) can also pass through placenta (Ou et al., 2014). However, due to high perfusion some Hg^0 is transported through the placental barrier and can also be oxidised in amniotic fluid or in foetal tissues. This is supported by a study on pregnant rats that showed that Hg^0 from dental amalgams was accumulated in the placenta but some part also crossed the placenta and was oxidised in the foetal liver (Takahashi et al., 2003). However, Hg(II) present in amniotic fluid could be from foetal urine and swallowed by the foetus combined with other nutrients (Underwood et al., 2005). If Hg^0 is transported and oxidised in foetal liver, we assume, that some part of Hg(II) is also transported to the meconium. Once in the meconium Hg(II) is unlikely to re-cross the membranes and is probably accumulated in the meconium until defecation after birth.

The main drawback of our study is the lack of precise data on dental amalgam fillings (as one of the important sources of Hg(II) in meconium) for the Croatian population, meaning that we were unable to include both populations in linear regression models. Further, MB was not available for the Slovenian participants. Additionally, we did not have the exact date and hour of meconium collection after birth; this is

problematic, as it was shown, that Hg concentrations depend on the time of meconium collection (Ortega Garcia et al., 2006). To precisely follow the Hg kinetics in meconium and investigate occurrence of demethylation process, it would be reasonable to sample MB several times during gestation.

4. Conclusions

This is the first study to perform Hg speciation in meconium on such large sample size. Our results showed that Hg(II) presented the majority (82%–100%) of measured THg in the meconium. To identify possible sources of Hg species in meconium we examined the correlations between Hg in meconium with Hg species in MH, MB, and personal characteristic obtained from the questionnaires. We observed significant correlation between meconium and MH Hg levels, as the latter, like meconium, indicates a long-term exposure. Furthermore, from the observed associations, we can suggest that high Hg(II) content in the meconium results from the transport of Hg^0 vapour from maternal dental amalgam fillings through the placental barrier and the subsequent partitioning of Hg(II) in foetal tissues, body fluids, and meconium. Correlation between maternal seafood intake and Hg(II) levels in meconium was not confirmed with linear regression, therefore there is certain evidence of MeHg demethylation, but in our study it is unlikely that MeHg biotransformation would significantly contribute to high Hg (II) content found in meconium.

Observed associations between meconium MeHg and seafood intake, and meconium Hg(II) and dental amalgams were significant only when meconium Hg species were taken into account in contrast to meconium THg measurements. Based on our data we can conclude that meconium is a suitable biomarker to assess prenatal Hg exposure to MeHg and Hg^0 under the condition that Hg speciation analysis is performed. With this approach, we will characterize the levels of prenatal Hg exposure from different sources. Sampling of meconium is non-invasive and simple, however, methods used so far are not standardized, therefore the results obtained in different studies are not entirely comparable. Further studies are also needed to test the hypothesis of MeHg demethylation, which would limit the use of meconium as biomarker of prenatal MeHg exposure.

Author contributions

A.T. wrote the paper and performed all meconium Hg measurements. I.F. contributed to the protocols and interpretation of the data. V.F. helped with Hg measurements. I.Ž. helped with statistics and performed PCA analysis. J.S.T. helped with statistics. I.P. and Z.Š. contributed to the study design and sampling in Croatia. M.H. contributed to the study design and protocols, supervised all Hg measurements, and interpreted the data.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by: the EU through the 6th FP, FOOD-CT-2006-016253, PHIME project; and the 7th FP no.603946, HEALS project, CROME-LIFE + project; a programme funded by the Slovenian Research Agency (ARRS) P1-0143 and project NEURDODYS J7-9400. University of Rijeka, Grant number: uniri-biomed-18-120; Long term outcome of children prenatally exposed to methyl-mercury: genetic and environmental factors.

The authors are grateful to all pregnant women and their children who participated in this study and the personnel of the "Jožef Stefan" Institute, University Medical Centre Ljubljana, Slovenia, and the University Hospital Centre Rijeka, Croatia, who collected biological samples and collaborated in conducting the interviews with the pregnant women.

References

Akagi, H., 1997. Analytical method for evaluating human exposure to mercury due to gold mining. In: Proceedings of the International Workshop on Health and Environmental Effects of Mercury Due to Mining Operations. National Institute for Minamata Disease, Manila.

Al-Saleh, I., Shinwari, N., Mashhour, A., El, G., Mohamed, D., Rabah, A., 2011. Heavy metals (lead, cadmium and mercury) in maternal, cord blood and placenta of healthy women. Int. J. Hyg Environ. Health 214, 79–101. https://doi.org/10.1016/j.ijheh.2010.10.001.

Bakdash, A., Burger, P., Goecke, T.W., Fasching, P.A., Reulbach, U., Bleich, S., Hastedt, M., Rothe, M., Beckmann, M.W., Pragst, F., Kornhuber, J., 2010. Quantification of fatty acid ethyl esters (FAEE) and ethyl glucuronide (EtG) in meconium from newborns for detection of alcohol abuse in a maternal health evaluation study. Anal. Bioanal. Chem. 396, 2469–2477. https://doi.org/10.1007/s00216-010-3474-5.

Bearer, C.F., 2003. Meconium as a biological marker of prenatal exposure. Ambul. Pediatr. 3, 40–43. https://doi.org/10.1367/1539-4409(2003)003<0040:MAABMO>2.0.CO;2.

Bogdanović Pristov, J., Spasojević, I., Miković, Ž., Mandić, V., Cerovic, N., Spasić, M., 2009. Antioxidative defense enzymes in placenta protect placenta and fetus in inherited thrombophilia from hydrogen peroxide. Oxid. Med. Cell Longev. 2, 14–18.

Byczkowski, J.Z., Lipscomb, J.C., 2001. Physiologically based pharmacokinetic modelingof the lactational transfer of methylmercury. Risk Anal. 21.

Caito, S.W., Jackson, B.P., Punshon, T., Scrimale, T., Grier, A., Gill, S.R., Love, T.M., Watson, G.E., Wijngaarden, E. Van, Rand, M.D., 2018. Variation in methylmercury metabolism and elimination status in humans following fish consumption. Toxicol. Sci. 161, 443–453. https://doi.org/10.1093/toxsci/kfx226.

Clarkson, T.W., 1997. The toxicology of metal. Crit. Rev. Clin. Lab. Sci. 34, 369–403.

Clarkson, T.W., Magos, L., Greenwood, M.R., 1972. The transport of elemental mercury into fetal tissue. Biol. Neonate 21, 239–244.

Concheiro, M., Huestis, M.A., 2018. Drug exposure during pregnancy: analytical methods and toxicological findings. Bioanalysis 2. https://doi.org/10.4155/bio-2017-0260.

Du, H., Ma, M., Igarashi, Y., Wang, D., 2019. Biotic and abiotic degradation of methylmercury in aquatic ecosystems: a review. Bull. Environ. Contam. Toxicol. 102, 605–611. https://doi.org/10.1007/s00128-018-2530-2.

EPA Method 7473, 1998. Mercury in solids and solutions by thermal decomposition, amalgamation, and atomic absorption spectrophotometry. [WWW Document]. URL. https://www.epa.gov/homeland-security-research/epa-method-7473-sw-846-mercury-solids-and-solutions-thermal-decomposition.

Gilbert, W.M., Brace, R.A., 1989. The missing link in amniotic fluid volume regulation: intramembranous absorption. Obstet. Gynecol. 74, 748–754.

Gundacker, C., Fröhlich, S., Graf-Rohrmeister, K., Eibenberger, B., Jessenig, V., Gicic, D., Prinz, S., Wittmann, K.J., Zeisler, H., Vallant, B., Pollak, A., Husslein, P., 2010. Perinatal lead and mercury exposure in Austria. Sci. Total Environ. 408, 5744–5749. https://doi.org/10.1016/j.scitotenv.2010.07.079.

Hammerschmidt, C.R., Finiguerra, M.B., Weller, R.L., Fitzgerald, W.F., 2013. Methylmercury accumulation in plankton on the continental margin of the Northwest Atlantic Ocean. Environ. Sci. Technol. 47, 3671–3677.

Horvat, M., Prosenc, A., Smrke, J., Konda, D., Byrne, A.R., Stegnar, P., Bergic, I., 1991. Trace element levels in the hair, blood, cord blood and placenta of pregnant women from central Slovenia. In: Aitio, A., Aro, A., Järvisalo, J., Vainio, H. (Eds.), Trace Elements in Health and Disease. The Royal Society of Chemistry, pp. 83–94.

Horvat, M., Snoj Tratnik, J., Miklavčič, A., 2012. Mercury: biomarkers of exposure and human biomonitoring. In: Knudsen, L.E., Merlo, D.F. (Eds.), Biomarkers and Human Biomonitoring. Volume 1: Ongoing Programs and Exposures. The Royal Society of Chemistry, Cambridge, UK, pp. 381–417.

Itoh, N., Fujita, Y., Nakanishi, H., Kawai, Y., Mayumi, T., Hwang, G.S., Min, K., Onosaka, S., Muto, N., Tanaka, K., 1996. Binding of Cd to metallothionein in the placenta of Cd-treated mouse. J. Toxicol. Sci. 21, 19–27.

Jiang, C., Hsi, H., Fan, C., Chien, L., 2014. Fetal exposure to environmental neurotoxins in Taiwan. PLoS One 9. https://doi.org/10.1371/journal.pone.0109984.

Jiang, C.B., Yeh, C.Y., Lee, H.C., Chen, M.J., Hung, F.Y., Fang, S.S., Chien, L.C., 2010. Mercury concentration in meconium and risk assessment of fish consumption among pregnant women in Taiwan. Sci. Total Environ. 408, 518–523. https://doi.org/10.1016/j.scitotenv.2009.10.043.

Kajiwara, A., Yasutake, Y., Adachi, K., Hirayama, T., 1996. Methylmercury transport across the placenta via neutral amino acid carrier. Arch. Toxicol. 70, 310–311. https://doi.org/10.1007/s002040050279.

Khan, M. a K., Wang, F., 2009. Mercury-selenium compounds and their toxicological significance: toward a molecular understanding of the mercury-selenium antagonism. Environ. Toxcol. Chem. 28, 1567–1577. https://doi.org/10.1897/08-375.1.

Knezović, Z., Trgo, M., Sultović, D., 2016. Monitoring mercury environment pollution through bioaccumulation in meconium. Process Saf. Environ. Prot. 101, 2–8. https://doi.org/10.1016/j.psep.2016.01.013.

Kuhnert, P., Kuhnert, B.R., Erhard, B.S., 1981. Comparison of mercury levels in maternal blood, fetal cord blood, and placental tissues. Am. J. Obstet. Gynecol. 139, 209–213. https://doi.org/10.1016/0002-9378(81)90448-8.

Li, H., Lin, X., Zhao, J., Cui, L., Wang, L., Gao, Y., Li, B., Chen, C., Li, Y.F., 2018. Intestinal methylation and demethylation of mercury. Bull. Environ. Contam. Toxicol. https://doi.org/10.1007/s00128-018-2512-4.

Liang, L., Bloom, N.S., Horvat, M., 1994. Simultaneous determination of mercury

speciation in biological materials by GC/CVAFS after ethylation and room-temperature precollection. Clin. Chem. 40, 602–607.
Magos, L., Halbach, S., Clarkson, T.W., 1978. Role of catalase in the oxidation of mercury vapor. Biochem. Pharmacol. 27, 1373–1377.
Miklavčič, A., Casetta, A., Snoj Tratnik, J., Mazej, D., Krsnik, M., Mariuz, M., Sofianou, K., Špirić, Z., Barbone, F., Horvat, M., 2013. Mercury, arsenic and selenium exposure levels in relation to fish consumption in the Mediterranean area. Environ. Res. 120, 7–17. https://doi.org/10.1016/j.envres.2012.08.010.
Miklavčič, A., Cuderman, P., Mazej, D., Snoj Tratnik, J., Krsnik, M., Planinšek, P., Osredkar, J., Horvat, M., 2011. Biomarkers of low-level mercury exposure through fish consumption in pregnant and lactating Slovenian women. Environ. Res. 111, 1201–1207. https://doi.org/10.1016/j.envres.2011.07.006.
Morgan, D.L., Chanda, S.M., Price, H.C., Fernando, R., Liu, J., Brambila, E., O'Connor, R.W., Beliles, R.P., Barone, S., 2002. Disposition of inhaled mercury vapor in pregnant rats: maternal toxicity and effects on developmental outcome. Toxicol. Sci. 66, 261–273. https://doi.org/10.1093/toxsci/66.2.261.
Mover, H., Ar, A., 1997. Antioxidant enzymatic activity in embryos and placenta of rats chronically exposed to hypoxia and hyperoxia. Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 117, 151–157.
Oliveira, C.S., Joshee, L., Zalups, R.K., Pereira, M.E., Bridges, C.C., 2015. Disposition of inorganic mercury in pregnant rats and their offspring. Toxicology 335, 62–71. https://doi.org/10.1016/j.tox.2015.07.006. Disposition.
Ortega Garcia, J.A., Carrizo Gallardo, D., Ferris i Tortajada, J., Garcia, M.M.P., Grimalt, J.O., 2006. Meconium and neurotoxicants: searching for a prenatal exposure timing. Arch. Dis. Child. 91, 642–646. https://doi.org/10.1136/adc.2005.084129.
Ostrea Jr., E., Bielawski, D.M., Posecion Jr., N.C., 2006. Meconium analysis to detect fetal exposure to neurotoxicants. Arch. Dis. Child. 91, 628–629. https://doi.org/10.1136/adc.2006.095059.
Ostrea Jr., E.M., Morales, V., Ngoumgna, E., Prescilla, R., Tan, E., Hernandez, E., Ramirez, G.B., Cifra, H.L., Manlapaz, M.L., 2002. Prevalence of fetal exposure to environmental toxins as determined by meconium analysis. NeuroToxicology 23, 329–339. https://doi.org/10.1016/S0161-813X(02)00077-3.
Ou, L., Chen, L., Chen, C., Yang, T., Wang, H., Tong, Y., Hu, D., Zhang, W., Long, W., Wang, X., 2014. Associations of methylmercury and inorganic mercury between human cord blood and maternal blood: a meta-analysis and its application. Environ. Pollut. 191, 25–30. https://doi.org/10.1016/j.envpol.2014.04.016.
Ou, L., Wang, H., Chen, C., Chen, L., Zhang, W., Wang, X., 2018. Physiologically based pharmacokinetic (PBPK) modeling of human lactational transfer of methylmercury in China. Environ. Int. 115, 180–187. https://doi.org/10.1016/j.envint.2018.03.018.
Park, B.Y., Lee, B.K., 2014. Use of meconium in perinatal epidemiology: potential benefits and pitfalls. Ann. Epidemiol. 24, 878–881. https://doi.org/10.1016/j.annepidem.2014.09.009.
Park, J., Zheng, W., 2012. Human exposure and health effects of inorganic and elemental mercury. J. Preven. Med. Public Health 45, 344–352.
Peng, S., Liu, L., Zhang, X., Heinrich, J., Zhang, J., Schramm, K., Huang, Q., Tian, M., Ali, S., Akber, M., Eqani, S., Shen, H., 2015. A nested case-control study indicating heavy metal residues in meconium associate with maternal gestational diabetes mellitus risk. Environ. Health 14, 1–10. https://doi.org/10.1186/s12940-015-0004-0.
Ramirez, G.B., Pagulayan, O., Stat, M.S., Akagi, H., Rivera, A.F., Eng, C., Lee, L.V., Berroya, A., Cristina, M., Cruz, V., Casintahan, D., 2003. Tagum Study II: follow-up study at two years of age after prenatal exposure to mercury. Pediatrics 111, 289–295. https://doi.org/10.1542/peds.111.3.e289.
Ramirez, G.B., Vince Cruz, M.C., Pagulayan, O., Ostrea, E., Dalisay, C., 2000. The Tagum study I: analysis and clinical correlates of mercury in maternal and cord blood, breast milk, meconium, and infants' hair. Pediatrics 106, 774–781.
Rand, M.D., Vorojeikina, D., van Wijngaarden, E., Jackson, B.P., Scrimale, T., Zareba, G., Love, T.M., Myers, G.J., Watson, G.E., 2016. Methods for individualized determination of methylmercury elimination rate and de-methylation status in humans following fish consumption. Toxicol. Sci. 149, 385–395. https://doi.org/10.1093/toxsci/kfv241.
Rothenberg, S.E., Keiser, S., Ajami, N.J., Wong, M.C., Gesell, J., Petrosino, J.F., Johs, A., 2017. The role of gut microbiota in fetal methylmercury exposure: insights from a pilot study. Toxicol. Lett. 3, 60–67. https://doi.org/10.1016/j.toxlet.2015.11.022. THE.
Rothenberg, S.E., Wagner, C.L., Hamidi, B., Alekseyenko, A.V., Azcarate-Peril, M.A., 2019. Longitudinal changes during pregnancy in gut microbiota and methylmercury biomarkers, and reversal of microbe-exposure correlations. Environ. Res. 172, 700–712. https://doi.org/10.1016/j.envres.2019.01.014.
Rowland, I.R., Davies, M.J., Grasso, P., 1977. The effect of elimination of the gastrointestinal flora on the accumulation of methylmercuric chloride by the rat. Biochem. Soc. Trans. 5, 423–425.
Rowland, I.R., Robinson, R.D., Doherty, R.A., Landry, T., 1983. Are developmental changes in methylmercury metabolism and excretion mediated by the intestinal microflora? In: Clarkson, T.W., Nordberg, G.F., Sager, P.R. (Eds.), Reproductive and Developmental Toxicity of Metals. Springer, Boston, MA, pp. 745–758. https://doi.org/10.1007/978-1-4615-9346-1_32.
Stigter, R.H., Mulder, E.J.H., Bruinse, H.W., Visser, G.H.A., 2011. Fetal urine production in late pregnancy. Obstet. Gynecol. https://doi.org/10.5402/2011/345431. 2011.
Suzuki, T., Takemoto, T.-I., Shishido, S., Kani, K., 1977. Mercury in human amniotic fluid. Scand. J. Work Environ. Health 3, 32–35.
Takahashi, Y., Tsuruta, S., Arimoto, M., Tanaka, H., Yoshida, M., 2003. Placental transfer of mercury in pregnant rats which received dental amalgam restorations. Toxicology 185, 23–33. https://doi.org/10.1016/S0300-483X(02)00588-7.
Trdin, A., Snoj Tratnik, J., Mazej, D., Fajon, V., Krsnik, M., Osredkar, J., Prpić, I., Špirić, Z., Petrović, O., Marc, J., Neubauer, D., Kodrič, J., Kobal, A.B., Barbone, F., Falnoga, I., Horvat, M., 2019. Mercury speciation in prenatal exposure in Slovenian and Croatian population - PHIME study. Environ. Res. https://doi.org/10.1016/j.envres.2019.108627.
Underwood, M.A., Gilbert, W.M., Sherman, M.P., 2005. State of the art amniotic fluid: not just fetal urine anymore. J. Perinatol. 341–348. https://doi.org/10.1038/sj.jp.7211290.
Unuvar, E., Ahmadov, H., Riza Kiziler, A., Aydemir, B., Toprak, S., Ulker, V., Ark, C., 2007. Mercury levels in cord blood and meconium of healthy newborns and venous blood of their mothers: clinical, prospective cohort study. Sci. Total Environ. 374, 60–70. https://doi.org/10.1016/j.scitotenv.2006.11.043.
Valent, F., Mariuz, M., Bin, M., Little, D., Mazej, D., Tognin, V., Tratnik, J., McAfee, A.J., Mulhern, M.S., Parpinel, M., Carrozzi, M., Horvat, M., Tamburlini, G., Barbone, F., 2013a. Associations of prenatal mercury exposure from maternal fish consumption and polyunsaturated fatty acids with child neurodevelopment: a prospective cohort study in Italy. J. Epidemiol. 23, 360–370. https://doi.org/10.2188/jea.JE20120168.
Valent, F., Horvat, M., Sofianou-Katsoulis, A., Spiric, Z., Mazej, D., Little, D., Prasouli, A., Mariuz, M., Tamburlini, G., Nakou, S., Barbone, F., 2013b. Neurodevelopmental effects of low-level prenatal mercury exposure from maternal fish consumption in a mediterranean cohort: Study rationale and design. https://doi.org/10.2188/jea.JE20120030.
Watson, A.L., Skepper, J.N., Jauniaux, E., Burton, G.J., 1998. Changes in concentration, localization and activity of catalase within the human placenta during early gestation. Placenta 19, 27–34. https://doi.org/10.1016/S0143-4004(98)90095-9.
Yoshida, M., 2002. Placental to fetal transfer of mercury and fetotoxicity. Tohoku J. Exp. Med. 196, 79–88.

3.3 Trace Elements and *APOE* Polymorphisms in Pregnant Women and Their Newborns

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In order to determine the relation between prenatal exposure to various contaminants and the possible health effects later in life, it is essential that we include all the factors that could modify this link, positively or negatively. The environment provided by the mother during pregnancy is often not included in epidemiological studies assessing the child's neurodevelopment, although maternal blood is the only source of nutrients but also potentially toxic compounds. *ApoE* genotypes were hypothesized to behave differently in relation to metal kinetics (especially Hg and Pb) and particularly to oxidative species (e.g. H_2O_2), which can also be a product of metal exposure. *ε4* carriers are believed to bind and eliminate reactive oxygen species and Hg less efficiently. Therefore, in this paper, maternal and child's *ApoE* polymorphisms were studied in relation to levels of selected elements (Hg, Pb, Cd, As, Se, Mn, Cu, Zn) in maternal blood, cord blood, maternal milk, and urine.

The Croatian participants were recruited within the PHIME project. This subgroup was selected because it contained the entire dataset of selected elements measured in maternal blood, milk, urine, and mixed cord blood. Apolipoprotein E genotypes were determined using TaqMan® pre-designed SNP genotyping assay for rs429358 and rs7412. According to their genotype, mothers and newborns were divided into two groups, *ε4* carriers (genotypes *APOE ε3/ ε4* and *ε4/ ε4*) and *ε4*-non carriers (genotypes *APOE ε2/ ε2*, *ε2/ ε3*, *ε3/ ε3*).

According to the determined genotypes, *ε4* was present in 16.7% and 20.1% of included mothers and newborns, respectively. Furthermore, in mothers, who were *ε4* carriers, the authors found statistically significant higher levels of blood MeHg, As, and Se; plasma Se; urine As; hair THg; cord blood MeHg, As, and Zn. However, only in the case of maternal plasma Se the difference between *ε4* carriers and non-carriers was confirmed with multiple linear regression. The authors reported geometrical means of maternal plasma Se being 62.6 ng/mL in *ε4* carriers in comparison with 54.9 ng/mL in *ε4* non-carriers ($p < 0.001$). All multiple linear regression models were adjusted for possible covariates that could affect levels of selected elements; these included maternal age, BMI, smoking status, seafood intake, and parity. The covariates for cord blood were extended with estimated gestational age, birth weight, gender of newborns, and newborn's genotype.

The authors suggested that higher levels of maternal plasma Se in *ε4* carriers could be linked to the beneficial effects of *ε4* during pregnancy. However, literature suggestions of possible *ε4* allele associations with Hg levels in biological samples were not confirmed in this study. Further studies are needed to fully understand the physiology of maternal ApoE, which would enable a detailed interpretation of the observed associations between maternal plasma Se and ApoE.

The candidate performed ApoE genotyping, and helped with data preparation and statistical analysis.

Trace elements and *APOE* polymorphisms in pregnant women and their new-borns

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Abstract

We investigated the relationship between lipid binding glycoprotein apolipoprotein E (apoE; gene *APOE*) polymorphisms (*ε4* allele carriers *versus* no carriers = *ε4+/ε4-*) and trace elements (TEs) (e.g., (methyl)mercury, arsenic, lead, cadmium, selenium, manganese, copper, and zinc) in mothers (N = 223) and their new-borns (N = 213) exposed to potentially toxic metal(loid)s from seafood consumption. The apoE isoform encoded by the *ε4* allele is believed to have beneficial effects in early life but represents a risk factor for age-associated diseases. Under certain conditions ε4 carriers are more susceptible to oxidative stress and metal(loid) toxicity. DNA from Croatian pregnant women (N = 223, third trimester) and their new-borns (N = 176), was genotyped for *APOE* by TaqMan® SNP assay - rs429358 and rs7412. Seafood intake data and TE levels in maternal urine, milk, hair, peripheral venous blood, mixed cord blood, and new-borns' urine were available from previous studies. We compared TEs between *ε4+* and *ε4-* carriers using Mann-Whitney U tests and applied multiple linear regression models to analyse the TE's dependence on the presence of allele ε4 (genotypes ε3/ε4, ε4/ε4) in combination with other explanatory variables. We identified 17% (n=37) and 20% (n=35) *ε4* allele carriers in mothers and new-borns, respectively. The Mann-Whitney U test showed that mothers with the ε4 allele had significantly higher mean levels of (methyl)mercury in peripheral venous blood, cord blood, and hair; arsenic in urine and cord blood; and selenium in peripheral venous blood and plasma. However, taking confounders into account, only the maternal plasma selenium remained statistically significant in the linear regression models (ε4 carriers vs non-carriers: 62.6 vs 54.9 ng/mL, $p < 0.001$). Literature suggestions of possible ε4 allele impact on Hg levels were not observed, while superior selenium status observed in healthy pregnant women carrying allele ε4 could be linked to the proposed APOE ε4 beneficial effects early in life.

Keywords: apolipoprotein E polymorphism; ε4 allele; pregnancy; selenium; mercury; trace elements

1. Introduction

Apolipoprotein E (apoE, gene *APOE*) is a pleiotropic lipid binding plasma (and cellular) glycoprotein that plays a central role in general and neuronal lipid metabolism by directing lipid transfer, uptake, and excretion (Giau et al., 2015; Huang and Mahley, 2014). Some studies also point to its antioxidative, metal-binding, and immunomodulatory/anti-inflammatory roles (Egert et al., 2012; Jofre-Monseny et al., 2008; Vitek et al., 2009). It has three major isoforms — apoE2, apoE3, and apoE4 — encoded by the alleles ε2, ε3, and ε4, respectively. In general, the alleles frequencies are from 0-14% for ε2, 49-90% for ε3 and 5-37% for ε4 (Giau et al., 2015). Protein isoforms differ in one amino acid at two positions (apoE2: 112cys, 158cys; apoE3: 112cys, 158arg; and apoE4: 112arg, 158arg) and differently affect peripheral lipid and brain neuronal homeostasis (Huang and Mahley, 2014). The apoE4 isoform is believed to have several age-related disadvantages over apoE3 and apoE2, mostly attributed to its comparatively lower antioxidative effect (Miyata and Smith, 1996; Xu et al., 2014), and lower degradation and clearance capacity for β-amyloid in the brain, with the consequent higher risk of developing Alzheimer's disease (Giau et al., 2015). Alternatively, many consider apoE4 to be a global activator of the innate immune function, providing a high survival value in a population exposed to high levels of infectious diseases (Vitek et al., 2009). Further, experimental and epidemiological evidence suggest that apoE allele ε4 is associated with higher levels of serum cholesterol, vitamin D (Huebbe et al., 2011), and higher bone Ca assimilation (Egert et al., 2012) in the general population, and higher progesterone levels in women (Jasienska et al., 2015). These factors can be beneficial in early life and promote higher fertility in women. Specifically, optimal cholesterol levels are important for prenatal and postnatal neurodevelopment (myelination, synaptogenesis) and steroid hormone and vitamin production; while higher vitamin D and calcium levels are essential for bone growth. Smith et al. (2019) and Tuminello and Han (2011) recently published literature reviews of studies that support the hypothesis of the ε4 allele's beneficial effects during different life stages. However, a few studies have suggested that *APOE* allele *ε4* carriers may be more susceptible to (Me)Hg toxicity during early neuronal development (Ng et al., 2013; Snoj Tratnik et al., 2017), whereas the opposite was found for lead (Pb) (Wright et al., 2003). Similar inconsistencies relating to other apoE effects suggest that either apoE4 or *APOE* ε4 may interact with various other factors (e.g., related gene polymorphisms, epigenetics, essential nutrient deficiency, pollutant exposure, or viral infections); consequently, its effects on a particular condition, such as Alzheimer's disease (AD) or neurodevelopment, will depend on a person's risk profile (Haas and Lathe, 2018; Nehls, 2016; Rea et al., 2016; Tuminello and Han, 2011)

The potential higher susceptibility to metal toxicity and oxidative stress of *ε4* carriers is based on experimental studies. *In vitro* studies have shown that different isoforms have different antioxidant properties for protecting against hydrogen-peroxide-induced oxidative stress, with ε4 having the least and ε2 having the most protective effect. Further, experimental studies also showed that apoE can bind metal ions such as copper, zinc, and iron (Miyata and Smith, 1996) and modify the expression of metal-regulating proteins, metallothioneins (Augsten et al., 2011; Florianczyk, 2007; Graeser et al., 2012). Previous research also suggests that apoE isoforms can affect the metabolic fate of metals and/or metal-related oxidative stress involved in AD and other disease aetiology (Egert et al., 2012; Jofre-Monseny et al., 2008; Xu et al., 2014). Mercury is among the metals of interest (Godfrey et al., 2003; Mutter et al., 2004). The general population is exposed to inorganic mercury (iHg) through dental amalgam fillings and to methylmercury (MeHg) through seafood consumption. Marine fish have some of the highest levels of nonessential metal(loid)s such as arsenic (although mostly in the nontoxic arsenobetaine form), mercury (mostly as

methylmercury), and moderate amounts of lead and cadmium (Bosch et al., 2016). Marine fish also contain high levels of beneficial nutrients such as long-chain polyunsaturated fatty acids, zinc, and vitamin D, which are important for optimal prenatal neurodevelopment (Julvez et al., 2016) and the health of ageing individuals (Morris et al., 2016). Fish are also a source of the essential element selenium (Morris et al., 2016), which has several important physiological functions mediated by selenoproteins in the form of selenocysteine(s) (Pieczyńska and Grajeta, 2015; Rayman, 2012), such as redox regulation (i.e., thyoredoxine reductases), antioxidative functions (i.e., glutathione peroxidases, selenoprotein P), selenium transfer (selenoprotein P) and thyroid hormone regulation (i.e., iodothyronine deiodinases). Furthermore, the well-known mutual antagonism between selenium and arsenic or between selenium and mercury can diminish the toxic effects of mercury and arsenic (Falnoga and Tušek-Žnidarič, 2007; La Port, 2011; Liu et al., 2018; Zeng et al., 2005; Zhang et al., 2014).

Given the suggested mutual metabolic interferences between apoE and metals, and the impact of either on maternal health during pregnancy and prenatal neurodevelopment, we focused our attention on apoE isoforms and various trace elements (TEs). This study aimed to assess whether there is a relationship between *APOE* gene polymorphisms (*ε4* carriers *versus* no carriers) and trace elements in Croatian pregnant mothers and their new-borns from the coastal region of the Adriatic Sea, who have been chronically exposed to low to moderate amounts of environmental mercury and arsenic from seafood consumption (Miklavčič et al., 2013; Bernhard, 1988; Kosta et al., 1978). The trace elements included in this study were mercury (Hg; as total mercury (THg) and methyl mercury (MeHg)), arsenic (As), lead (Pb), and cadmium (Cd), along with the essential TEs selenium (Se), copper (Cu), zinc (Zn), manganese (Mn), calcium (Ca), magnesium (Mg), and iron (Fe),

2. Materials and Methods

2.1. Study population

Mothers (n = 223; 19-44 years) and their new-borns (n = 213; 102 girls, 101 boys, 10 with missing sex classification) from the Adriatic coastal region of Croatia (Rijeka and its surroundings) were recruited in 2007–2009 as part of the wider birth cohort study PHIME (EU 6th Framework Programme).

The PHIME project (Public Health Impact of Long-term Low-level Mixed Element Exposure in Susceptible Population Strata) was designed to study metal exposure and its possible negative effects in people living in the Mediterranean area. Participants were recruited from four countries (Slovenia, Croatia, Italy, and Greece) (Valent et al., 2013b). In our previous investigations, we used cord blood Hg levels to estimate the impact of prenatal mercury exposure on motor, cognitive, and language performance in 18-month old children of the Croatian participants (Prpić et al., 2017), combined Slovenian-Croatian (Snoj Tratnik et al., 2017) and all PHIME participants (Barbone et al., 2019). Studies confirmed slight statistically significant alterations in fine motor skills associated with Hg levels in the Croatian and Slovenian-Croatian cohorts (Barbone et al., 2019; Prpić et al., 2017; Snoj Tratnik et al., 2017) and Hg-associated cognitive score alterations together with a co-effect of children's *APOE* ε4 allele in the Slovenian-Croatian cohort (Snoj Tratnik et al., 2017). In the full set of PHIME participants, a similar alteration was observed in gross motor skills, but the significance was borderline (Barbone et al., 2019). Regarding other exposure biomarkers, a positive relationship was found between language composite scores and maternal hair Hg (in the full PHIME dataset) and with maternal venous blood Hg (Croatian sample) (Barbone et al., 2019). However, the maternal APOE allelic effect on Hg or other TE levels was not tested in any of the previous studies.

In the present study we used only the Croatian dataset, which has the most complete data. The study design (settings, recruitment, exclusion criteria, questionnaires, and biological sampling) are described in detail in previous publications (Miklavčič et al., 2013; Valent et al., 2013a, 2013b). The study was conducted in accordance with the Declaration of Helsinki and its later amendments; the Ethics Committee of the University Hospital Centre Rijeka approved the protocol for the Croatian participants (No. 2170-29-02/1-07-1). Mothers gave their informed consent; recruitment and sampling took place at the University Hospital Rijeka. Participants were sampled during the third trimester of pregnancy for maternal peripheral venous blood (whole blood, plasma, and serum), morning urine, and hair (1–3 cm closest to scalp); at delivery for mixed cord blood (whole blood, plasma and serum), cord tissue, and new-borns' first urine; and one month after delivery for maternal breast milk.

2.2. Study database

Table 1 summarises the basic study protocols for the collected samples, including sampling time, analysed TEs with limits of detection, and DNA extracts. The obtained TE database consisted of nonessential Hg (as THg and MeHg), As, Pb, and Cd, and essential Se, Cu, Zn, Mn, Ca, Mg, and Fe. The TE levels were determined at the Jožef Stefan Institute (JSI, Ljubljana, Slovenia) and the University Medical Centre Ljubljana (UMCL, Institute of Clinical Chemistry and Biochemistry, Ljubljana, Slovenia) using the methods described below.

161 **Table 1.** Basic sampling and analytical data for Croatian cohort of mothers (N=223) and their new-borns (N=213) (PHIME project subgroup).
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Sampling time and participants' samples	TE analysed$_{(LOD)}$	DNA extraction	N of analysed samples (%)
3rd TRIMESTER			
Maternal hair	$Hg_{(0.2\ ng/g)}$, $MeHg^{d}_{(0.02\ ng/g)}$		222 (99.6)
Maternal blood[a]	$Hg_{(0.02\ ng/g)}$, $MeHg_{(0.02\ ng/g)}$, $Pb_{(1.3\ ng/g)}$, $Cd_{(0.12\ ng/g)}$, $As_{(0.13\ ng/g)}$, $Se_{(5\ ng/g)}$, $Mn_{(2\ ng/g)}$, $Cu_{(11ng/g)}$, $Zn_{(20\ ng/g)}$		223 (100)
Maternal plasma	$Se_{(0.16\ ng/mL)}$, $Zn_{(1.6\ ng/mL)}$		212 (95.1)
Maternal serum	Fe, Mg, Ca		209 (93.7)
Maternal leukocytes	-	Blood DNA	223 (100)
Maternal urine[b]	$Hg_{(0.01\ ng/mL)}$, $MeHg^{d}_{(0.003\ ng/mL)}$, $Pb_{(0.1\ ng/mL)}$, $Cd_{(0.04ng/mL)}$, $As_{(0.4\ ng/mL)}$, $Se_{(2.8\ ng/mL)}$, $Mn_{(0.2\ ng/mL)}$, $Cu_{(1\ ng/mL)}$, $Zn_{(2\ ng/mL)}$, SG,		220 (98.7)
AT DELIVERY			
Cord blood[c]	$Hg_{(0.02\ ng/g)}$, $MeHg_{(0.02\ ng/g)}$ $Pb_{(1.3\ ng/g)}$, $Cd_{(0.12\ ng/g)}$, $As_{(0.13\ ng/g)}$, $Se_{(5\ ng/g)}$, $Mn_{(2\ ng/g)}$, $Cu_{(11\ ng/g)}$, $Zn_{(20\ ng/g)}$		205 (91.9)
Cord plasma	$Se_{(0.16\ ng/mL)}$, $Zn_{(1.6\ ng/mL)}$		190 (85.2)
Cord serum	Fe, Mg, Ca		184 (82.5)
Cord tissue	-	Tissue DNA	176 (82.6)
New-born's first urine	$Hg_{(0.02\ ng/mL)}$, SG		126 (59.2)
1 MONTH POST-DELIVERY			
Maternal breast milk	$Hg_{(0.045\ ng/g)}$, $MeHg^{d}_{(0.003\ ng/g)}$, $Pb_{(0.4\ ng/g)}$, $Cd_{(0.04\ ng/g)}$, $As_{(0.04\ ng/g)}$, $Se_{(0.9\ ng/g)}$, $Mn_{(0.4\ ng/g)}$, $Cu_{(6\ ng/g)}$, $Zn_{20ng/g)}$		123 (55.2)

163 N – Sample size; LOD – Limit of detection; SG – Specific gravity; a – Peripheral venous blood;
164 b – Morning urine; c - Mixed venous-arterial blood; d – MeHg analysed in cases when total Hg in maternal hair exceeded 1 μg/g.

Hg levels in maternal hair, blood, and cord blood and in the new-borns' urine were determined by thermal combustion at 650°C, amalgamation and analysis using a direct mercury analyser (DMA; Milestone, USA) (EPA and US EPA (US Environmental Protection Agency), 2007; Miklavčič et al., 2013). Hg in maternal milk and urine was determined using a semi-automated mercury analyser based on cold vapour atomic absorption spectrometry (CVAAS, Model Hg-201; Sanso Seisakusho Co. Ltd., Japan) (Miklavčič et al., 2013).

MeHg in hair was measured by gas chromatography with electron capture detection (GC-ECD, Hewlett-Packard Model 5890; HP/Agilent Technologies; USA) (Horvat and Byrne, 1990). MeHg in maternal blood, cord blood, and breast milk was measured by cold vapour atomic fluorescence detection (CV AFS, Tekran 2700 instrument; Tekran Instruments Corporation, Canada and Brooks Rand Model III, Brooks Rand Instruments, USA) (Liang et al., 1994).

Hg^{2+} levels in blood, cord blood, hair, and milk were obtained by subtracting MeHg from THg.

As, Cd, Pb, Se, Cu, and Zn in blood, cord blood, breast milk, and urine were previously prepared (Barany et al., 1997) and analysed by inductively coupled plasma mass spectrometry (ICP-MS; 7500ce, Agilent, Tokyo, Japan) equipped with an ASX-510 Auto sampler (Cetac). The mass spectral interferences were eliminated using an Octapole Reaction System (ORS) with helium, or hydrogen in case of Se determination. Urine samples only were analysed without digestion.

Se in plasma was measured using a Zeeman electrothermal AAS (Varian SpektrAA-800 ETAA spectrometer; Varian Australia Pty. Ltd., Mulgrave, Victoria, Australia) (Kobal et al., 2004).

Zn in plasma was measured by flame AAS (FAAS, deuterium) using a Varian SpektrAA-250 Plus FAAS (Varian Australia Pty. Ltd., Mulgrave, Victoria, Australia) (Tsalev and Zaprianov, 1983).

Ca, Mg, and Fe(III) in serum were determined spectrophotometrically using their complexes with diazonium salt xilidyl blue (for Mg at 505 - 660 nm), o-cresolphtalein (for Ca at 546 and 660 nm) and ferrozine (for Fe at 570 nm). All measurements were performed on a Roche Hitachi 917 analyser calibrated by C.f.a.s. (Roche, Switzerland). To control the method accuracy and precision, we used Control Sera presenting normal ranges (PerciNorm U, Roche) and pathological ranges (PerciPath U, Roche) of the analysed essential elements.

Urine concentrations were adjusted for specific gravity (SG) using an Atago® PAL-10S Refractometer (Japan). Adjustments were performed according to Santonen et al. (2015) as follows: $c_s = c_o \times (SG_{ref} - 1.000)/(SG_o - 1.000)$, where c_s is the corrected concentration; c_o, the observed concentration; SG_{ref}, the SG reference value; and SG_o, the observed specific gravity. SG_{ref} is a population reference value representing normal or undiluted urine. SG_{ref} was 1.021, that is, the middle value of the SG reference range of 1.018–1.024 for the general population. For multivariate statistics, we used raw urine data SG_o as the independent variable.

All measurements were performed under strict quality control procedures and gave comparable results. The limits of detection (LODs), calculated as three times the standard deviation of the blank sample, are included in Table 1. The reference materials Seronorm™ Trace Elements in Whole Blood L-1, and Seronorm™ Trace Elements in Serum L-1 were used to check the accuracy of the methods. Becton Dickinson tubes for trace elements (7 mL) were used for blood sampling.

Personal characteristics (e.g., age, pre-pregnancy body mass index (BMI), parity, estimated gestation week at sampling (EGW) and at delivery (EGA)), and lifestyle data (e.g., seafood consumption, smoking habits, education, employment, supplement intake) were obtained through two subsequent questionnaires: the first during pregnancy and the second

during breastfeeding. Parity was defined as the number of times a participant carried a pregnancy to a viable stage (> 20 weeks of gestation). Self-reported smokers during pregnancy (8.5%) and self-reported former smokers (32%; defined as those quitting smoking immediately before or at the beginning of the pregnancy) were combined in the group 'ever-smokers' (40.5%). Daily seafood intake was estimated from the second questionnaire with several questions on seafood that addressed the frequency (never, 1x/month, 1–3x/month, 1x/week, 2–4x/week, 5–6x/week, 1x/day, 2–3x/day, more than 3x/day) that 150-g servings of fish, crustaceans, molluscs (boiled, grilled, fried, baked, or in oil) was consumed. Selected characteristics data stratified by maternal genotyping results (presence versus absence of ε4 allele) are presented in the results (Section 3.2).

2.3. *APOE* genotyping

Archived high molecular DNA extracts (stored at -80°C) from maternal leukocytes and umbilical cord tissues were provided by the Faculty of Pharmacy, University of Ljubljana. DNA extraction was performed using a High Pure PCR Template Preparation Kit (Roche) and a QIA amp DNA Mini Kit (Qiagen, USA). The DNA was genotyped using a 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, USA) according to the given procedure. A LightCycler 480 II (Roche) was used to identify polymorphisms: rs429358 (c.334T > C; Cys112Arg) and rs7412 (c.472C > T; Arg158Cys). Based on these results, we determined the genotype of each individual. The distribution of the *APOE* genotype frequencies were in Hardy-Weinberg equilibrium ($p > 0.05$; Chi-square test). Mothers and new-borns were then divided into ε4 carriers (genotypes *APOE* ε3/*ε4* and *ε4/ε4*) versus *ε4* non-carriers (genotypes *APOE ε3/ε3*, *ε3/ε2*, and *ε2/ε2*). Carriers of the genotype *ε2/ε4* were excluded from both groups because specific properties of *ε4* are weakened when the *ε4* allele is combined with the *ε2* allele; apoE2/4 proteins are suspected to functionally resemble apoE3/3 proteins.

2.4. Statistical analyses

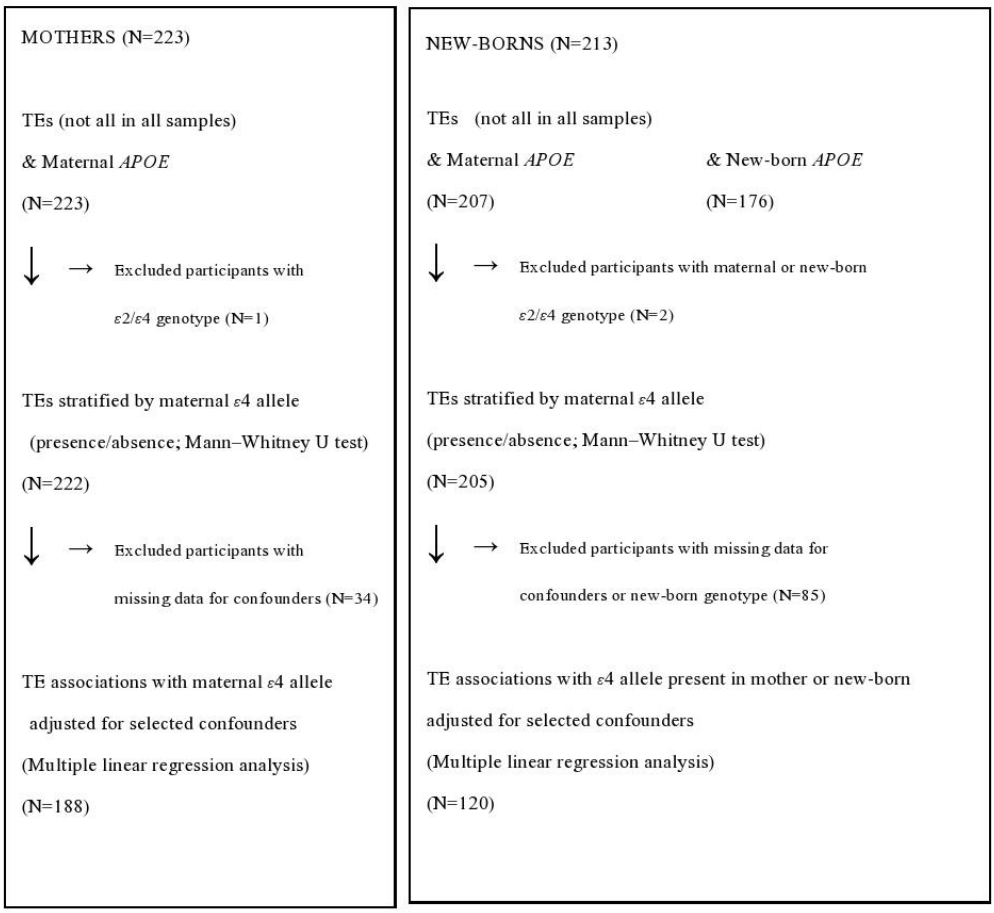
First, we divided all samples into two groups, *ε4+* and *ε4-*, according to maternal genotype. The distribution of TEs in the two groups was compared using the Mann-Whitney U test. The influence of *ε4* on TE concentration controlling for the other explanatory variables (obtained through questionnaires) was examined using multiple linear regression models. All dependent variables, such as TE levels in maternal blood, serum/plasma, hair, urine, and milk were normal log-transformed to avoid heteroscedasticity. The independent variables included the presence of maternal *APOE* allele ε4, presence of parity, maternal pre-pregnancy BMI, maternal age, seafood intake (servings/day), ever-smoking, and urine SG_o. We extended independent variables for mixed cord blood and cord plasma/serum with newborns' characteristics: EGA, birth weight, and sex. According to Gundacker et al. (2000), *"the maternal genotype determines the placental environment (...), while the foetal genotype determines the capacity of the placenta to cope with this environment"*. Given the supposed complementary effects of maternal/foetal genotype on (mixed) cord blood and placenta, we analysed the combined effect of maternal/foetal *APOE* allele ε4 using the presence of at least one allele ε4 in either the mother or new-born as an explanatory variable. A flow chart of the number of participants included in the statistical analyses is shown in **Figure 1**.

Multiple regression model diagnostics were performed based on an analysis of the residuals. All models were checked for collinearity. The *p*-values for the individual effect of each explanatory variable were corrected according to the simultaneous testing of numerous null hypotheses using the glht function from the multcomp package in the R statistical package (Team, 2017). All TEs were log-transformed for regression modelling, which means

that an estimate of the model parameter multiplied by 100 represents the percentage change in the TE levels for a one-unit increase in each numeric explanatory variable (e.g., 1 seafood serving/day, 1 year for age, etc.) given that all other explanatory variables remain constant. For binary explanatory variables (presence/absence), an estimate of the model parameter multiplied by 100 represents the percentage difference between the two groups. The coding for binary variables (0/1; no/yes) was as follows: *APOE* ε4 = 0 for non-carriers, and *APOE* ε4 = 1 for carriers; parity = 0 for absence of deliveries, and parity = 1 for 1–3 deliveries; ever-smoking = 0 for non-smoking, and ever-smoking = 1 for smoking during or before pregnancy; and sex = 0 for boys, and 1 for girls.

EGW at sampling in the third semester, supplement intake, education level, and employment data (yes/no) were not included in the explanatory variables to avoid over-fitting the regression models or diminishing the number of participants. EGW and supplement intake were available for a relatively low subset of samples (148 and 139, respectively). Further, we had a population with known low social differences: the majority of participants were employed (78%) and had finished at least middle school (98%). All participants had finished elementary school. In pre-testing models, the addition of EGW or education level (primary versus secondary or more) together with employment (yes/no) or supplement intake did not modify the associations between *APOE* and TEs.

284



285

286 **Figure 1.** Flow chart on participants included in statistical analyses.

287 **3. Results**

288 **3.1 Frequencies of *APOE* genotypes and alleles**

289 Frequencies of six possible genotypes (% ε2/ε2, % ε2/ε3, % ε2/ε4, % ε3/ε3, % ε3/ε4,
290 ε4/ε4) and individual alleles (% ε2, % ε3, % ε4) are listed in the supplemental material (**Table**
291 **S1**). The frequencies were similar for mothers and their new-borns and geographically
292 matched those reported in the literature (Giau et al., 2015; Huebbe and Rimbach, 2017).
293 Genotype ε3/ε3 was the most prevalent (74% of mothers, 68.2% of new-borns) followed by
294 ε3/ε4 (16.1% of mothers, 17.6% of new-borns) and ε2/ε3 (8.5% of mothers, 10.8% of new-
295 borns). The other three genotypes, ε2/ε2, ε2/ε4 and ε4/ε4 were present in minor or even zero
296 percentages (0.5%, 0.5%, and 0.5% of mothers; 0%, 1.1% and 2.4% of new-borns).
297 Excluding the ε2/*ε4* genotype (n = 3), we identified 16.7% (n = 37) and 20.1% (n = 35) of
298 mothers and new-borns, respectively, to be ε4 allele carriers.

299

300 **3.2 Participants' personal characteristics stratified by maternal ε4 allele**

301 **Table 2** lists the basic personal characteristics of the mothers and new-borns. Only one
302 mother and two new-borns were omitted for having the genotype ε2/ε4. For new-borns, data
303 for all cases with the identified genotype of their mother (but their own genotype could be
304 missing) were included. The average maternal age was 30.1 ± 4.7 years and EGA at delivery
305 was 39.4 ±1.2 weeks. The majority of women were *nulliparous* or *primiparous* (56% and
306 37%, respectively). Except for seafood intake (servings/day), the distribution of the personal
307 characteristics did not differ significantly by the presence or absence of maternal ε4 alleles.
308 The estimated seafood frequency intake was higher in mothers carrying the ε4 allele (p =
309 0.038, **Table 2**), whereas the number of ever-smokers was lower (35% ε4+ versus 42% ε4-).
310 Employment and education status were relatively high overall, as mentioned in section 2.4.
311

312 **Table 2.** Selected characteristics for Croatian cohort of mothers and new-borns stratified by maternal
313 *APOE* ε4 allele*.
314

Participants	All x ± SD (range)	N (%)	ε4+ x ± SD (range)	N (%)	ε4- x ± SD (range)	N (%)	p
MOTHERS (m)		222		37		185	
m Age (years)	30.1 ± 4.8 (19 - 44)	209	30.8 ± 4.6 (20 - 38)	36	29.9 ± 4.8 (19 - 44)	173	0.143
m BMI (kg/m^2)	23.0 ± 4.2 (17 - 41)	220	22.3 ± 3.2 (18 - 32)	36	23.2 ± 4.3 (17 - 41)	184	0.334
m Seafood intake (servings/day)	0.45 ± 0.3 (0- 2.2)	195	0.54 ± 0.3 (0.1 - 1.2)	33	0.43 ± 0.3 (0 - 2.2)	162	**0.038**
m Parity**	(0 - 3)	220	(0 – 2)	37	(0 – 3)	183	0.835
0		125 (56.8)		21 (56.8)		104 (56.8)	
1 - 3		95 (43.2)		16 (43.2)		79 (43.2)	
m Ever-smoking		222		37		185	0.463
Yes		90 (40.5)		13 (35.1)		77 (41.6)	
No		132 (59.5)		24 (64.9)		108 (58.4)	
m Education ***		195		32		163	0.352
Primary		4 (2.1)		0 (0)		4 (2.5)	
Secondary or more		191 (97.9)		32 (100)		159 (97.5)	
m Employment		192		31		161	0.556
Yes		174 (91.6)		29 (93.5)		145 (90.1)	
No		18 (9.4)		2 (6.5)		16 (9.9)	
m Use of supplements (Yes)		139		24		115	
m EGW (weeks)	37 ± 2.5 (35 - 41)	148	36.9 ± 1.8 (35 - 40)	22	37.1 ± 2.6 (35 - 41)	126	0.770
NEW-BORNS (nb)		207		34		173	
nb EGA (weeks)	39.4 ± 1.2 (35 - 41)	165	39.1 ± 1.4 (36-41)	25	39.5 ± 1.2 (35-41)	140	0.205
nb BW (kg)	3.52 ± 0.5 (1.8 - 4.8)	192	3.60 ± 0.6 (2.5-4.7)	33	3.50 ± 0.4 (2.4-4.8)	159	0.704
nb Sex		203					
Boys		101 (49.8)		18 (50)		83 (49.7)	
Girls		102 (50.2)		18 (50)		84 (50.3)	

315 x - arithmetical mean; SD - standard deviation; *p* - statistically significant difference between groups according to maternal genotype (ε4+ and ε4-),
316 Mann-Whitney U test for all except for parity, education, employment, and ever-smokers, where χ^2 test was used; BMI - pre-pregnant body mass index;
317 EGW - estimated gestation week at maternal sampling at 3rd trimester; EGA - estimated gestational age at delivery; BW - birth weight; * Excluded were
318 one mother with maternal *ε2/ε4* genotype and three new-borns with either maternal or new-born's *ε2/ε4* genotype; **Parity - number of deliveries after
319 20th gestation week; ***Education defined by two groups: primary with elementary diploma and secondary or higher (middle school diploma + high
320 school diploma + university degree).

321 **3.3. TE levels stratified by maternal ε4 allele**

322 **Table 3** lists mothers' and new-borns' TE levels stratified by the presence or absence of
323 maternal ε4 allele. The mean blood levels for potentially toxic metals such as Hg, Cd, and Pb
324 were low in general. MeHg accounted for an average of 80% of the total mercury in maternal
325 and cord blood (mean value). Almost all urine arsenic was in the nontoxic arsenobetaine form
326 (90% of total As) (Stajnko et al., 2019). Breast milk had low mean levels of Hg, Pb, Cd, and
327 As, meaning that breastfed infants were not at risk in either group. Selenium levels in breast
328 milk in both groups were almost equal. The mean values of all essential elements were within
329 UMCL laboratory reference levels.
330 Statistically significant differences in the distribution of TEs between the maternal ε4+
331 and ε4- groups were observed for As, (Me)Hg, Se, and Zn (**Table 3**). Mothers with the ε4
332 allele (genotypes *APOE* ε3/ε4 and ε4/ε4) had significantly higher levels ($p < 0.05$) of (Me)Hg,
333 As, and Se in blood; Se in plasma; Hg in hair; As in urine; and (Me)Hg and As in cord blood.
334 Although Zn levels in cord blood were significantly lower in the ε4+ group, this has low
335 importance because plasma/serum levels are the only recommended biochemical indicator
336 for Zn status assessment (Gibson et al., 2008). **Figure 2** shows the distribution of each
337 mentioned TE in boxplots. There was little difference in the Hg and As levels in maternal
338 blood (2.67 *vs* 2.03 ng/g for Hg and 3.31 *vs* 2.02 ng/g for As (geometrical means)), while the
339 differences in Se were more pronounced (99.4 *vs* 90.1 ng/mL for maternal blood and 61.7 *vs*
340 53.7 ng/mL for maternal plasma). Larger differences were observed in the distribution of Hg
341 in maternal hair (0.74 *vs* 0.48 μg/g) and As in maternal urine (42.6 *vs* 23.3 ng/mL).

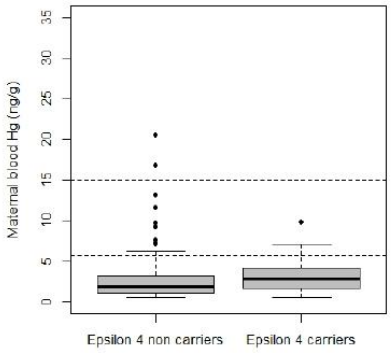
342 **Table 3.** TE levels in samples for Croatian cohort of mothers and their new-borns stratified by

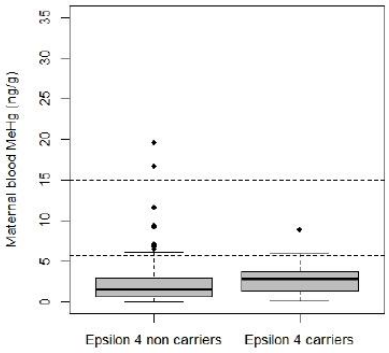
343 maternal *APOE* ε4 allele*.

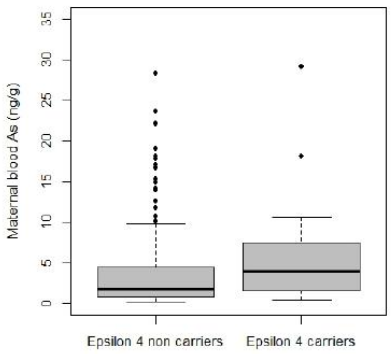
TE	ε4+ GM, range, Me, (N)	ε4- GM, range, Me, (N)	All GM (N)	p
MATERNAL BLOOD				
mB Hg ng/g	**2.67, 0.59-9.83, 2.85 (37)**	**2.03, 0.55-20.5, 1.93 (184)**	**2.12 (221)**	**0.0111**
mB MeHg ng/g	**2.13, 0.12-8.90, 2.84 (37)**	**1.38, 0.03-19.6, 1.64 (184)**	**1.47 (221)**	**0.0105**
mB IHg ng/g	0.17, 0.01-1.19, 0.33 (37)	0.18, 0.01-12.6, 0.37 (184)	0.12 (221)	0.8554
mB Pb ng/g	12.8, 5.25-53.6, 12.78 (35)	12.0, 3.58-87.6, 11.55 (180)	12.1 (215)	0.4909
mB Cd ng/g	0.50, 0.06-3.70, 0.47 (35)	0.47, 0.06-3.69, 0.51 (180)	0.48 (215)	0.7294
mB As ng/g	**3.31, 0.42-29.2, 3.96 (35)**	**2.02, 0.25-37.3, 1.76 (180)**	**2.19 (215)**	**0.0144**
mB Se ng/g	**99.4, 67.9-142, 99.4 (35)**	**90.1, 42.4-182, 89.2 (180)**	**91.6 (215)**	**0.0080**
mB Mn ng/g	13.7, 7.45-26.5, 13.8 (35)	15.3, 3.80-49.4, 15.0 (180)	15.1 (215)	0.0850
mB Cu µg/g	1.49, 1.06-2.02, 1.53 (35)	1.56, 0.91-2.51, 1.56 (180)	1.55 (215)	0.2719
mB, Zn µg/g	6.21, 4.58-10.27, 6.03 (35)	6.19, 2.90-11.04, 6.12 (180)	6.19 (215)	0.7283
MATERNAL PLASMA				
mP Se ng/mL	**61.7, 41.0-96.0, 62.0 (36)**	**53.7, 33.0-90.0, 54.0 (176)**	**55.0 (212)**	**0.0006**
mP Zn µg /mL	0.75, 0.52-1.08, 0.74 (36)	0.76, 0.46-1.14, 0.73 (176)	0.76 (212)	0.8137
MATERNAL SERUM				
mS Ca µg /mL	89.4, 29.7-104.6, 92.2 (35)	89.8, 50.5-117.4, 91.0 (174)	89.7 (209)	0.2357
mS Mg µg /mL	16.4, 5.10-20.7, 16.8 (35)	16.6, 9.48-20.9, 16.8 (174)	16.6 (209)	0.5745
mS Fe µg /mL	0.79, 0.20-4.94, 0.83 (35)	0.85, 0.22-2.94, 0.86 (174)	0.84 (209)	0.4169
MATERNAL URINE (SG corrected)				
mU Hg ng/mL	0.81, 0.16-5.94, 0.69 (33)	0.73, 0.03-30.8, 0.66 (141)	0.74 (180)	0.5431
mU Pb ng/mL	0.83, 0.08-3.58, 0.93 (31)	0.73, 0.04-8.33, 0.95 (141)	0.75 (172)	0.9238
mU Cd ng/mL	0.38, 0.03-1.48, 0.44 (31)	0.40, 0.04-1.98, 0.42 (141)	0.40 (172)	0.9033
mU As ng/mL	**42.6, 2.83-260, 66.4 (31)**	**23.3, 1.58-1679, 22.5 (141)**	**26.0 (172)**	**0.0233**
mU Se ng/mL	25.2, 5.98-56.6, 28.5 (31)	26.5, 3.71-73.8, 28.6 (141)	26.3 (172)	0.9381
mU Mn ng/mL	0.52, 0.08-19.1, 0.41 (31)	0.40, 0.06-22.7, 0.36 (144)	0.42 (176)	0.9142
mU Cu µg/mL	0.017, 0.002-0.057, 0.018 (31)	0.020, 0.008-0.545, 0.019 (141)	0.019 (172)	0.4245
mU Zn µg/mL	0.37, 0.59-1.36, 0.34 (31)	0.49, 0.066-2.96, 0.49 (141)	0.46 (172)	0.0828
mU SG	1.0163, 1.002-1.029, 1.016 (33)	1.0159, 1.005-1.035, 0.016 (149)	1.016 (182)	0.6529
MATERNAL MILK				
mM Hg ng/g	0.19, 0.04-1.82, 0.20 (25)	0.13, 0.01-2.36, 0.13 (98)	0.14 (123)	0.1411
mM MeHg ng/g	0.17, 0.09-0.33, 0.17 (6)	0.14, 0.04-0.55, 0.14 (19)	0.15 (25)	
mM IHg ng/g	0.09, 0.01-0.46, 0.12 (6)	0.07, 0.01-0.9, 0.09(19)	0.08 (25)	
mM Pb ng/g	0.92, 0.20-5.70, 1.08 (24)	0.54, 0.20-11.1, 0.52 (97)	0.60 (121)	0.0572
mM Cd ng/g	0.12, 0.02-1.12, 0.12 (24)	0.10, 0.02-6.54, 0.10 (97)	0.10 (121)	0.3690
mM As ng/g	0.25, 0.02-2.18, 0.21 (23)	0.21, 0.02-19.0, 0.21 (97)	0.22 (120)	0.6412
mM Se ng/g	18.6, 8.70-29.0, 18.5 (24)	18.3, 8.43-48.8, 17.5 (97)	18.4 (121)	0.4908
mM Mn ng/g	3.38, 1.10-22.0, 2.94 (24)	2.66, 0.67-17.1, 2,82 (97)	2.79 (121)	0.2608
mM Cu µg/mL	0.55, 0.24-0.86, 0.61 (24)	0.48, 0.14.1.05, 0.50 (97)	0.49 (121)	0.0898
mM Zn µg/mL	2.92, 0.73-9.73, 3.15 (24)	2.48, 0.21-9.37, 2.63 (97)	2.56 (121)	0.1936
MATERNAL HAIR				
mH Hg µg/g	**0.74, 0.08-3.45, 0.84 (37)**	**0.48, 0.02-8.71, 0.56 (185)**	**0.51 (222)**	**0.0107**
mH MeHg µg/g	1.76, 1.15-3.33, 1.74 (10)	1.78, 0.93-8.71, 1.65 (35)	1.78 (45)	
mH IHg ng/g	1.82; 0.1-135; 4.0 (10)	5.30; 0.1-1302; 30.5 (35)	4.18 (45)	
CORD BLOOD				
cB Hg ng/g	**4.01, 0.82-21.8, 3.96 (34)**	**2.74, 0.33-32.3, 2.83 (171)**	**2.92 (205)**	**0.0137**
cB MeHg ng/g	**3.59, 0.29-21.7, 3.96 (34)**	**2.26, 0.16-31.9, 2.70 (171)**	**2.44 (205)**	**0.0181**
cB IHg ng/g	0.07, 0.01-2.60, 0.08 (34)	0.07, 0.01-4.79, 0.10 (171)	0.07 (205)	0.7897
cB Pb ng/g	8.64, 3.23-17.7, 877 (34)	7.87, 1.82-34.1, 8.19 (168)	8.00 (202)	0.9407
cB Cd ng/g	0.08, 0.06-0.30, 0.06 (34)	0.09, 0.06-1.21, 0.06 (168)	0.09 (202)	0.9407
cB As ng/g	**2.60, 0.29-25.7, 2.59 (34)**	**1.84, 0.33-31.8, 1.42 (168)**	**1.95 (202)**	**0.0475**
cB Se ng/g	94.4, 64.7-132, 90.1 (34)	94.4, 54.8-163, 96.0 (168)	94.4 (202)	0.9231
cB Mn ng/g	29.7, 16.0-53.9, 29.2 (34)	30.7, 7.23-77.3, 31.6 (168)	30.5 (202)	0.5034
cB Cu µg/g	0.69, 0.50-0.99, 0.68 (34)	0.69, 0.36-1.16, 0.69 (168)	0.69 (202)	0.9078
cB, Zn µg/g	**2.13, 1.58-3.18, 2.07 (33)**	**2.30, 1.16-4.72, 2.21 (167)**	**2.27 (200)**	**0.0456**
CORD PLASMA				
cP Se ng/mL	41.1, 25.0-65.0, 41.5 (30)	40.8, 24.0-71.0, 42.0 (160)	40.8 (190)	0.9509
cP Zn µg /mL	0.91, 0.55-1.38, 0.92 (30)	0.99, 0.39-1.77, 0.99 (159)	0.98 (198)	0.1023
CORD SERUM				
cS Ca µg /mL	101.5, 63.3-144.7, 104.6 (31)	101.5, 60.1-127.1, 103.4 (153)	101.5(184)	0.7631
cS Mg µg /mL	20.3, 16.3-27.0, 19.7 (31)	19.9, 14.6-27.2, 19.7 (152)	19.9 (183)	0.6713

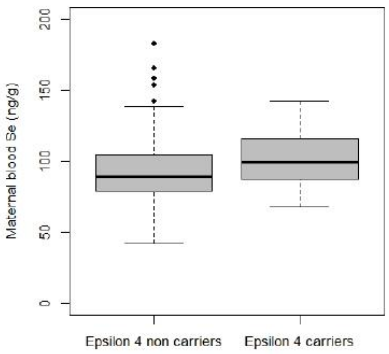
cS Fe µg /mL	1.45, 0.75-2.87, 1.44 (31)	1.33, 0.28-2.45, 1.37 (153)	1.35 (184)	0.3207
NEW-BORN URINE (SG corrected)				
nb U Hg ng/mL	0.45, 0.10-1.76, 0.60 (15)	0.31, 0.04-8.64, 0.37 (73)	0.33 (88)	0.0542
nb U SG	1.010, 1.002-1.019, 1.009 (18)	1.012, 1.002-1.029, 1.013 (83)	1.012 (101)	0.1285

GM - geometrical mean; Me – median; N – sample size; SG - specific gravity; p - statistical significance of difference between ε4 carriers and non-carriers obtained using the Mann-Whitney U test, bolded $p < 0.05$; m - maternal; c - cord; nb – new-born child, B - blood; P - plasma: S - serum; U - urine; M - milk; H – hair; * excluded were one mother with maternal *ε2/ε4* genotype and three new-borns with either maternal or new-born *ε2/ε4* genotype

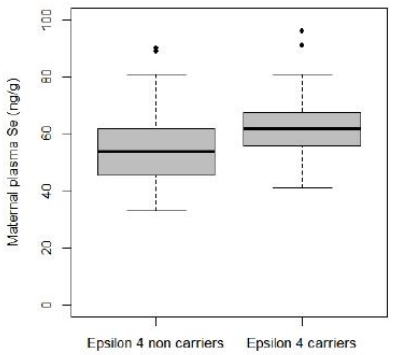

Maternal blood Hg (ng/g)
0
5
10
15
20
25
30
35
Epsilon 4 non carriers
Epsilon 4 carriers

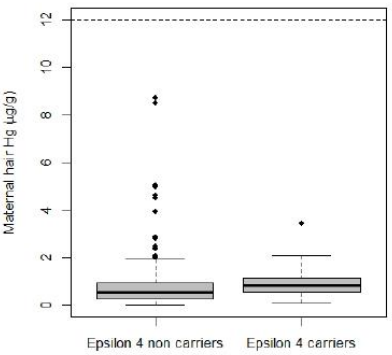

Maternal blood MeHg (ng/g)
0
5
10
15
20
25
30
35
Epsilon 4 non carriers
Epsilon 4 carriers

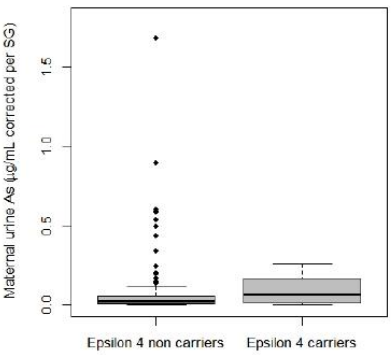

Maternal blood As (ng/g)
0
5
10
15
20
25
30
35
Epsilon 4 non carriers
Epsilon 4 carriers


Maternal blood Se (ng/g)
0
50
100
150
200
Epsilon 4 non carriers
Epsilon 4 carriers

348


Maternal plasma Se (ng/g)
0
20
40
60
80
100
Epsilon 4 non carriers
Epsilon 4 carriers


Maternal hair Hg (µg/g)
0
2
4
6
8
10
12
Epsilon 4 non carriers
Epsilon 4 carriers


Maternal urine As (µg/mL corrected per SG)
0.0
0.5
1.0
1.5
Epsilon 4 non carriers
Epsilon 4 carriers

349

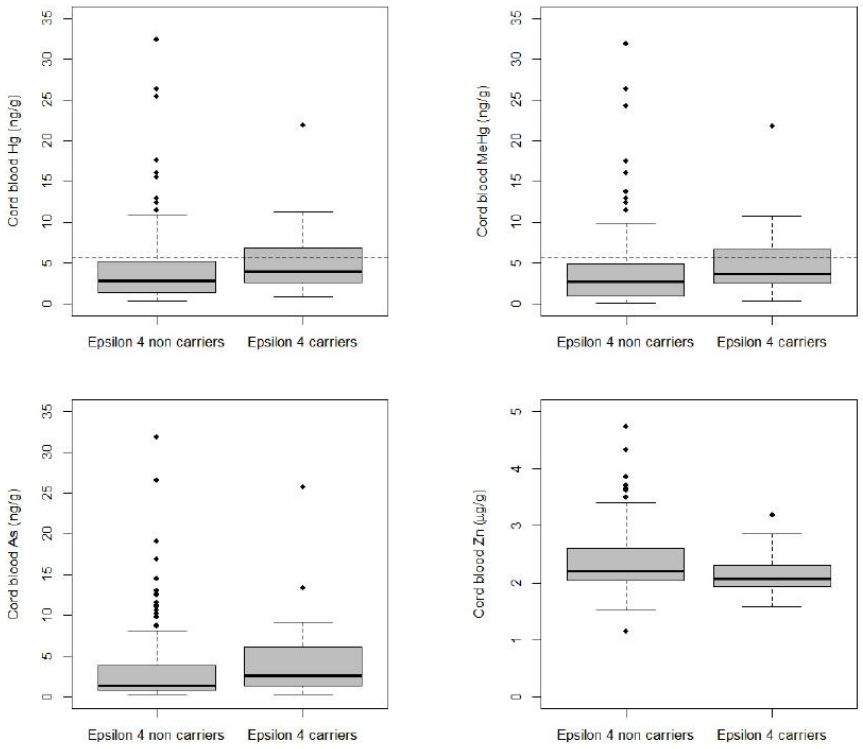


Figure 2. TEs in maternal blood, plasma, hair, urine, and cord blood stratified by the presence of maternal *APOE* ε4 *allele.* Boxplot data distribution showing the median, 25th, and 75th percentiles and outliers; horizontal dotted lines represent alert and intervention levels for maternal blood (5.8ng/mL; 15 ng/mL) and hair Hg (> 12 µg/g) regarding the new-born's health (Taylor et al., 2014; Ewers et al., 1999; JECFA, n.d.).

3.4. Linear regression models

Tables 4 and 5 summarise the statistically significant associations between TEs and *APOE* ε4 and other independent variables obtained by the linear regression models. For maternal samples, we used the presence of the maternal ε4 allele; for new-borns, the combined involvement of maternal and foetal ε4 allele was considered (at least one ε4 allele in mother or child versus none). Estimates for TEs without any statistically significant or marginally significant association are not presented (e.g., Pb, Mn, Cu, Zn, Ca, Mg, and Fe).

The differences previously observed by simple comparison shown in **Figure 2** and **Table 3** (Mann-Whitney U test) were nearly all lost in the regression models, except for maternal plasma selenium. The association between the ε4 allele and maternal plasma selenium persisted even after considering the influence of parity, maternal BMI, age, seafood intake, and ever-smoking (**Table 4**). The presence of the ε4 allele was associated with a 12% increase in the mean selenium level in maternal plasma (95% CI 1.2%–22.7%). Further, the regression models did not reveal any new influence of the maternal and/or new-born ε4 allele on the TE

levels in any sample type (**Tables 4 and 5**). However, they did confirm modifying effects of five explanatory variables on TE levels: positive effects of seafood intake > ever-smoking >> and age, and negative effect of parity >> and BMI on (Me)Hg, As, Se, or Cd levels.

In total, the models explained 10%–39% of the variation (R^2) in the TE levels: ~30% in the case of (Me)Hg in maternal and cord blood and maternal hair, ~20% for As in maternal and cord blood and maternal urine, and 12% for As in breast milk. They also explained 14% and 10% of the variation of Se in maternal blood and plasma, respectively, and ~22% and ~33% of the variation of Cd in maternal blood and urine, respectively.

The associations between *APOE* polymorphisms and metalloids were additionally tested by considering the presence of either foetal (new-born) or maternal ε4 allele separately (data not shown). No significant associations were observed.

3.4.1 *Influence of confounders*

Seafood intake had a significant positive influence on (Me)Hg levels in maternal and cord blood and on Hg in maternal hair. It also had a significant positive influence on As levels in maternal and cord blood, maternal urine, and breast milk. The influence of seafood on As excretion through urine and milk was most likely from biologically inactive and readily excreted arsenobetaine, known to be present in seafood (Taylor et al., 2017) and previously identified in high percentages of total As in the urine samples of the present study (Stajnko et al., 2019). In general, we estimated that an extra serving of seafood per day (150 g) resulted in a 92.4% (95% CI 53.5%-131.3%) increase in maternal blood Hg levels. For other mentioned samples and both metals, As and (Me)Hg, the increase was similar or even higher.

The impact of ever-smoking resulted in a 60.4% (95% CI 31.5%–89.3%) increase in the blood Cd level. At the same time, an increase in age (1 year) resulted in a 5.3% increase in urine Cd levels (95% CI 1.4%–9.2%).

Inverse associations were observed for parity (with maternal blood Hg, Se, and Cd and cord blood Hg) and to a lesser degree for BMI (with hair Hg, blood Hg, and blood As). The presence of previous deliveries (1-3) was associated with a 31.5% decrease in the average Hg level in maternal blood and a 47.8% decrease in cord blood. The decreased level of maternal blood Cd was almost the same, 30.2%, although only marginally significant, and that of maternal blood Se was lower (10.4%). Notably, parity did not affect maternal plasma Se levels; therefore, erythrocytes are the main candidates for Se decrease in maternal blood. They contain Gpx1; when the Se supply for selenoprotein synthesis is limited, Gpx1 is among those selenoproteins that can disappear fast (Brigelius-Flohe and Maiorino, 2013; Burk and Hill, 2015).

Table 4. Influence of maternal APOE allele (ε4 +), parity, BMI, age, seafood intake, and ever-smoking on mothers' TE levels (estimated by multiple regression models[a]).

Metal(loid)	m ε4 + (yes)	Parity (yes)	m BMI (kg/m²)	m Age (years)	Seafood intake (serv./day)	Ever-smoking (yes)	Constant	N	R^2 (%)	Chi^2
mB Hg	.123 (.119)	**-.315** (-.569, -.060)**	-.026° (-.053, .002)	**.039*** (.012, .066)**	**.924*** (.535, 1.313)**	-.010 (.091)	-.103 (.391)	187	31	**69.2***(df =6)
mB MeHg	.162 (.177)	-.357° (-.734, .020)	**-.050** (-.091, -.009)**	**.064*** (.024, .104)**	**1.414*** (.838, 1.990)**	-.016 (.136)	-.819 (.580)	187	33	**75.5***(df =6)
mH Hg	.183 (.178)	-.252 (.143)	**-.053** (-.100, -.012)**	**.052** (.011, .092)**	**1.239*** (.656, 1.823)**	.036 (.030)	5.450 (.586)	188	28	**61.8***(df =6)
mB As	.329 (.211)	-.400 (.168)	-.045 ° (-.093, .003)	.037 (.018)	**1.248*** (.567, 1.930)**	-.048 (.158)	.326 (.677)	183	23	**48.5***(df =6)
mU As	.435 (.290)	-.227 (.242)	-.064 (.028)	.028 (.027)	**1.210* (.133, 2.288)**	-.151 (.233)	-71.07 (18.7)	148	19	**30.4***(df = 7)#
mM As	.136 (.438)	-.412 (.378)	.008 (.042)	-.009 (.041)	**2.325** (.561, 4.088)**	-.002 (.377)	-2.215 (1.550)	109	12	**14.4*** (df = 6)
mB Se	.077 (.042)	**-.104* (-.193, -.016)**	.004 (.003)	**.012** (.003, .021)**	.114 (.051)	0.010 (.032)	4.138 (.135)	183	14	**27.0***** (df = 6)
mP Se	**.120*(.012, .227)**	-.032 (.033)	.001 (.004)	.007 (.004)	.079 (.050)	.024 (.031)	3.717 (.138)	179	10	**18.9**** (df = 6)
mB Cd	.059 (.145)	-.302 ° (-.608, 0.003)	-.019 (.012)	.003 (.012)	.137 (.176)	**.604*** (0.315, 0.893)**	-.468 (.464)	183	22	**45.5***** (df = 6)
mU Cd	-.096 (.157)	-.110 (.130)	-.002 (.015)	**.053** (.014, .092)**	-.069 (.217)	.292 (.126)	87.3 (10.1)	148	39	**73.8***** (df = 7)#

[a] Summarised data with statistically significant effects of independent variables on TE levels; °p < 0.10, * p < 0.05, ** p < 0.01; and *** p < 0.001; 95% confidence limits for the parameters are added if the effect was statistically significant and the standard error of the estimated parameter otherwise; R^2 - percentage of variability of metal(loid) level explained by the model; Chi^2 - significance of the model; excluded was one mother with maternal *ε2/ε4* genotype; m - maternal; B - blood; P - plasma; M - milk; U - urine; H - hair; N - number of observations; # for urine samples, an additional independent variable SG was included in the multiple regression models; its effects are **73.1***** (95% CI: 23.5–122.7) for As, **83.1***** (95% CI: 56.3–110.0) for Cd; statistically significant results are indicated in bold.

415 **Table 5.** Influence of maternal and/or new-born APOE allele (ε4 +), parity, m BMI, m age, seafood intake, m
416 ever-smoking, nb birth weight, nb sex, nb EGA on new-borns' TE levels in cord blood (estimated by multiple
417 regression models[a]).

	cB Hg	cB MeHg	cB As
m or/and nb ε4 + (yes)	-.025 (.167)	-.030 (.203)	-.074 (.210)
m Parity (yes)	**-.478** (-.883, -.073)**	**-.551* (-1.042, -.059)**	-.264 (.194)
m BMI (kg/m^2)	-.026 (.016)	-.037 (.020)	-.043 (.021)
m Age (years)	.035 (.016)	.040 (.019)	.042 (.021)
m Seafood intake (serv./day)	**1,143*** (.536, 1.751)**	**1.341*** (.604, 2.078)**	**.895* (.095, 1.695)**
m Ever-smoking (yes)	-.097 (.146)	-.083 (.176)	-.076 (.192)
nb EGA (weeks)	-.074 (.067)	-.097 (.080)	.035 (.087)
nb Birth weight (kg)	.0002 (.0002)	.0003 (.0002)	.0004 (.0002)
nb Sex (girl)	.037 (.142)	-.021 (.172)	.017 (.188)
Constant	2.700 (2.526)	3.088 (3.061)	-2.508 (3.290)
N	120	120	119
R^2 (%)	32	31	19
Chi^2 (df = 9)	**46.4*** **	**45.3*** **	**24.4*** **

418 [a] Summarised data with statistically significant effects of independent variables on TE levels; * $p < 0.05$; ** $p <$
419 0.01; and *** $p < 0.001$; 95% confidence limits for the parameters are added if the effect was statistically significant
420 and standard error of the estimated parameter otherwise; R^2 - percentage of variability of level explained by the
421 model; Chi^2 - significance of the model; * excluded were one mother with maternal *ε2/ε4* genotype and three new-borns
422 with either maternal or new-born's *ε2/ε4* genotype; cB - cord blood; m - maternal; nb - new-born; N - number of
423 observations; statistically significant results are indicated in bold.

425 **4. Discussion**

426 **4.1 TEs and APOE**

427 *APOE* gene isoforms are supposed to behave differently in relation to metal kinetics,
428 oxidative stress, age-related neurodegeneration, and (neonatal) neurodevelopment (Cardoso
429 et al., 2017; Jofre-Monseny et al., 2008; Miyata and Smith, 1996; Ng et al., 2013; Smith et
430 al., 2019; Wright et al., 2003; Xu et al., 2014). Studies suggest that young individuals with
431 at least one copy of the ε4 allele may have several advantages that could facilitate survival in
432 harsh environments (Smith et al., 2019), while older individuals may be at higher risk for
433 age-related diseases, such as AD and cardiovascular disease (Smith et al., 2019). Modifying
434 affects or interactions of the ε4 allele related to various metal(loid)s are less clear. In the
435 present study, we observed significant differences between Hg, As, Se, and Zn distributions
436 in the peripheral venous blood, peripheral venous plasma, hair, urine, and cord blood of
437 Croatian pregnant mothers carrying the *APOE* ε4 allele compared to those without the *APOE*
438 ε4 allele (but not for all elements in all samples) (**Table 3, Figure 2**). The mean blood levels
439 for potentially toxic metals such as Hg, Cd, and Pb in maternal or cord blood in either group
440 did not exceed the existing guideline values for pregnant women (5.8 ng/mL,1 ng/mL for Cd,
441 50 ng/mL for Pb) (Taylor et al., 2014) and As blood levels were also similar to those of the
442 general population. Only four mothers (from the ε4 group) exceeded the threshold or
443 intervention blood value for Hg (15 ng/g) (Ewers et al., 1999). Furthermore, after controlling
444 for seafood intake and other confounders, multiple linear regression analysis revealed no
445 associations between investigated TEs and the presence of the ε4 allele, except for Se in
446 peripheral venous plasma (**Table 4 and 5**). This positive association was even stronger when
447 we included only nulliparous women (data not shown) and excluded multiparous; but the
448 number of nulliparous was too small for reliable assessment. The observed association is
449 interesting, although it requires further study involving larger populations and attention on
450 *nulliparous* cohorts to omit the masking effects of the inverse association found between the
451 parity and blood elements Se, Hg, and Cd.

452 Parity affects the placenta by improving placental function via increased foetal-maternal
453 contact, higher vascularisation, increased perfusion, and increased villi surface density (Prior
454 et al., 2014). It can also have an independent positive effect on foetal weight and pregnancy;
455 pregnancy complications associated with placental dysfunction occur more frequently in
456 *nulliparous* than in *parous* women. Thus, the parity-related decrease in maternal and cord
457 blood (Me)Hg, Cd, and Se levels found in the present study may have resulted from an
458 increase in the placenta's ability to detoxify, perhaps by accumulating and/or excreting
459 various stress and foetal waste products including nonessential TEs, possibly also at the cost
460 of essential ones (e.g., Se) needed for detoxification. For instance, Kuhnert et al. (1982)
461 observed a parity-related increase in Cd levels in the placenta of pregnant women together
462 with a reduced amount of Zn; and Horvat et al. (1988) reported a tendency for Hg
463 accumulation and relatively high Se levels in the placental tissues of pregnant women. By
464 contrast, higher placental efficiency is also known to reduce systemic oxidative stress, which
465 is believed to be present during normal and pathological pregnancy (Leal et al., 2011). Such
466 reduction might decrease demands for Se and might explain the observed inverse relationship
467 between parity (0–3) and whole blood Se.

468

469 *4.1.1 Se and APOE during pregnancy*

470 In the present study, the maternal plasma selenium level means (61.7 ng/g for ε4+, 53.7
471 ng/g for ε4-) were within the European mean levels for pregnant women and close to the
472 level believed to be sufficient for normal GPx activity (2/3 of maximal activity, 62 ng/mL)
473 (Thomson, 2004).

Higher Se levels in pregnant women (in blood or plasma) were evidented to have several beneficial effects. Selenium was found to have a positive influence on reproduction, pregnancy-related diseases such as pre-eclampsia, miscarriage, preterm birth, thyroid autoimmunity, and neonatal diseases accompanied by oxidative stress (Pieczyńska and Grajeta, 2015; Rayman, 2016). Its antioxidative effect is known to be of great importance during embryogenesis (Ufer and Wang, 2011). It should also be pointed out that in a previous study of the present population group, authors observed a positive correlation between maternal blood selenium levels and a child's cognitive abilities (BSID-III scores) (n=154; r=0.176, p=0.029; multivariate analyses were not performed) (Močenić et al., 2019).

Similarly, positive influences have been suggested for the ε4 allele, mostly attributed to higher cholesterol levels, vitamin D levels, and bone Ca assimilation, which also can have a positive influence on fertility (particularly in highly infectious environments), neurodevelopment, bone growth, and lower susceptibility to infectious diseases (Dieckmann et al., 2012; Huebbe et al., 2011; Jasienska et al., 2015; Smith et al., 2019). Accordingly, it is possible that sufficient nutritional intake of Se is a necessary condition for optimal functioning of redox and antioxidative processes and protection against lower antioxidative capacity of ε4 allele. In other words, Se deficiency could pose a trigger for negative effects of *APOE* ε4.

4.1.2 Maternal versus foetal APOE

In two recent studies, the authors investigated the role of child *APOE* polymorphism and the relationship between prenatal Hg exposure (mixed cord blood levels) on child behaviour or neurodevelopment at around age two (Ng et al., 2013; Snoj Tratnik et al., 2017). Various confounders, including mixed cord blood Pb, mixed cord blood Se, and mixed cord plasma Se, were considered. However, because of the mixed cord blood/plasma samples the results remain debatable. It is possible that in such cases maternal levels of trace elements would be more powerful confounders. Unfortunately, the importance of maternal characteristics (Gundacker et al., 2000) for a child's prenatal neurodevelopment are often underestimated. In addition, in these studies we (Snoj Tratnik et al., 2017), and others (Ng et al., 2013), did not consider the maternal *APOE* genotype, whose important role in foetal lipid metabolism has been discussed in some older studies (Descamps et al., 2004; Witsch-Baumgartner et al., 2004; Witsch-Baumgartner and Lanthaler, 2015). For example, Witsch-Baumgartner et al. (2004) noted that the importance of the maternal genotype is, "*consistent with the finding that there is no difference in cholesterol concentrations of cord blood from new-borns with different APOE genotypes, suggesting that the embryo's APOE genotype does not significantly modulate lipoprotein concentrations prenatally*". Descamps et al. (2004) supported this suggestion, observing that maternal genetic variations of *APOE* and some other genes influence foetal lipoprotein concentrations independently of the genetic status of the foetus. However, in the case of pathological maternal levels, the foetal genotype may modulate maternal lipoprotein metabolism (Descamps et al., 2005). It should also be noted that, according to emerging data, the foetal apoE role differs from the new-born apoE role, and a lot remains to be discovered (Augsten et al., 2011).

4.2 Metabolic pathways of Se and apolipoprotein E

We also tried to elucidate a possible link between metabolic pathways of Se and apolipoprotein E. There are only a few studies covering selenium's relationship with apolipoproteins, including apoE and the lipoprotein receptors (apoER2, gene *LRP8*; megalin, gene *LRP2*) (Burk et al., 2014; Cardoso et al., 2017; Gabre-Medhin et al., 1988). It is also interesting that in some tissues, selenoprotein P (the main supplier of Se to extrahepatic tissues) and apoE are using the same two receptors, but different binding sites, for transfer of

524 selenium and lipids, respectively. That is, there is apoER2 in the brain, placenta, bone
525 marrow, and testis (Burk et al., 2014; Burk and Hill, 2015; Jinyuan Mao et al., 2016; Rayman,
526 2016), and megalin in the kidneys (renal proximal convoluted cells) and brain (Burk and Hill,
527 2015), and probably also in the placenta (Storm et al., 2016). Further, vitamins A and D
528 regulate megalin expression (Liu et al., 1998). Regulation of megalin (mRNA and protein
529 expression) was studied in a cell line derived from rat kidney proximal tubule cells, in human
530 JEG-3 cells, and in the mouse embryonal carcinoma cell line F9. Animal studies have also
531 shown that megalin (expressed by renal PCT cells) is important for retaining both vitamin D
532 *via* the vitamin D binding protein (Kim and Kim, 2014) and selenium in the form of
533 selenoprotein P (Burk and Hill, 2015). Accordingly, if kidney megalin is induced by vitamin
534 D in humans (being higher in ε4 allele carriers), this could be one reason for the observed
535 association between Se and *APOE* allele ε4.
536 Overall, we concluded that the superior Se status observed in healthy pregnant women
537 carrying the ε4 allele could be linked to suggested beneficial effects of *APOE* ε4 during
538 pregnancy. We also suppose that the reported apoE4 associations with low nail Se levels in
539 an elderly Chinese population (Gao et al., 2009) and low post-mortem brain Se levels of AD
540 patients (Cardoso et al., 2017) could be related to their general poor nutritional status
541 including low Se and vitamin D levels. As already discussed by other research groups, it
542 seems that the *APOE* allele ε4 could be a risk allele either for reproduction,
543 neurodevelopment, or age-related neurodegenerative diseases, especially, if not alone, in
544 combination with several other factors (Cardoso et al., 2017; Morris et al., 2016; Nehls, 2016;
545 Rea et al., 2016) including epigenetics, polymorphisms in related genes, and the simultaneous
546 deficiency of basic nutrients such as vitamin D and essential fatty acids, and/or selenium.
547
548 **4.3 Study limitation**
549 The main shortcomings of our study are the relatively low number of participants, low
550 concentration levels of potentially toxic elements, and mixed cord blood/serum/plasma
551 samples. Given the difficulties in obtaining separated venous/arterial cord samples in wider
552 epidemiological studies with low metal(loid) exposure, mixed samples are usually used as an
553 approximation of in utero exposure. There is no consensus regarding the type of studies for
554 which mixed samples are acceptable and when they are likely to introduce unacceptable
555 biases. Accordingly, for mixed cord blood and, in particular, mixed cord plasma/serum
556 levels, elaborate comments on distribution differences in trace elements between ε4+/ε4-
557 carriers are not possible. Although parity-related higher perfusion in the placenta (Prior et
558 al., 2014) may lead to an increase in Se transfer to the foetus, we did not observe any effect
559 of parity or apoE on Se levels in cord blood or cord plasma (**Tables 4 and 5**). Better insight
560 would be possible with data on TE levels in the placenta.
561 For those nonessential elements that accumulate mostly in erythrocytes (e.g., Hg, Pb,
562 and Cd), the mixed cord blood levels can be taken as a possible approximation, although
563 erythrocyte levels would be more reliable. In contrast, mixed cord plasma/serum samples are
564 more problematic. Individual cord venous/arterial differences are lost; therefore, the samples
565 are not truly representative and thus more or less unsuitable for reliable
566 comparison/association studies, although they are used in many studies as a rough substitute.
567 Additionally, Vitamin D status (25-hydroxysterol measurement), uncertainty of self-
568 reported data for smoking and seafood intake, and selenoproteins gene polymorphisms may
569 have introduced residual confounding. For instance, a recent study by Mao et al. (2016) in
570 the UK, *"shows that women who carry the SEPP1 rs3877899 A allele were better able to*
571 *maintain selenium status during pregnancy"* ; however, they did not follow either the vitamin
572 D status or the presence of the ε4 allele.
573

5. Conclusion

Our study indicates that genetic variations in the *APOE* gene (ε4+ versus ε4-) potentially influence selenium levels in pregnant women at the third trimester, while the effect of maternal/foetal *APOE* variations on cord blood should be re-evaluated on separated venous/arterial cord blood samples. Previous research suggestions of possible ε4 allele impact on Hg levels were not observed, while superior selenium status observed in healthy pregnant women carrying allele ε4 could be linked to the *APOE* ε4 beneficial effects early in life. Follow-up research with the same and larger *nonparous* cohorts with additional potential confounders are ongoing.

Acknowledgements

This work was supported by the Slovenian Research Agency, Slovenia, through research programme P1 0143 and projects J3-6104, J3-0161, and J7-9400; University of Rijeka (grant no. 13.06.1.2.25); and the Ministry of Science and Education of Republic of Croatia (grant no. 062-0000000-3395). We would like to thank Dr. Mateja Blaš for assisting Prof. Dr. Damijana Kastelec and all other co-workers and participants involved in this study.

Conflicts of Interest

The authors declare no conflict of interest.

593 **6. References**

594

595 Augsten, M., Hackl, H., Ebner, B., Chemelli, A., Glatter, O., Marsche, G., Lang, U., Desoye, G.,
596 Wadsack, C., 2011. Fetal HDL/apoE: a novel regulator of gene expression in human placental
597 endothelial cells. Physiol Genomics 43, 1255–1262.
598 https://doi.org/10.1152/physiolgenomics.00109.2011
599 Barany, E., Bergdahl, I.A., Schütz, A., Skerfving, S., Oskarsson, A., 1997. Inductively Coupled
600 Plasma Mass Spectrometry for Direct Multi-Element Analysis of Diluted Human Blood and
601 Serum. J Anal Spectrom 12, 1005–1009. https://doi.org/10.1039/a700904f
602 Barbone, F., Rosolen, V., Mariuz, M., Parpinel, M., Casetta, A., Sammartano, F., Ronfani, L., Vecchi,
603 L., Bin, M., Castriotta, L., Valent, F., Latesha, D.A., Mazej, D., Snoj Tratnik, J., Miklavčič, A.,
604 So, K., Špirič, Z., Krsnik, M., Neubauer, D., Kodrič, J., Prpič, I., Petrovič, O., Vlašič, I., Horvat,
605 M., 2019. Prenatal mercury exposure and child neurodevelopment outcomes at 18 months:
606 Results from the Mediterranean PHIME cohort. Int. J. Hyg. Environ. Heal. 222, 9–21.
607 https://doi.org/10.1016/j.ijheh.2018.07.011
608 Bernhard, M., 1988. UNEP Report: Mercury in the Mediterranean.
609 Bosch, A.C., O'Neill, B., Sigge, G.O., Kerwath, S.E., Hoffman, L.., 2016. Heavy metals in marine
610 fish meat and consumer health: a review. J Sci Food Agric 96, 32–48.
611 https://doi.org/10.1002/jsfa.7360
612 Brigelius-Flohe, R., Maiorino, M., 2013. Glutathione peroxidases. Biochim Byophys Acta 1839,
613 3289–3303. https://doi.org/10.1016/j.bbagen.2012.11.012
614 Burk, R.F., Hill, K.E., 2015. Regulation of selenium metabolism and transport. Annu Rev Nutr 35,
615 109–134. https://doi.org/10.1146/annurev-nutr-071714-034250
616 Burk, R.F., Hill, K.E., Motley, A.K., Winfrey, V.P., Kurokawa, S., Mitchell, S.L.., Zhang, W., 2014.
617 Selenoprotein P and apolipoprotein E receptor-2 interact at blood brain barrier and also within
618 the brain to maintain an essential selenium pool that protects against neurodegeneration. FASEB
619 J 28, 3579–3588. https://doi.org/10.1096/fj.14-252874
620 Cardoso, B.R., Hare, J.D., Lind, M., McLean, C.A., Volitakis, I., Laws, S.M., Masters, C.L., Bush,
621 A.I., Roberts, B.R., 2017. The APOE ε4 allele is associated with lower selenium levels in the
622 brain: Implications for Alzheimer's disease. Neurosci 8, 1459–1464.
623 https://doi.org/10.1021/acschemneuro.7b00014.
624 Descamps, O.S., Bruniaux, M., Guilmot, P.F., Tonglet, R., Heller, F.., 2004. Lipoprotein
625 concentrations in newborns are associated with allelic variations in their mothers.
626 Atherosclerosis 172, 287–298. https://doi.org/10.1016/j.atherosclerosis.2003.11.002
627 Descamps, O.S., Bruniaux, M., Guilmot, P.R., Tonglet, R., Heller, F.R., 2005. Lipoprotein
628 metabolism of pregnant women is associated with both their genetic polymorphisms and those
629 of their newborn children. JLR 46, 2405–2414. https://doi.org/10.1194/jlr.M500232-JLR200
630 Dieckmann, M., Beil, T.F., Mueller, B., A., B., Marshall, R.P., Koehne, T., Amling, M., Ruether, W.,
631 Cooper, J.A., Humphries, S.E., Herz, J., Niemeier, A., 2012. Human apolipoprotein E isoforms
632 differentially affect bone mass and turnover in vivo. J Bone Min. Res.
633 https://doi.org/10.1002/jbmr.1840
634 Egert, S., Rimbach, G., Huebbe, P., 2012. ApoE genotype : from geographic distribution to function
635 and responsiveness to dietary factors Proceedings of the Nutrition Society Proceedings of the

636 Nutrition Society. Proc Nutr Soc 410–424. https://doi.org/10.1017/S0029665112000249
637 EPA, US EPA (US Environmental Protection Agency), 2007. Mercury in solids and solutions by
638 thermal decomposition, amalgamation, and atomic absorption spectrophometry - method 7473
639 - total Mercury. SW-846, Test Methods Eval. Solid Waste, Phys. Methods 1–17.
640 Ewers, U., Krause, C., Schulz, C., Wilhellm, M., 1999. Reference values and human biological
641 monitoring values for environmental toxins - Report on the work and recommendations of the
642 Commission on Human Biological Monitoring of the German Federal Environmental Agency.
643 Int. Arch. Occup. Environ. Heal. 72, 255–260. https://doi.org/10.1007/s004200050369
644 Falnoga, I., Tušek-Žnidarič, M., 2007. Selenium–Mercury Interactions in Man and Animals. Biol
645 Trace Elem. Res 119, 212–220. https://doi.org/10.1007/s12011-007-8009-3
646 Florianczyk, B., 2007. Metallothioneins and its role in metal regulation, binding of reactive oxygene
647 species, apoptosis and cell differentiation. JPCCR 016–018.
648 Gabre-Medhin, M., Ewald, U., Tuvemo, T., 1988. Serum selenium is related to low density
649 lipoproteins in healthy children but not in children with diabetes. Ups J Med Sci 93, 57–62.
650 https://doi.org/10.1517/03009734000000038
651 Gao S, Jin Y, Hall KS, Liang C, Unverzagt FW, Ma F, Cheng Y, Shen J, Cao J, Matesan J, Li P, Bian
652 J, Hendrie HC, Murrell, J., 2009. Selenium level is associated with apoE epsilon4 in rural elderly
653 Chinese. Public Heal. Nutr 12, 2371–2376. https://doi.org/10.1017/S1368980009005102
654 Giau, V.V., Bagyinszky, E., An, S.S.A., Kim, S., 2015. Role of apolipoprotein E in neurodegenerative
655 diseases. Neuropsychiatr Dis Treat 11, 1723–1737. https://doi.org/10.2147/NDT.S84266
656 Gibson, R.S., Hess, S.Y., Holtz, C., Brown, K., H., 2008. Indicators of zinc status at the population
657 level: a review of the evidence. Br J Nutr 99. https://doi.org/10.1017/S0007114508006818
658 Godfrey, M.E., Wojcik, D.P., Krone, C.A., 2003. Apolipoprotein E genotyping as potential biomarker
659 for mercury neurotoxicity. J Alzheimer's Dis 5, 189–95. https://doi.org/10.3233/JAD-2003-
660 5303
661 Graeser, A., Huebbe, P., Storm, N., Hoppner, W., Wagner, A.E., Rimbach, G., Doring, F., 2012.
662 Apolipoprotein E genotype affects tissue metallothionein levels : studies in targeted gene
663 replacement mice. Genes Nutr 7, 247–255. https://doi.org/10.1007/s12263-012-0282-x
664 Gundacker, C., Neesen, J., Straka, E., Ellinger, I., Dolzing, H. Hengestschläger, M., 2000. Genetics
665 of the human placenta: implications for toxicokinetics. Arch Toxicol 90, 2563–2581.
666 https://doi.org/10.1006/s00204-016-1816-6
667 Haas, J.G., Lathe, R., 2018. Microbes and Alzheimer's Disease: New findings call for a Paradigm
668 Change. Trends Neurosci. 41570–573. https://doi.org/10.1016/j.tins.2018.07.001
669 Horvat, M., Byrne, A.R., 1990. A modified method for the determination of methylmercury by gas
670 chromatography. Talanta 37, 207–212. https://doi.org/10.1016/0039-9140(90)80024-A
671 Horvat, M., Stegnar, P., Byrne, A.R., Dermelj, M., 1988. A study of trace elements in human placenta,
672 blood and hair from the Yugoslav Central Adriatic., in: Brätter, P., Schramel, P. (Eds.), Trace
673 Element Analytical Chemistry in Medicine and Biology, 5. Proceedings of the Fifth
674 International Workshop. Walter de Gruyter: Berlin New York, pp. 243–550.
675 Huang, Y., Mahley, R.W., 2014. Apolipoprotein E: Structure and function in lipid metabolism,
676 neurobiology, and Alzheimer's diseases. Neurobiol Dis 72, 3–12.
677 https://doi.org/10.1016/j.nbd.2014.08.025
678 Huebbe, P., Nebel, A., Siegert, S., Moehring, J., Boesch-Saadatmandi, C., Most, E., Pallauf, J., Egert,

679 S., Müller, M.J., Schreiber, S., Nöthlings, U., Rimbach, G., 2011. APOE ε4 is associated with
680 higher vitamin D levels in targeted replacement mice and humans. FASEB J 25, 3262–70.
681 https://doi.org/10.1096/fj.11-180935
682 Huebbe, P., Rimbach, G., 2017. Evolution of human apolipoprotein E (APOE) isoforms: Gene
683 structure, protein function and interaction with dietary factors. Ageing Re Rev 37, 146–161.
684 https://doi.org/10.1016/j.arr.2017.06.002
685 Jasienska, G., Ellison, P.T., Galbarczyk, A., Jasienski, M., Kalemba-Drozdz, M., Nenko, I., Thune,
686 I., Ziomkiewicz, 2015. Apolipoprotein R (ApoE) polymorphism is related to differences in
687 potential fertility in women: a case of antagonistic pleotropy? A Proc R Soc 282.
688 https://doi.org/20142395
689 JECFA, n.d. Summary and conclusions of the sixty-first meeting of the Joint FAO/WHO Expert
690 Committee on Food Additives. [WWW Document].
691 Jofre-Monseny, L., Minihane, A.M., Rimbach, G., 2008. Impact of apoE genotype on oxidative stress,
692 inflammation and disease risk. Mol Nutr Food Res 52, 131–145.
693 https://doi.org/10.1002/mnfr.200700322
694 Julvez, J., Méndez, M., Fernandez-Barres, S., Romaguera, D., Vioque, J., Llop, S., Ibarluzea, J.,
695 Guxen, M., Avella-Garcia, C., Tardón, A., Riano, I., Andiarena, A., Robinson, O., Arija, V.,
696 Esnaola, M., Ballester, F., Sunyer, J., 2016. Maternal Consumption of seafood in pregnancy and
697 child neuropsychological development: A longitudinal study based on population with high
698 consumption levels. Am J Epidemiol 3, 169–182. https://doi.org/10.1093/aje/kwv195
699 Kim, C.S., Kim, S.W., 2014. Vitamin D and chronic kidney disease. Korean J Intern Med 29, 414–
700 427. https://doi.org/10.3904/kjim.2014.29.4.416
701 Kobal, A.B., Horvat, M., Prezelj, M., Sešek Briški, A., Kersnik, M., Dizdarevič, T., Mazej, D.,
702 Falnoga, I., Sibilj, V., Arnerič, N., Kobal, N., Osredkar, J., 2004. The impact of long-term past
703 exposure to elemental mercury on antioxidative capacity and lipid peroxidation in mercury
704 miners. J Trace Elem Med Biol 17, 261–274.
705 Kosta, L., Ravnik, V., Byrne, A.R., Štirn, J., Dermelj, M., Stegnar, P., 1978. Some trace elements in
706 the waters, marine organisms and sediments of the Adriatic by neutron activation analysis. J
707 Radioanal Chem 44, 317–332.
708 Kuhnert, B.R., Bottoms, S.F., Erhard, P., 1982. Cadmium levels in maternal blood, fetal cord blood,
709 and placental tissues of pregnant women who smoke. Am J Obs. Gynecol 142, 2021–1025.
710 La Port, P.., 2011. Selenium in the detoxification of arsenic: Mechanisms and clinical efficiency.
711 Dissertation, The University of Chicago. https://doi.org/347891
712 Leal, C.A.M., Schetinger, M.R.C., Leal, D.B.R., Morsch, V.M., Schafer da Silva, A., Rezer, J.F.P.,
713 2011. Oxidative stress and antioxidant defenses in pregnant women. Free Radic Res Commun.
714 16, 230–236. https://doi.org/10.1179/1351000211Y.0000000013
715 Liang, L., Bloom, N.S., Horvat, M., 1994. Simultaneous determination of mercury speciation in
716 biological materials by GC/CVAFS after ethylation and room-temperature precollection. Clin.
717 Chem. 40, 602–607.
718 Liu, W., Carling, T., Rastad, J., Akerström, G., Yu, W., R., Juhlin, C., Ridefelt, P., Hellman, P., 1998.
719 Regulation of gp330/megalin expression by vitamins A and D. Eur J Clin Investig 28, 100–107.
720 https://doi.org/10.1046/j.1365-2362.1998.00253.x
721 Liu, Y., Zhang, W., Zhao, J., Lin, X., Liu, J., Cui, L., Gao, Y., Zhang, T.L., Li, B., Li, Y.F., 2018.

722 Selenoprotein P as the major transporter for mercury and serum from methylmercury-poisoned
723 rats. J. Trace Elem. Med. Biol. 50, 589–595. https://doi.org/10.1016/j.jtemb.2018.04.013
724 Mao, J., Vanderlelie, J.J., Perkins, A.V., Redman, C.G.W., Ahmadi, K.R., Rayman, M., 2016. Genetic
725 polymorphisms that affect selenium status and response to selenium supplementation in United
726 Kingdom pregnant women. Am J Clin Nutr 104, 100–106.
727 https://doi.org/10.3945/ajcn.115.114231
728 Mao, J., Vanderlelie, J.J., Perkins, A. V., Redman, C.W.G., Ahmadi, K.R., Rayman, M.P., 2016.
729 Genetic polymorphisms that affect selenium status and response to selenium supplementation
730 in United Kingdom pregnant women. Am. J. Clin. Nutr.
731 https://doi.org/10.3945/ajcn.115.114231
732 Miklavčič, A., Casetta, A., Snoj Tratnik, J., Mazej, D., Krsnik, M., Mariuz, M., Sofianou, K., Špirić,
733 Z., Barbone, F., Horvat, M., 2013. Mercury, arsenic and selenium exposure levels in relation to
734 fish consumption in the Mediterranean area. Environ. Res. 120, 7–17.
735 https://doi.org/10.1016/j.envres.2012.08.010
736 Miyata, M., Smith, J.D., 1996. Apolipoprotein E allele-specific antioxidant activity and effects on
737 cytotoxicity by oxidative insults and beta-amyloid peptides. Nat Genet 14, 55–61.
738 https://doi.org/10.1038/ng0996-55
739 Močenić, I., Kolić, I., Radić Nišević, J., Belančić, A., Snoj Tratnik, J., Mazej, D., Falnoga, I., Vlašić-
740 Cicvarić, I., Štimac, T., Špirić, Z., Horvat, M., Prpić, I., 2019. Prenatal selenium status, neonatal
741 cerebellum measures and child nevrodevelopment at the age of 18 months. Env. Res 176.
742 https://doi.org/10.1016/j.envres.2019.108529
743 Morris, M.C., Brockman, J., Schneider, J.A., Wang, Y., Beneett, D.D., Tangney, C.C., van de Rest,
744 O., 2016. Association of Seafood consumption, brain mercury levels, and APO ε4 status with
745 brain neuropathology in older adults. JAMA 315, 489–497.
746 https://doi.org/10.1001/jama.2015.19451.
747 Mutter, J., Naumann, J., Sadaghiani, C., Schneider, R., Walach, H., 2004. Alzheimer disease:
748 Mercury as pathogenetic factor and apolipoprotein E as a moderator. Neuro Endocrinol Let 25,
749 331–339.
750 Nehls, M., 2016. Unified theory of Alzheimer's disease (UTAD): implications for prevention and
751 curative therapy. Mol Psychiatry 15, 1–52. https://doi.org/10.1186/s40303-016-0018-8
752 Ng, S., Lin, C.-C., Hwang, Y.-H., Hsieh, W.-S., Liao, H.-F., Chen, P.-C., 2013. Mercury, APOE, and
753 children's neurodevelopment. Neurotoxicology 37, 85–92.
754 https://doi.org/10.1016/j.neuro.2013.03.012
755 Pieczyńska, J., Grajeta, H., 2015. The role of selenium in human conception and pregnancy. J. Trace
756 Elem. Med. Biol. 29, 31–38. https://doi.org/10.1016/j.jtemb.2014.07.003
757 Prior, T., Mullins, E., Bennet, E., Kumar, S., 2014. Influence of parity on fetal hemodynamics and
758 amniotic fluid volume at term. Ultrasound Obs. Gynecol 44, 688–692.
759 https://doi.org/10.1002/uog.13332
760 Prpić, I., Milardović, A., Vlašić-Cicvarić, I., Špirić, Z., Radić Nišević, J., Vukelić, P., Snoj Tratnik,
761 J., Mazej, D., Horvat, M., 2017. Prenatal exposure to low-level methylmercury alters the child's
762 fine motor skills at the age of 18 months. Environ. Res. 152, 369–374.
763 https://doi.org/10.1016/j.envres.2016.10.011
764 Rayman, M.P., 2012. Selenium and human health. Lancet 379, 1256–1268.

765 https://doi.org/10.1016/S0140-6736(11)61452-9
766 Rayman, M.R., 2016. Is adequate selenium important for healthy human pregnancy?, in: Hatfield,
767 D.L., Schweizer, U., Tsuji, P.A., Gladyshe, V.N. (Eds.), Selenium: Its Molecular Biology and
768 Role in Human Health. Springer Science, NY, USA, pp. 353–364.
769 Rea, I.M., Dellet, M., Mills, K.I., 2016. ACUME2 Project. Living long and ageing well: is
770 epigenomics the missing link between nature and nurture? Biogerontology 17, 33–54.
771 https://doi.org/10.1007/s10522-015-9589-5
772 Santonen, T., Aitio, A., Fowler, B.., Nordberg, M., 2015. Biological Monitoring and Biomarkers., in:
773 Nordberg, G.A., Fowler, B.A., Nordberg, M. (Ed.), Handbook on the Toxicology of Metals.
774 Elsevier, London, UK, pp. 155–171.
775 Smith, J.S., Ashford, J.W., Perffeti, T.A., 2019. Putative survival advantageous in young
776 apolipoprotein e4 carriers are associated with increased neuronal stress. J Alzheimers Dis 885–
777 923. https://doi.org/10.3233/JAD-181089
778 Snoj Tratnik, J., Falnoga, I., Trdin, A., Mazej, D., Fajon, V., Miklavčič, A., Kobal, A.B., Sešek Briški,
779 A., Krsnik, M., Neubauer, D., Kodrič, J., Stropnik, S., Gosar, D., Lešnik Musek, P., Marc, J.,
780 Jurkovič Mlakar, S., Petrović, O., Vlašič Cicvarić, I., Prpič, I., Milardovič, A., Radić Nišević,
781 J., Vuković, D., Fišić, E., Špirić, Z., Horvat, M., 2017. Prenatal mercury exposure,
782 neurodevelopment and apolipoprotein E genetic polymorphism. Environ. Res. 152, 375–385.
783 https://doi.org/10.1016/j.envres.2016.08.035
784 Stajnko, A., Falnoga, I., Tratnik, J.S., Mazej, D., Jagodic, M., Krsnik, M., Kobal, A.B., Prezelj, M.,
785 Kononenko, L., Horvat, M., 2017. Low cadmium exposure in males and lactating females–
786 estimation of biomarkers. Environ. Res. 152. https://doi.org/10.1016/j.envres.2016.09.025
787 Stajnko, A., Šlejkovec, Z., Mazej, D., France-Štiglic, A., BRIŠKI, A.S., Prpić, I., Špirić, Z., Horvat,
788 M., Falnoga, I., 2019. Arsenic metabolites; selenium; and AS3MT, MTHFR, AQP4, AQP9,
789 SELENOP, INMT, and MT2A polymorphisms in Croatian-Slovenian population from PHIME-
790 CROME study. Environ. Res. 170, 301–319. https://doi.org/10.1016/j.envres.2018.11.045
791 Storm, T., Christensen, E.I., Christensen, J.N., Kjaergaard, T., Uldbjerg, N., Larsen, A., Honoré, B.,
792 Madsen, M., 2016. Megalin Is Predominantly Observed in Vesicular Structures in First and
793 Third Trimester Cytotrophoblasts of the Human Placenta Journal of Histochemistry and
794 Cytochemistry. J Histochem Cytochem 64. https://doi.org/10.1369/0022155416672210
795 Taylor, C.M., Golding, J., Edmond, A.D., 2014. Lead, cadmium and mercury levels in pregnancy:
796 the need for international concern. J Dev Orig Heal. Dis 5, 16–30.
797 https://doi.org/10.1017/S2040174413000500
798 Taylor, V., Goodale, B., Raab, A., Schwertdle, T., Reimer, K., Conklin, S., Karagas, M.R.,
799 Francesconi, K.A., 2017. Human exposure to organic arsenic species through seafood. Sci Tot
800 Env. 580, 266–282. https://doi.org/10.1016/j.scitotenv.2016.12.113
801 Team, R.C., 2017. R: A language and environment for statistical computing. [WWW Document]. R
802 Found. Stat. Comput. Vienna, Austria.
803 Thomson, C.D., 2004. Assessment of requirements for selenium and adequacy of selenium status: a
804 review. Eur. J Clin Nutr 58, 391–402. https://doi.org/10.1038/sj.ejcn.1601800
805 Tsalev, D.L., Zaprianov, Z.K.Z., 1983. Determination of individual Elements, in: Atomic Absorption
806 Spectrometry in Occupational and Environmental Health Practice. Florida CRC Press, Florida,
807 pp. 219–221.

808 Tuminello, E.R., Han, S.D., 2011. The apolipoprotein E antagonistic pleiotropy hypothesis: Review
809 and recommendations. Int. J .Alzheimer's Dis. 2011, 1–12.
810 https://doi.org/10.4061/2011/726197
811 Ufer, C., Wang, C.C., 2011. The roles of glutathione peroxidases during embryo development. Front
812 Mol Neurosci 4. https://doi.org/10.3389/fnmol.2011.00012
813 Valent, F., Horvat, M., Sofianou-Katsoulis, A., Spiric, Z., Mazej, D., Little, D., Prasouli, A., Mariuz,
814 M., Tamburlini, G., Nakou, S., Barbone, F., 2013a. Neurodevelopmental Effects of Low-level
815 Prenatal Mercury Exposure From Maternal Fish Consumption in a Mediterranean Cohort: Study
816 Rationale and Design. J. Epidemiol. https://doi.org/10.2188/jea.JE20120030
817 Valent, F., Mariuz, M., Bin, M., Little, D., Mazej, D., Tognin, V., Tratnik, J., McAfee, A.J., Mulhern,
818 M.S., Parpinel, M., Carrozzi, M., Horvat, M., Tamburlini, G., Barbone, F., 2013b. Associations
819 of Prenatal Mercury Exposure From Maternal Fish Consumption and Polyunsaturated Fatty
820 Acids With Child Neurodevelopment: A Prospective Cohort Study in Italy. J. Epidemiol.
821 https://doi.org/10.2188/jea.JE20120168
822 Vitek, M.P., Brown, C.M., Colton, C. a, 2009. APOE genotype-specific differences in the innate
823 immune response. Neurobiol. Aging 30, 1350–60.
824 https://doi.org/10.1016/j.neurobiolaging.2007.11.014
825 Witsch-Baumgartner, M., Gruber, M., Kraft, H.G., Rosi, M., Clayton, P., Giros, M., Haas, D., Kelley,
826 R.I., Krajewska-Walasek, M. Utermann, G., 2004. Maternal apo E genotype is a modifier of the
827 Smith-Lemli-Opitz syndrome. J Med Genet 41, 577–584.
828 https://doi.org/10.1136/jmg.2004.018085
829 Witsch-Baumgartner, M., Lanthaler, B., 2015. Birthday of a syndrome: 50 years anniversary of
830 Smith–Lemli–Opitz Syndrome. Eur J Hum Genet 23, 277–278.
831 https://doi.org/org/10.1038/ejhg.2014.87
832 Wright, R.O., Hu, H., Silverman, E.K., Tsaih, S.W., Schwartz, J., Bellinger, D., Palazuelos, E., Weiss,
833 S.T., Hernandez-Avila, M., 2003. Apolipoprotein E genotype predicts 24-month Bayley Scales
834 Infant Development Score. Pediatr. Res. 54, 819–825.
835 https://doi.org/10.1203/01.PDR.0000090927.53818.DE
836 Xu, H., Finkelstein, D.I., Adlard, P., 2014. Interactions of metals and apolipoprotein e in Alzheimer's
837 disease. Front Aging Neurosci 6, 1–7. https://doi.org/10.3389/fnagi.2014.00121
838 Zeng, H., Uthus, E.O., Combs, G.F.J., 2005. Mechanistic aspects of the interaction between selenium
839 and arsenic. Inorg Biochem 99, 1269–1274. https://doi.org/10.1016/j.jinorgbio.2005.03.006
840 Zhang, H., Feng, X., Chan, H.M., Larssen, T., 2014. New insights into traditional health risk
841 assessments of mercury exposure: Implications of selenium. Env. Sci Technol 48, 1206–1212.
842 https://doi.org/10.1021/es4051082
843

3.4 Prenatal Mercury Exposure, Neurodevelopment and Apolipoprotein E Genetic Polymorphism

Snoj Tratnik, J., Falnoga, I., Trdin, A., Mazej, D., Fajon, V., Miklavčič, A., Kobal, A.B., Osredkar, J., Sešek Briški, A., Krsnik, M., Neubauer, D., Kodrič, J., Stropnik S., Gosar, D., Lešnik Musek, P., Marc, J., Jurkovič Mlakar, S., Petrović, O., Vlašić-Cicvarić, I., Prpič, I., Milardović, A., Radić-Nišević, J., Vuković, D., Fišić, E., Špirić, Z., Horvat, M. (2017). *Environmental research*, 152, 375-385. https://doi.org/10.1016/j.envres.2016.08.035

Apolipoprotein E genetic polymorphisms were most commonly studied in relation to neurodegenerative diseases (e.g. Alzheimer's disease) and lipid metabolism. On the other hand, *ApoE* is believed to be involved in processes associated with neurodevelopment. As recent studies (Ng et al., 2015; 2013) reported negative association between mixed cord blood Hg levels with child development and *ApoE* genotypes were hypothesized to modify this association, the authors aimed to evaluate prenatal Hg exposure and child's neurodevelopment (assessed with Bayley-III), taking into account genetic polymorphism of *ApoE* and other relevant confounders.

The study population were children from Slovenia and Croatia (N = 601 and N = 243, respectively), who were included in the PHIME study. The prenatal exposure to Hg was assessed through measurements of THg in maternal hair and mixed cord blood. Apolipoprotein E genotypes were determined using TaqMan® pre-designed SNP genotyping assay for rs429358 and rs7412. As described in Section 3.3., newborns were divided into two groups, *ε4* carriers and *ε4*-non-carriers. Children neurodevelopment was assessed using Bayley Scales of Infant and Toddler development third edition (Bayley-III). Cognitive, language, motor (fine and gross) were administered according to the standardised procedure.

The authors reported 72% of children being *ε4*-non-carriers. The obtained results showed that children having at least one *ε4* allele had marginally significant higher levels of mixed cord blood THg (1.98 ng/g vs. 2.37 ng/g, p = 0.079). However, after considering possible covariates (maternal age, child's gender, birth weight, maternal educational level, smoking status) the authors observed a Hg-related decrease in cognitive score in children carrying at least one *ApoE ε4* allele. The Hg related decrease in fine motor skills was genotype independent. Anyway, all observed results were inside normal levels.

However, as Hg exposure was assessed through THg measurements in maternal hair and mixed cord blood, the results did not make clear what is the contribution of different Hg species to negative association between Hg and neurodevelopment. Maternal blood levels were not included as the results were available only for Croatian mothers. The authors recognized the sample size as the main limitation of the study. Moreover, multiple linear regression models were in some cases statistically non-significant, therefore the results should be interpreted carefully.

The candidate performed ApoE genotyping, determined the genotypes, divided children into two groups of *ε4* carriers vs. *ε4* non-carriers and contributed to the preparation of the manuscript.

Environmental Research 152 (2017) 375–385

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Prenatal mercury exposure, neurodevelopment and apolipoprotein E genetic polymorphism

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ARTICLE INFO

Article history:
Received 13 March 2016
Received in revised form
21 June 2016
Accepted 30 August 2016
Available online 9 September 2016

Keywords:
Mercury
Selenium
Lead
Cord blood
Neurodevelopment
Apolipoprotein E
Genetic polymorphism

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°) 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° 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° 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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http://dx.doi.org/10.1016/j.envres.2016.08.035
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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.

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%)

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.

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 ε3/ε3 genotypes, considered as a wild (reference) genotype (72 %); the frequency of genotypes ε2/ε2 and ε2/ε3 was 9 %, and the frequency of susceptible genotypes ε3/ε4 and ε4/ε4 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 ε2/ε4 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 (± 13.0) and Croatian 106.7 (± 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** *model R^2=0.026, p=0.009*	0.40 (−1.34, 2.14), 0.650 *model R^2=0.0006, p=0.900*	−1.03 (−2.27, 0.20), 0.102 *model R^2=0.013, p=0.099*	**−0.51 (−0.80, −0.26), <0.001** *model R^2=0.051, p < 0.001*	0.18 (−0.06, 0.42), 0.143 *model R^2=0.007, p=0.295*
	ε2, ε3 carriers	289	−1.77 (−3.58, 0.04), 0.055 *model R^2=0.013*	0.67 (−1.32, 2.65), 0.508 *model R^2=0.002*	−0.87 (−2.29, 0.55), 0.227 *model R^2=0.005*	**−0.45 (−0.74, −0.15), 0.003** *model R^2=0.031*	0.17 (−0.10, 0.44), 0.210 *model R^2=0.006*
	ε4 carriers	68	**−3.98 (−7.72, −0.25), 0.037** *model R^2=0.064*	−0.64 (−4.29, 3.02), 0.729 *model R^2=0.002*	−1.65 (−4.13, 0.83), 0.189 *model R^2=0.026*	**−0.76 (−1.28, −0.24), 0.005** *model R^2=0.116*	0.22 (−0.35, 0.78), 0.449 *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 *model R^2=0.09, p < 0.001*	−0.32 (−2.42, 1.77), 0.762 *model R^2=0.063, p=0.015*	**−1.74 (−3.14, −0.34), 0.015** *model R^2=0.051, p=0.023*	**−0.49 (−0.78, −0.21), 0.001** *model R^2=0.095, p < 0.001*	−0.11 (−0.39, 0.17), 0.443 *model R^2=0.092, p < 0.001*
	ε2, ε3 carriers	281	−0.19 (−2.19, 1.81), 0.849 *model R^2=0.09, p < 0.001*	−0.07 (−2.48, 2.33), 0.951 *model R^2=0.062, p=0.031*	−1.59 (−3.21, 0.03), 0.054 *model R^2=0.036, p=0.175*	**−0.46 (−0.79, −0.13), 0.007** *model R^2=0.062, p=0.900*	−0.10 (−0.41, 0.20), 0.499 *model R^2=0.092, p < 0.001*
	ε4 carriers	65	−4.04 (−8.26, 0.19), 0.061 *model R^2=0.15, p=0.087*	−1.91 (−6.43, 2.62), 0.400 *model R^2=0.13, p=0.500*	−2.41 (−5.33, 0.51), 0.104 *model R^2=0.133, p=0.295*	**−0.61 (−1.18, −0.03), 0.039** *model R^2=0.0006, p=0.001*	−0.21 (−0.94, 0.52), 0.572 *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 *model R^2=0.12, p < 0.001*	−1.03 (−3.46, 1.40), 0.406 *model R^2=0.091, p=0.016*	−1.16 (−2.76, 0.44), 0.153 *model R^2=0.056, p=0.100*	**−0.33 (−0.66, −0.01), 0.043** *model R^2=0.103, p < 0.001*	−0.10 (−0.41, 0.22), 0.553 *model R^2=0.078, p=0.029*
	ε2, ε3 carriers	232	−0.49 (−2.76, 1.78), 0.949 *model R^2=0.12, p < 0.001*	−0.59 (−3.42, 2.45), 0.682 *model R^2=0.074, p=0.0102*	−0.84 (−2.68, 1.00), 0.369 *model R^2=0.049, p=0.253*	−0.29 (−0.66, 0.08), 0.122 *model R^2=0.086, p=0.002*	**−0.06 (−0.41, 0.29), 0.742** *model R^2=0.093, p=0.017*
	ε4 carriers	51	**−5.44 (−10.7, 0.19), 0.043** *model R^2=0.19, p=0.207*	−2.61 (−7.41, 2.20), 0.276 *model R^2=0.323, p=0.202*	−2.54 (−5.94, 0.86), 0.139 *model R^2=0.224, p=0.258*	−0.51 (−1.16, 0.13), 0.117 *model R^2=0.208, p=0.018*	−0.26 (−1.09, 0.57), 0.528 *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** *model R^2=0.024, p=0.014*	0.63 (−0.96, 2.22), 0.436 *model R^2=0.002, p=0.733*	−0.83 (−1.95, 0.29), 0.145 *model R^2=0.013, p=0.095*	**−0.39 (−0.62, −0.16), 0.001** *model R^2=0.039, p=0.001*	0.14 (−0.08, 0.36), 0.211 *model R^2=0.007, p=0.293*
	ε2, ε3 carriers	259	−1.25 (−2.94, 0.44), 0.147 *model R^2=0.007*	1.08 (−0.77, 2.93), 0.251 *model R^2=0.005*	−0.64 (−1.94, 0.67), 0.338 *model R^2=0.003*	**−0.31 (−0.58, 0.04), 0.027** *model R^2=0.017*	0.13 (−0.12, 0.38), 0.292 *model R^2=0.004*
	ε4 carriers	63	**−4.21 (−7.27, −1.15), 0.008** *model R^2=0.101*	−0.84 (−3.93, 2.25), 0.590 *model R^2=0.004*	−1.47 (−3.62, 0.68), 0.177 *model R^2=0.027*	**−0.66 (−1.09, −0.23), 0.003** *model R^2=0.123*	0.17 (−0.34, 0.67), 0.513 *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 *model R^2=0.090, p < 0.001*	0.53 (−1.41, 2.47), 0.589 *model R^2=0.065, p=0.013*	−1.19 (−2.40, 0.01), 0.052 *model R^2=0.044, p=0.027*	**−0.36 (−0.63, −0.10), 0.008** *model R^2=0.080, p < 0.001*	−0.11 (−0.37, 0.16), 0.428 *model R^2=0.084, p < 0.001*
	ε2, ε3 carriers	276	0.19 (−1.69, 2.07), 0.840 *model R^2=0.092, p < 0.001*	0.64 (−1.64, 2.91), 0.581 *model R^2=0.062, p=0.035*	−0.97 (−2.39, 0.45), 0.178 *model R^2=0.028, p=0.241*	**−0.32 (−0.63, −0.01), 0.043** *model R^2=0.048, p=0.010*	−0.14 (−0.43, 0.14), 0.323 *model R^2=0.097, p < 0.001*
	ε4 carriers	66	−3.23 (−6.89, 0.44), 0.083 *model R^2=0.134, p=0.116*	0.05 (−3.82, 3.93), 0.979 *model R^2=0.142, p=0.402*	−2.07 (−4.39, 0.26), 0.081 *model R^2=0.111, p=0.275*	−0.48 (−0.98, 0.02), 0.061 *model R^2=0.209, p=0.006*	0.02 (−0.68, 0.72), 0.962 *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 *model R^2=0.118, p < 0.001*	−0.01 (−2.32, 2.30), 0.671 *model R^2=0.097, p=0.015*	−1.04 (−2.43, 0.34), 0.139 *model R^2=0.055, p=0.081*	−0.29 (−0.59, 0.01), 0.059 *model R^2=0.098, p < 0.001*	−0.12 (−0.42, 0.18), 0.441 *model R^2=0.079, p=0.039*
	ε2, ε3 carriers	226	−0.15 (−2.37, 2.06), 0.893 *model R^2=0.122, p < 0.001*	0.26 (−2.52, 3.03), 0.856 *model R^2=0.082, p=0.086*	−0.64 (−2.30, 1.02), 0.445 *model R^2=0.046, p=0.243*	−0.23 (−0.59, 0.12), 0.197 *model R^2=0.080, p=0.006*	−0.10 (−0.44, 0.25), 0.585 *model R^2=0.092, p=0.028*
	ε4 carriers	51	−4.02 (−8.54, 0.50), 0.080 *model R^2=0.172, p=0.285*	−0.50 (−4.75, 3.74), 0.810 *model R^2=0.295, p=0.281*	−2.35 (−4.84, 0.14), 0.063 *model R^2=0.244, p=0.099*	−0.40 (−0.95, 0.16), 0.157 *model R^2=0.278, p=0.022*	−0.15 (−0.87, 0.56), 0.663 *model R^2=0.130, p=0.817*
	Interaction p* *Apoe × Hg*	280	0.034	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 ε2 and ε3 carriers (cord blood model $\beta = -0.82$, $p = 0.008$; hair model $\beta = -0.82$, $p = 0.008$), but not in ε4 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* ε4 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* ε4 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 ε4 carriers compared to ε2 and ε3 carriers. Similarly, ε4 carriers cognitively outperformed non-carriers in several studies involving a young population (Tuminello and Han, 2011). The authors suggested that the *Apoe* ε4 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* ε4 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* ε4 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 ε4 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 ε4 carriers, while in girls in the attention and motor domains, but with improved performance in ε4 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.

Role of funding source

This study was part of the EU 6th Framework Programme, Public Health Impact of Long-term Low-level Mixed Element Exposure in Susceptible Population Strata (PHIME), CROME-LIFE+ project and the EU 7th Framework Programme, Health and Environment-wide Associations based on Large population Surveys (HEALS). The aim of the PHIME project was to improve the integrated health risk-assessment of long-term, low-level environmental exposure to toxic and essential metals via food. The study design was established and the subjects were recruited within this project. CROME (Cross-Mediterranean Environment and Health Network), is an on-going LIFE+ project employing a technically feasible integrated methodology for interpretation of human biomonitoring data and has provided the basic protocol for assessing the role of metal- and neurodevelopment-related genetic polymorphisms. HEALS is an 'exposome' project in which a study design and testing for environment wide assessment study (EWAS) is one of the objectives. The study has been approved by the National Ethics Committees of the Republic of Slovenia and Croatia.

Declaration of interest

The authors report no declaration of interest.

Acknowledgements

This work was supported by the EU through the Sixth Framework Programme for RTD (FOOD-CT-2006-016253, PHIME project), CROME-LIFE+ project, 7th Framework Programme (no. 603946, HEALS project) and programme funded by the Slovenian Research Agency (P1-0143). It reflects only the authors' views. The Community is not liable for any use that may be made of the information contained therein.

The authors are grateful to all pregnant women and their children who participated in this study, the personnel of the "Jožef Stefan" Institute, University Medical Centre Ljubljana, Slovenia and University Hospital Centre Rijeka, Croatia, who collected biological samples and collaborated in conducting the interviews with the pregnant women, the psychology students and psychologists of the University Medical Centre Ljubljana and University Hospital Centre Rijeka for conducting the neurodevelopmental tests on children.

References

Akagi, H., 1997. Analytical methods for evaluating human exposure to mercury due to gold mining. In: Proceedings of the International Workshop. Health and Environmental Effects of Mercury due to Mining Operations. National Institute for Minamata Disease, Minamata, pp. 131–141.

Andersen, H.R., Nielsen, J.B., Grandjean, P., 2000. Toxicologic evidence of developmental neurotoxicity of environmental chemicals. Toxicology 144, 121–127. http://dx.doi.org/10.1016/S0300-483X(99)00198-5.

Aylward, G., 2013. Continuing issues with the Bayley-III: where to go from here. J. Dev. Behav. Pediatr. 34, 697–701.

Barany, E., Bergdahl, I. a, Schuetz, A., Skerfving, S., Oskarsson, A., 1997. Inductively coupled plasma mass spectrometry for direct multi-element analysis of diluted human blood and serum. J. Anal. At. Spectrom. 12, 1005–1009. http://dx.doi.org/10.1039/a700904f.

Barcelos, G.R.M., Grotto, D., de Marco, K.C., Valentini, J., Lengert, A.V.H., de Oliveira, A.Á.S., Garcia, S.C., Braga, G.Ú.L., Schläwicke Engström, K., Cólus, I.M.D.S., Broberg, K., Barbosa, F., 2013. Polymorphisms in glutathione-related genes modify mercury concentrations and antioxidant status in subjects environmentally exposed to methylmercury. Sci. Total Environ. 463–464, 319–325. http://dx.doi.org/10.1016/j.scitotenv.2013.06.029.

Basu, N., Goodrich, J.M., Head, J., 2014. Ecogenetics of mercury: From genetic polymorphisms and epigenetics to risk assessment and decision-making. Environ. Toxicol. Chem. 33, 1248–1258. http://dx.doi.org/10.1002/etc.2375.

Bayley, 2006. Bayley Scales of Infant and Toddler Development. Hartcourt Assessment, Inc, San Antonio, TX (Third Edition).

Björkman, L., Lundekvam, B.F., Laegreid, T., Bertelsen, B.I., Morild, I., Lilleng, P., Lind, B., Palm, B., Vahter, M., 2007. Mercury in human brain, blood, muscle and toenails in relation to exposure: an autopsy study. Environ. Health 6, 30. http://dx.doi.org/10.1186/1476-069X-6-30.

Buttini, M., Orth, M., Bellosta, S., Akeefe, H., Pitas, R.E., Wyss-Coray, T., Mucke, L., Mahley, R.W., 1999. Expression of human apolipoprotein E3 or E4 in the brains of Apoe-/- mice: isoform-specific effects on neurodegeneration. J. Neurosci. 19, 4867–4880.

Cace, I.B., Milardovic, a, Prpic, I., Krajina, R., Petrovic, O., Vukelic, P., Spiric, Z., Horvat, M., Mazej, D., Snoj, J., 2011. Relationship between the prenatal exposure to low-level of mercury and the size of a newborn's cerebellum. Med. Hypotheses 76, 514–516. http://dx.doi.org/10.1016/j.mehy.2010.12.005.

Chen, C., Yu, H., Zhao, J., Li, B., Qu, L., Liu, S., Zhang, P., Chai, Z., 2006. The roles of serum selenium and selenoproteins on mercury toxicity in environmental and occupational exposure. Environ. Health Perspect. 114, 297–301. http://dx.doi.org/10.1289/ehp.7861.

Davidson, P.W., Myers, G.J., Weiss, B., 2004. Mercury exposure and child development outcomes. Pediatrics 113, 1023–1029.

Falnoga, I., Tušek-Žnidarič, M., 2007. Selenium-mercury interactions in man and animals. Biol. Trace Elem. Res. 119, 212–220. http://dx.doi.org/10.1007/s12011-007-8009-3.

Grandjean, P., Landrigan, P.J., 2006. Developmental neurotoxicity of industrial chemicals. Lancet 368, 2167–2178. http://dx.doi.org/10.1016/S0140-6736(06)69665-7.

Grandjean, P., Weihe, P., White, R., Debes, F., Araki, S., Yokoyama, K., Murata, K., Sorensen, N., Dahl, R., Jorgensen, P., 1997. Cognitive deficit in 7 year-old children with prenatal exposure to methylmercury. Neurotoxicol. Teratol. 19, 417–428.

Gundacker, C., Gencik, M., Hengstschläger, M., 2010. The relevance of the individual genetic background for the toxicokinetics of two significant neurodevelopmental toxicants: mercury and lead. Mutat. Res. 705, 130–140. http://dx.doi.org/10.1016/j.mrrev.2010.06.003.

Horvat, M., Lupšina, V., Pihlar, B., 1991a. Determination of total mercury in coal fly ash by gold amalgamation cold vapour atomic absorption spectrometry. Anal. Chim. Acta 24, 71–79.

Horvat, M., Stegnar, P., Byrne, A., Dermelj, M., 1988. A study of trace elements in human placenta, blood and hair from the Yugoslav Central Adriatic. In: Brätter, P., Schramel, P. (Eds.), Trace Element Analytical Chemistry in Medicine and Biology. Walter de Gruyter, Berlin New York, p. 243.

Horvat, M., Prosenc, A., Smrke, J., Konda, D., Byrne, A., Stegnar, P., 1991b. Trace elements level in the hair, blood, cord blood and placenta of pregnant women from central Slovenija. In: Aito, A., Aro, A. (Eds.), Trace Elements in Health and Disease. The Royal Society of Chemistry, Cambridge, pp. 83–91.

Julvez, J., Grandjean, P., 2013. Genetic susceptibility to methylmercury developmental neurotoxicity matters. Front. Genet. 4, 278. http://dx.doi.org/10.3389/fgene.2013.00278.

Julvez, J., Smith, G.D., Golding, J., Ring, S., Pourcain, B.S., Gonzalez, J.R., Grandjean, P., 2013. Prenatal methylmercury exposure and genetic predisposition to cognitive deficit at age 8 years. Epidemiology 24, 643–650. http://dx.doi.org/10.1097/EDE.0b013e31829d5c93.

Karagas, M.R., Choi, A.L., Oken, E., Horvat, M., Schoeny, R., Kamai, E., Cowell, W., Grandjean, P., Korrick, S., 2012. Evidence on the human health effects of low-level methylmercury exposure. Environ. Health Perspect. 120, 799–806. http://dx.doi.org/10.1289/ehp.1104494.

Kobal, A.B., Horvat, M., Prezelj, M., Briški, a S., Krsnik, M., Dizdarevič, T., Mazej, D., Falnoga, I., Stibilj, V., Arnerič, N., Kobal, D., Osredkar, J., 2004. The impact of long-term past exposure to elemental mercury on antioxidative capacity and lipid peroxidation in mercury miners. J. Trace Elem. Med. Biol. 17, 261–274.

http://dx.doi.org/10.1016/S0946-672X(04)80028-2.
Landrigan, P.J., Todd, A.C., 1994. Lead poisoning. West. J. Med. 161, 153–159.
Llop, S., Engström, K., Ballester, F., Franforte, E., Alhamdow, A., Pisa, F., Tratnik, J.S., Mazej, D., Murcia, M., Rebagliato, M., Bustamante, M., Sunyer, J., Sofianou-Katsoulis, A, Prasouli, A., Antonopoulou, E., Antoniadou, I., Nakou, S., Barbone, F., Horvat, M., Broberg, K., 2014. Polymorphisms in ABC transporter genes and concentrations of Mercury in newborns – evidence from two Mediterranean birth cohorts. PLoS One 9, e97172. http://dx.doi.org/10.1371/journal.pone.0097172.
Mahley, R.W., 1988. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. Science 240, 622–630.
Mauch, D.H., Nägler, K., Schumacher, S., Göritz, C., Müller, E.C., Otto, a, Pfrieger, F.W., 2001. CNS synaptogenesis promoted by glia-derived cholesterol. Science 294, 1354–1357. http://dx.doi.org/10.1126/science.294.5545.1354.
Miklavčič, a, Cuderman, P., Mazej, D., Snoj Tratnik, J., Krsnik, M., Planinšek, P., Osredkar, J., Horvat, M., 2011. Biomarkers of low-level mercury exposure through fish consumption in pregnant and lactating Slovenian women. Environ. Res. 111, 1201–1207. http://dx.doi.org/10.1016/j.envres.2011.07.006.
Miklavčič, A., Casetta, A., Snoj Tratnik, J., Mazej, D., Krsnik, M., Mariuz, M., Sofianou, K., Špirić, Z., Barbone, F., Horvat, M., 2013. Mercury, arsenic and selenium exposure levels in relation to fish consumption in the Mediterranean area. Environ. Res. 120, 7–17. http://dx.doi.org/10.1016/j.envres.2012.08.010.
Ng, S., Lin, C.-C., Hwang, Y.-H., Hsieh, W.-S., Liao, H.-F., Chen, P.-C., 2013. Mercury, APOE, and children's neurodevelopment. Neurotoxicology 37, 85–92. http://dx.doi.org/10.1016/j.neuro.2013.03.012.
Ng, S., Lin, C.-C., Jeng, S.-F., Hwang, Y.-H., Hsieh, W.-S., Chen, P.-C., 2015. Mercury, APOE, and child behavior. Chemosphere 120, 123–130. http://dx.doi.org/10.1016/j.chemosphere.2014.06.003.
Oken, E., Wright, R.O., Kleinman, K.P., Bellinger, D., Amarasiriwardena, C.J., Hu, H., Rich-Edwards, J.W., Gillman, M.W., 2005. Maternal fish consumption, hair mercury, and infant cognition in a U.S. cohort. Environ. Health Perspect. 113, 1376–1380. http://dx.doi.org/10.1289/ehp.8041.
Oken, E., Radesky, J.S., Wright, R.O., Bellinger, D.C., Amarasiriwardena, C.J., Kleinman, K.P., Hu, H., Gillman, M.W., 2008. Maternal fish intake during pregnancy, blood mercury levels, and child cognition at age 3 years in a US cohort. Am. J. Epidemiol. 167, 1171–1181. http://dx.doi.org/10.1093/aje/kwn034.
Prpic, I., Milardovic, A., Vlašić-Cicvarić, I., Špirić, Z., Nišević, J.R., Vukelić, P., Tratnik, J. S., Mazej, D., Horvat, M., 2016. Prenatal exposure to low-level methylmercury alters the child's fine motor skills at the age of 18 months. Environ. Res. (this issue)
Renzoni, a, Zino, F., Franchi, E., 1998. Mercury levels along the food chain and risk for exposed populations. Environ. Res. 77, 68–72. http://dx.doi.org/10.1006/enrs.1998.3832.
Rice, D., Barone, S., 2000. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. Environ. Health Perspect. 108, 511–533. http://dx.doi.org/10.1289/ehp.00108s3511.
Sanders, T., Liu, Y., Buchner, V., Tchounwou, P.B., 2009. Neurotoxic effects and biomarkers of lead exposure: a review. Rev. Environ. Health 24, 15–45. http://dx.doi.org/10.1515/REVEH.2009.24.1.15.
Schläwicke Engström, K., Strömberg, U., Lundh, T., Johansson, I., Vessby, B., Hallmans, G., Skerfving, S., Broberg, K., 2008. Genetic variation in glutathione-related genes and body burden of methylmercury. Environ. Health Perspect. 116, 734–739. http://dx.doi.org/10.1289/ehp.10804.
Skröder, H.M., Hamadani, J.D., Tofail, F., Persson, L.Å., Vahter, M.E., Kippler, M.J., 2014. Selenium status in pregnancy influences children's cognitive function at 1.5 years of age. Clin. Nutr. 34, 1–8. http://dx.doi.org/10.1016/j.clnu.2014.09.020.
Trdin, A., 2015. Connection Between Mutations in the Gene for Apolipoprotein E with Concentrations of Mercury in the Mothers and Newborns (Master's Thesis) http://dx.doi.org/10.1017/CBO9781107415324.004.
Tuminello, E.R., Han, S.D., 2011. The Apolipoprotein E Antagonistic Pleiotropy Hypothesis: Review and Recommendations. Int. J. Alzheimers Dis. 2011, 1–12. http://dx.doi.org/10.4061/2011/726197.
UNEP/WHO, 2008. Guidance for Identifying Populations at Risk from Mercury Exposure. Geneva, Switzerland.
US EPA, 2001. Water quality criterion for the protection of human health: methylmercury. In: Borum, D., Manibusan, M., Schoeny, R., Winchester, E. (Eds.), Washington DC.
Vahter, M., Mottet, N., Friberg, L., Lind, B., Shen, D., Burbacher, T., 1994. Speciation of mercury in the primate blood and brain following long-term exposure to methyl mercury. Toxicol. Appl. Pharmacol. 124, 221–229.
Valent, F., Horvat, M., Sofianou-Katsoulis, A., Spiric, Z., Mazej, D., Little, D., Prasouli, A., Mariuz, M., Tamburlini, G., Nakou, S., Barbone, F., 2013. Neurodevelopmental effects of low-level prenatal mercury exposure from maternal fish consumption in a Mediterranean cohort: study rationale and design. J. Epidemiol. 23, 146–152. http://dx.doi.org/10.2188/jea.JE20120030.
Višnjevec, A.M., Kocman, D., Horvat, M., 2014. Human mercury exposure and effects in Europe. Environ. Toxicol. Chem. 33, 1259–1270. http://dx.doi.org/10.1002/etc.2482.
Wang, Y., Goodrich, J.M., Gillespie, B., Werner, R., Basu, N., Franzblau, A., 2012. An investigation of modifying effects of metallothionein single-nucleotide polymorphisms on the association between mercury exposure and biomarker levels. Environ. Health Perspect. 120, 530–534. http://dx.doi.org/10.1289/ehp.1104079.
Woods, J.S., Heyer, N.J., Russo, J.E., Martin, M.D., Farin, F.M., 2014. Genetic polymorphisms affecting susceptibility to mercury neurotoxicity in children: summary findings from the Casa Pia Children's Amalgam clinical trial. Neurotoxicology 44, 288–302. http://dx.doi.org/10.1016/j.neuro.2014.07.010.
Wright, R.O., Hu, H., Silverman, E.K., Tsaih, S.W., Schwartz, J., Bellinger, D., Palazuelos, E., Weiss, S.T., Hernandez-Avila, M., 2003. Apolipoprotein E genotype predicts 24-month bayley scales infant development score. Pediatr. Res. 54, 819–825. http://dx.doi.org/10.1203/01.PDR.0000090927.53818.DE.
Yoshida, M., 2002. Placental to fetal transfer of mercury and fetotoxicity. Tohoku J. Exp. Med. . http://dx.doi.org/10.1620/tjem.196.79
Zannis, V.I., Breslow, J.L., 1981. Human very low density lipoprotein apolipoprotein E isoprotein polymorphism is explained by genetic variation and posttranslational modification. Biochemistry 20, 1033–1041. http://dx.doi.org/10.1021/bi00507a059.

Chapter 4

Discussion and Summary

This dissertation contributes to the scientific knowledge about the assessment of prenatal Hg exposure, highlights the limits of the current approach and recognizes the opportunities for its improvement. Despite its global relevance, there has been limited synthesis of the available literature on the possible negative health effects because of prenatal Hg exposure in the general population. Reliable exposure assessment in this low-level and long-term exposure to various Hg species is many times challenged by selected Hg measurement methods and suitability of included exposure or susceptibility biomarkers.

The studied population from Slovenia and Croatia (N = 584 and N = 234, respectively) were pregnant women with their newborns exposed to MeHg from seafood consumption and Hg^0 vapour released from dental amalgams. On average, MeHg in maternal blood from Croatia presented 86% of measured THg, which is in general consistent with the assumption of most of the epidemiological studies that MeHg presents the majority of measured THg in blood (Basu et al., 2018; Berglund et al., 2005; Horvat et al., 2012). Moreover, MeHg in cord blood collected in Slovenian and Croatian participants presented 98% of THg. Participants from Croatia had higher levels of THg in maternal hair (604 ng/g vs. 298 ng/g) and cord blood (2.94 ng/g vs. 1.55 ng/g) in comparison with the Slovenian population, which is related to the fact that women from Croatia ate fish more frequently (0.45 meals per day vs. 0.28 meals per day). However, only about 10 % of the studied population exceeded the thresholds of 1 µg/g in hair (NRC, 2000c) and 5.8 µg/L in cord blood (Rice, 2004; US EPA, 1997), which is currently the exposure considered safe for prenatal development. Moreover, 23% of women from Croatia had blood THg ≥ 3.5 µg/L, which is the threshold considered relevant for preventing foetal neurotoxicity (Mahaffey et al., 2004, 2009). With this study (Chapter 3.1) we confirmed the first hypothesis that on average the majority of measured THg will be present in its organic (MeHg) form. However, we showed important individual differences in proportions of THg as MeHg (%MeHg). The proportion of %MeHg in maternal blood varied between 4%-100% and that in cord blood it varied between 8%-100%. Additionally, at lower THg concentrations the variability of %MeHg was higher. The direct measurements of MeHg showed better prediction values when studying associations between the child's cognitive score and cord blood MeHg levels in comparison with THg cord blood levels. With this we confirmed the second hypothesis that Hg speciation provides more detailed information about exposure to different Hg species, which was shown to be especially important at an individual level, when studying associations between prenatal Hg exposure and its effects on child development.

Analytical techniques are rapidly developing and in recent years, measurements near limits of detection are achievable not only with measurements of THg, but also with measurements of MeHg. Methodologies to accurately speciate Hg in human samples are

available and cost-effective, and no longer represent a barrier for improved study protocols. Measurements of THg were shown to be appropriate for biomonitoring purposes, but when assessing the relation between Hg exposure and possible adverse effect(s) later in life, they can act as an additional source of uncertainty and as such contribute to misinterpretations. It was shown that the proportion of THg as MeHg can vary considerably at an individual level, especially at lower levels of exposure. Moreover, it is clear that different Hg species have different toxicokinetics profiles and target organs therefore they are hardly treated as comparable (Clarkson, 2002; Wells et al., 2017).

Assessment of Hg in whole blood provides information about Hg exposure for the past ~3 months. Moreover, Hg in blood is in a constant dynamic state of metabolism, distribution, and elimination. For these reasons, measurements of Hg in maternal blood or cord blood are not appropriate for the assessment of Hg exposure during early months of gestation. Measurements of Hg in maternal hair are frequently used for the indirect long-term exposure assessment, although the relation between Hg levels in maternal hair, as a mechanism of Hg elimination, and amount of Hg that presented the foetal exposure is questionable (Ruggieri et al., 2017). In this context, meconium represents a foetal excretory product and is therefore a direct measure of exposure. Meconium starts to form in the beginning of the 2nd trimester and the majority accumulates after 38 weeks of pregnancy (Concheiro & Huestis, 2018). This means that meconium analysis would provide the cumulative exposure primarily during the last trimester of pregnancy, but could also give insights to earlier exposures. Our study (Chapter 3.2) was the first one to perform Hg speciation on such a large sample size (N = 488). As expected, THg levels found in meconium (11.1 ng/g) are on average higher than those found in blood or cord blood and that the majority of THg is in its inorganic Hg(II) form (82%-100%). Consistently, multielemental analysis of selected samples (N = 20) showed that levels of Se are higher in meconium (range: 227 – 738 ng/g) than those measured in maternal or cord blood. On the other hand, levels of As (range: 0.35 – 5.45 ng/g) and Cd (range: 0.10 – 0.60 ng/g) were comparable between different biological samples (maternal blood, cord blood, meconium) whereas levels of Pb (range: 0.90 – 9.85 ng/g) were the lowest in meconium.

All Hg species, to which general population is exposed to, can pass the placental barrier and have the potential to be accumulated in meconium, therefore their toxicokinetics and biotransformation mechanisms should be established. Our results showed that both, MeHg from seafood and Hg^0 released from dental amalgams, were transported to the foetal side. Association showed that Hg^0 vapor released from dental amalgams was transported through the placental barrier and later accumulated in meconium. However, it was impossible to estimate whether oxidation of Hg^0 to Hg(II) occurred in maternal or foetal tissues. This partially confirms our third hypothesis, as we identified that meconium is a promising new biomarker of prenatal Hg exposure. However, high Hg(II) content in meconium was related to Hg^0 oxidation and accumulation of Hg(II), whereas MeHg demethylation to Hg(II), as an effective mechanism of MeHg elimination from the adult body, could neither be confirmed nor excluded during prenatal life, because some of the possible mechanisms (e.g. the role of microbiome, selenium or ROS) were impossible to test or prove.

Certain bacteria, which are also found in intestinal flora, can cleave the C-Hg bond (Rand et al., 2016; Rowland, Davies, & Evans, 1980). Moreover, in mammals MeHg demethylation can occur in organs such as the liver and brain (Friberg & Mottet, 1989; Shapiro & Chan, 2008; Vahter et al., 1995). Besides the role of microbiome, in vitro and animal experiments have shown that demethylation occurs as a result of the interaction of MeHg with reactive oxygen species (ROS) (Nagano et al., 2010; Shapiro & Chan, 2008; Vahter et al., 1995) or possibly with selenium (Khan & Wang, 2010). Although prenatal life was shown to be influenced with microbiome (Schoenmakers, Steegers-Theunissen, & Faas, 2019), it is hard to determine which microbial composition is detrimental or optimal

and what is the exact function of the microbiome at each location. In contrast, during pregnancy the formation of ROS is an unavoidable consequence of rapid aerobic metabolism (Watson, Skepper, Jauniaux, & Burton, 1998) and levels of selenium, essential element for human health, are particularly well maintained (Rayman, 2012). During gestation, blood-brain-barrier is immature (Goasdoué, Miller, Colditz, & Björkman, 2017) thus allowing more exchange (influx and efflux) of various compounds. Therefore, brain MeHg demethylation including the role of different levels of ROS and/or selenium should be considered in future studies.

Although sampling of the meconium is easy and non-invasive, methods of its collection are far from standardized. In most cases, meconium is excreted in the first 72 hours after birth and sampled from diaper (Concheiro & Huestis, 2018). Concentrations in meconium were shown to be correlated with the time of its excretion after birth (Ortega Garcia et al., 2006); therefore, this information should be taken into account when assessing levels of exposure. Moreover, sampling from diaper makes it impossible to compare the water content and is in some way pointless to lyophilize the samples before the measurements, since the majority of water (and dissolved trace elements) was probably absorbed in the diaper. For these reasons, it could be useful to sample meconium directly in plastic containers. Additionally, to follow the dynamics of Hg levels found in meconium precisely, sampling of excreted meconium at different points inside 72 hours could be interesting. Correlation between meconium Hg concentrations excreted during different time frames with Hg levels found in maternal blood, preferably sampled several times during pregnancy, could explain prenatal Hg toxicokinetics and biotransformation mechanisms in detail.

It is clear that complex interactions between genetic and environmental factors determine a high degree of variability in an individual response to a given exposure. Regarding in vitro experiments (Miyata & Smith, 1996) protein isoforms of Apolipoprotein E were hypothesized to act differently in relation to metal kinetics (Miyata & Smith, 1996) and to be involved in neurodevelopment (Huang & Mahley, 2014b). During prenatal life, the developing child depends on nutrients provided by the mother. In this context, human placenta is essential for foetal health and development and although placenta is of maternal and foetal origin, the environment provided by the mother during pregnancy is often not considered. In this context, maternal *ApoE* polymorphisms were included to study how they could affect the levels of selected elements in maternal blood, cord blood, maternal urine, and milk (Chapter 3.3). In vitro studies showed that structural differences between isoforms are the reason for their different metals and ROS binding capacity. Variants of ApoE with Cys in their structure (ApoE2 and ApoE3, encoded with alleles *ε2* and *ε3,* respectively) were hypothesized to bind metals more efficiently in comparison with ApoE4 (encoded with *ε4* allele), which would contribute to lower capacity of *ε4* carriers to bind Hg and locally protect specific cells (particularly in the brain) against its toxic effects. After we took into account other covariates that could contribute to levels of Hg found in maternal blood or cord blood (seafood intake, age, parity, smoking, pre-pregnancy BMI, child's gender, gestational age, birth weight), we did not find statistically significant association between Hg levels in any of the studied biological matrices and *ApoE* genetic polymorphisms. The observed higher levels of Hg in maternal blood in ApoE *ε4* carriers were not confirmed by regression analysis. Surprisingly, the distribution difference between *ε4* carriers and non-carriers persisted only for plasma Se levels, geometrical means of plasma Se being 62.6 ng/mL in *ε4* carriers in comparison with 54.9 ng/mL in *ε4* non-carriers ($p < 0.001$). Selenium was found to have a beneficial effect on reproduction, pregnancy-related diseases like pre-eclampsia, miscarriage, preterm birth, thyroid autoimmunity, and neonatal diseases accompanied with oxidative stress (Rayman, 2012). Therefore, the superior Se status observed in healthy pregnant women carrying at least one *ε4* allele could be linked to beneficial effects of *ApoE*.

As ApoE isoforms are believed to be differentially involved in metal kinetics in neurobiology, we evaluated the association between prenatal Hg exposure and the neurodevelopment of the child, taking into account the ApoE genetic polymorphisms (Chapter 3.4). In this evaluation children who were *ε4* allele carriers had marginally statistically significant higher levels of Hg in cord blood (1.98 ng/g vs. 2.37 ng/g). After taking into account covariates (mother's age, child's gender, birth weight, educational level of the mother, smoking) we observed a Hg-associated decrease in cognitive score (inside normal levels) of children having at least one *ε4* allele. Additionally, Hg-associated decreases in fine motor scores were genotype independent. With this, our last hypothesis was only partially confirmed and needs further research.

The inconsistent conclusions related to the last two papers (Chapter 3.3 and Chapter 3.4) could point to several roles of ApoE in different life stages. Clearly, homeostasis mechanisms are different in prenatal life (development) or during pregnancy, as the latter is the time of rapid changes in metabolism, oxidative stress, and other physiological and anatomical changes. On the other hand, we could speculate that the child's genotype plays a central role in the newborn's neurodevelopment and metabolism of Hg and the maternal genotype provides the basic environment, in which the baby develops and has access to the needed nutrients. However, as seen from methods used in both studies, the covariates included in the evaluation of the role of ApoE are different and were subjectively selected based on different points of view. Moreover, child's neurodevelopment was associated with Hg measurements assessed in mixed cord blood, which can be an additional source of uncertainty. Moreover, with mixed cord blood the real prenatal exposure is concealed and also contamination with maternal blood cannot be excluded. The reliable interpretation of the presented results will be possible when the physiology of ApoE will be studied in detail. As mentioned throughout the introduction, many inconsistencies and controversial conclusions exist in relation to prenatal Hg exposure and its possible negative effects later in life. Moreover, the assessment of neurodevelopmental outcomes in children aged 18 months is far from a controlled laboratory technique, although the psychologists are well trained and the Bayley-III test is standardized. However, the influence of maternal and parental intelligence and mental health are usually not included in such studies, but can clearly be considered as one of the most important factors (Barbone et al., 2019).

Studies addressing the issue of Hg-related adverse effects provided numerous valuable information, but at this point available data should be summarized to recognize the issues and uncertainties related to prenatal Hg exposure. Firstly, researchers could comment on the selection of used analytical techniques. Toxicokinetics mechanisms differ between Hg species, and although all can be toxic for human health, the mechanisms behind and target organs are often not comparable. As Hg speciation methods are available and cost-effective, they could be performed on all epidemiological studies addressing the health effects of Hg. Speciation analysis is especially encouraged when assessing the exposure in a general population, as the contribution of different Hg species to the total Hg levels found in their body can vary considerably at an individual level. Secondly, critical evaluation of available biomarkers should be done. Researchers assessing Hg exposure must be aware of the limitations of each biomarker. Although maternal hair and blood are the easiest to collect, they present an indirect measure of exposure. In the case of pregnancy, where haematological changes are known to occur, normalization for hematocrit would enable a more accurate comparison of the measured concentrations. As foetal and maternal haemoglobin have a different binding capacity for Hg and cord blood has higher haematocrit levels (which also differ according to gestational age), adjustment for haematocrit or haemoglobin could be useful also in the case of cord blood. Separation to venous and arterial cord blood is encouraged, as only with this approach we will assess the amount of Hg, which is passing from the mother to the child or the amount of Hg that was

metabolized in foetal tissues and is returning back to the maternal side. Thirdly, meconium as a novel long-term exposure biomarker should be discussed. Further studies should evaluate the mechanisms of intestinal prenatal MeHg demethylation and the involvement of microbiome, selenium, and reactive oxygen species. Studies could assess the relation between Hg species found in meconium and the child's neurodevelopment. A closer look into microbiome with its ~ 3 million genes possibly involved in Hg metabolism could give new insights into Hg toxicokinetics. Fourthly, genetic susceptibility biomarkers could answer to some of the complex interactions between external and internal exposure. However, the physiological role of the selected genetic factors and their role(s) in Hg toxicokinetics or toxicodynamics should be clear. Finally yet importantly, all individual characteristics and habits (e.g. seafood intake, number of dental amalgams, nutritional status, co-exposure to other potentially toxic substances, smoking, age, gender, etc.), and maternal and parental intelligence should be included in the evaluation of this sensitive topic.

These steps could be the starting point of standardized protocol for the assessment of prenatal Hg exposure and are encouraged to be used in further epidemiological studies. Re-evaluation of the observed results with considering all of the listed shortcomings will be done in our future studies. Only precise Hg measurements in relevant biological matrices including all possible influencing factors will enable the establishment of thresholds that will undoubtedly be safe for human health.

Chapter 5

Conclusions

The first two goals of the dissertation were to define the exposure to different Hg species (MeHg, Hg(II)) in a population of mothers and their newborns from Slovenia and Croatia and to compare associations between different blood Hg species with the children's neurodevelopmental outcomes. The hypotheses related to those goals were that the majority of total Hg will be present in organic form (MeHg) and that blood methyl-Hg measurements in combination with THg levels will provide clearer information about the exposure to different Hg species.

The studied population of pregnant women from Slovenia and Croatia were exposed to low-to-moderate levels of Hg especially through seafood intake and dental amalgam fillings. The majority of THg in maternal blood and cord blood was present in its organic MeHg form, but substantial individual variations in proportion of THg as MeHg were observed, especially at lower levels of exposure. Hg speciation provided better prediction values when studying associations between the child's cognitive score and cord blood MeHg levels in comparison with cord blood THg levels. Hg speciation analysis should be included in future studies, especially for the assessment of health effects after prenatal Hg exposure and when studying Hg toxicokinetics mechanisms.

The next goal was to study meconium as a biomarker of prenatal long-term Hg exposure and discuss possible mechanisms of Hg biotransformation during pregnancy with hypothesis that Hg speciation in meconium will help to estimate long-term foetal exposure to Hg and explain Hg biotransformation processes during pregnancy.

Meconium showed to be a promising new biomarker of prenatal long-term Hg exposure. Hg levels found in meconium were higher compared to those found in maternal blood or cord blood and the majority of Hg measured in meconium was in its inorganic Hg(II) form. Both MeHg and Hg^0 vapor are transported through the placental barrier and are later accumulated in meconium. However, the hypothesis of prenatal intestinal MeHg demethylation could be neither confirmed nor excluded; further studies are needed to establish Hg biotransformation. Oxidation of Hg^0 to Hg(II) and accumulation of the latter in meconium seems to have a major contribution of high Hg(II) content found in meconium.

The last two goals were related to ApoE genetic polymorphisms how their polymorphisms are associated with selected elements, and, on the other hand, what is the relation between prenatal Hg exposure, child's neurodevelopment, and ApoE genetic polymorphisms. The hypothesis was that the presence of ApoE *ε4* allele would be associated with higher levels of Hg in maternal and cord blood, which could have a synergistic modifying effect on a child's neurodevelopment.

Women from Croatia, who were ApoE *ε4* carriers, had higher levels of plasma Se in comparison with non-carriers. Higher levels of Se found in ApoE *ε4* carriers could be linked to ApoE beneficial effects in early life. However, the hypothesis of higher levels of Hg in

maternal blood in ApoE *ε4* carriers was not confirmed, whereas newborns, who were ApoE *ε4* carriers had marginally significant higher levels of cord blood Hg, in case of combined Slovenian and Croatian population. We identified a negative association between Hg and the cognitive performance of children who were *ε4* carriers, while the decrease in fine motor skills was genotype independent. Further studies are needed to study the physiological role of ApoE in Hg and Se kinetics, and its involvement in neurodevelopment.

References

Andreoli, V., & Sprovieri, F. (2017). Genetic aspects of susceptibility to mercury toxicity: An overview. *International Journal of Environmental Research and Public Health*, *14*(93). https://doi.org/10.3390/ijerph14010093

ATSDR. (1999). Toxicological profile for mercury. Retrieved 22 July 2019, from U.S. Department of Health and Human Services. website: https://www.atsdr.cdc.gov/toxprofiles/tp46.pdf

Bakir, F., Damluji, S. F., Amin-Zaki, L., Murtadha, M., Khalidi, A., Al-Rawi, N. Y., … Doherty, R. A. (1973). Methylmercury Poisoning in Iraq: an interuniversity report. *Science*, *181*(4096), 230–241.

Ballatori, N. (2002). Transport of toxic metals by molecular mimicry. *Environmental Health Perspectives*, *110*(suppl 5), 689–694. https://doi.org/10.1289/ehp.02110s5689

Ballatori, N., & Clarkson, T. W. (1985). Biliary secretion of glutathione and of glutathione-metal complexes. *Fundamental and Applied Toxicology : Official Journal of the Society of Toxicology*, *5*(5), 816–831. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/4065458

Barbone, F., Rosolen, V., Mariuz, M., Parpinel, M., Casetta, A., Sammartano, F., … Horvat, M. (2019). Prenatal mercury exposure and child neurodevelopment outcomes at 18 months: Results from the Mediterranean PHIME cohort. *International Journal of Hygiene and Environmental Health*, *222*(2019), 9–21. https://doi.org/10.1016/j.ijheh.2018.07.011

Basu, N., Goodrich, J. M., & Head, J. (2014). Ecogenetics of mercury: from genetic polymorphisms and epigenetics to risk assessment and decision-making. *Environmental Toxicology and Chemistry*, *33*(6), 1248–1258. https://doi.org/10.1002/etc.2375

Basu, N., Horvat, M., Evers, D. C., Zastenskaya, I., Weihe, P., & Tempowski, J. (2018). A state-of-the-science review of mercury biomarkers in human populations worldwide between 2000 and 2018. *Environmental Health Perspectives*, *126*(October), 1–14. https://doi.org/10.1289/EHP3904

Berglund, M., Lind, B., Björnberg, K. A., Palm, B., Einarsson, Ö., & Vahter, M. (2005). Inter-individual variations of human mercury exposure biomarkers: a cross-sectional assessment. *Environmental Health*, *4*(20). https://doi.org/10.1186/ 1476-069X-4-20

Berlin, M., Zalups, R. K., & Fowler, B. A. (2015). Mercury. In G. F. Nordberg, B. A. Fowler, & M. Nordberg (Eds.), *Handbook on the Toxicology of Metals. Volume II, Specific metals* (4th., pp. 1013–1075). London, UK: Academic Press.

Bernhard, D., Rossmann, A., & Wick, G. (2005). Metals in cigarette smoke. *International*

Union of Biochemistry and Molecular Biology: Life, *57*(12), 805–809. https://doi.org/10.1080/15216540500459667

Borsonelo, E. C., & Galduróz, J. C. F. (2008). The role of polyunsaturated fatty acids (PUFAs) in development, aging and substance abuse disorders: review and propositions. *Prostaglandins, Leukotrienes, and Essential Fatty Acids*, *78*(4–5), 237–245. https://doi.org/10.1016/j.plefa.2008.03.005

Branco, V., Caito, S., Farina, M., Teixeira, J., & Carvalho, C. (2017). Biomarkers of mercury toxicity: Past, present, and future trends. *Journal of Toxicology and Environmental Health, Part B*, *20*(3), 119–154. https://doi.org/10.1080/10937404.2017.1289834

Bridges, C. C., & Zalups, R. K. (2005). Molecular and ionic mimicry and the transport of toxic metals. *Toxicology and Applied Pharmacology*, *204*(3), 274–308. https://doi.org/10.1016/j.taap.2004.09.007

Broberg, K., Engstorm, K., & Ammer, S. (2014). Gene-Environment Interactions for Metals. In G. Nordberg, B. Fowler, & M. Nordberg (Eds.), *Handbook on the Toxicology of Metals* (4th ed., pp. 214–241). United Kingdom: Academic Press.

Burk, R. F., & Hill, K. E. (2005). Selenoprotein P: An Extracellular Protein with Unique Physical Characteristics and a Role in Selenium Homeostasis. *Annual Review of Nutrition*, *25*(1), 215–235. https://doi.org/10.1146/annurev.nutr.24.012003.132120

Cace, I. B., Milardovic, A., Prpic, I., Krajina, R., Petrovic, O., Vukelic, P., … Snoj, J. (2011). Relationship between the prenatal exposure to low-level of mercury and the size of a newborn's cerebellum. *Medical Hypotheses*, *76*(4), 514–516. https://doi.org/10.1016/j.mehy.2010.12.005

Caito, S. W., Jackson, B. P., Punshon, T., Scrimale, T., Grier, A., Gill, S. R., … Rand, M. D. (2018). Variation in methylmercury metabolism and elimination status in humans following fish consumption. *Toxicological Sciences*, *161*(2), 443–453. https://doi.org/10.1093/toxsci/kfx226

Caldwell, K. L., Mortensen, M. E., Jones, R. L., Caudill, S. P., & Osterloh, J. D. (2009). Total blood mercury concentrations in the U.S. population: 1999-2006. *International Journal of Hygiene and Environmental Health*, *212*(6), 588–598. https://doi.org/10.1016/j.ijheh.2009.04.004

Carneiro Hornos, M. H., Grotto, D., & Barbosa Jr, F. (2014). Inorganic and methylmercury levels in plasma are differentially associated with age, gender, and oxidative stress markers in a population exposed to mercury through fish consumption. *Journal of Toxicology and Environmental Health, Part A*, *77*, 69–79. https://doi.org/10.1080/15287394.2014.865584

Cave, M., Appana, S., Patel, M., Falkner, K. C., McClain, C. J., & Brock, G. (2010). Polychlorinated Biphenyls, Lead, and Mercury are associated with liver disease in American adults: NHANES 2003–2004. *Environmental Health Perspectives*, *118*(12), 1735–1742. https://doi.org/10.1289/ehp.1002720

Clarkson, T. W. (2002). The three modern faces of mercury. *Environmental Health Perspectives*, *110*(1), 11–23. https://doi.org/10.1289/ehp.02110s111

Clarkson, T. W., & Magos, L. (2006). The Toxicology of Mercury and Its Chemical Compounds. *Critical Reviews in Toxicology*, *36*(8), 609–662.

https://doi.org/10.1080/10408440600845619

Clarkson, T. W., Magos, L., & Greenwood, M. R. (1972). The transport of elemental mercury into fetal tissue. *Biology of the Neonate*, *21*, 239–244.

Concheiro, M., & Huestis, M. A. (2018). Drug exposure during pregnancy: analytical methods and toxicological findings. *Bioanalysis*, *2*. https://doi.org/10.4155/bio-2017-0260

Custodio, H. M., Harari, R., Gerhardsson, L., Skerfving, S., & Broberg, K. (2005). Genetic influences on the retention of inorganic mercury. *Archives of Environmental & Occupational Health*, *60*(1), 17–23. https://doi.org/10.3200/AEOH.60.1.17-23

de Chaves, E. P., & Narayanaswami, V. (2008). Apolipoprotein E and cholesterol in aging and disease in the brain. *Future Lipidology*, *3*(5), 505–530. https://doi.org/10.2217/17460875.3.5.505

Driscoll, C. T., Mason, R. P., Chan, H. M., Jacob, D. J., & Pirrone, N. (2013). Mercury as a global pollutant: Sources, pathways, and effects. *Environmental Science & Technology*, *47*(10), 4967–4983. https://doi.org/10.1021/es305071v

Du, H., Ma, M., Igarashi, Y., & Wang, D. (2019). Biotic and abiotic degradation of methylmercury in aquatic ecosystems: A review. *Bulletin of Environmental Contamination and Toxicology*, *102*(5), 605–611. https://doi.org/10.1007/s00128-018-2530-2

EFSA Scientific Committee. (2015). Statement on the benefits of fish/seafood consumption compared to the risks of methylmercury in fish/seafood. *EFSA Journal 2015*, *13*(1). https://doi.org/10.2903/j.efsa.2015.3982

Egert, S., Rimbach, G., & Huebbe, P. (2012). ApoE genotype: from geographic distribution to function and responsiveness to dietary factors. *Proceedings of the Nutrition Society*, 410–424. https://doi.org/10.1017/S0029665112000249

Eisenberg, D. T. A., Kuzawa, C. W., & Hayes, M. G. (2010). Worldwide allele frequencies of the human Apolipoprotein E gene: Climate, local adaptations, and evolutionary history. *American Journal of Physical Anthropology*, *111*(October 2009), 100–111. https://doi.org/10.1002/ajpa.21298

EPA. (2015). EPA: How people are exposed to mercury. Retrieved 22 July 2019, from https://www.epa.gov/mercury/how-people-are-exposed-mercury

Falnoga, I., & Tušek-Žnidarič, M. (2007). Selenium–Mercury interactions in man and animals. *Biological Trace Element Research*, *119*(3), 212–220. https://doi.org/10.1007/s12011-007-8009-3

Friberg, L., & Mottet, N. K. (1989). Accumulation of methylmercury and inorganic mercury in the brain. *Biological Trace Element Research*, *21*(1), 201–206. https://doi.org/10.1007/BF02917253

Getz, G. S., & Reardon, C. A. (2009). Apolipoprotein E as a lipid transport and signaling protein in the blood, liver, and artery wall. *Journal of Lipid Research*, *50*. https://doi.org/10.1194/jlr.R800058-JLR200

Giau, V. V., Bagyinszky, E., An, S. S. A., & Kim, S. (2015). Role of apolipoprotein E in neurodegenerative diseases. *Neuropsychiatric Disease and Treatment*, *11*, 1723–1737. https://doi.org/10.2147/NDT.S84266

Gibb, H., & O'Leary, K. G. (2014). Mercury Exposure and Health Impacts among

Individuals in the Artisanal and Small-Scale Gold Mining Community: A Comprehensive Review. *Environmental Health Perspectives*, *122*(7), 667. https://doi.org/10.1289/EHP.1307864

Goasdoué, K., Miller, S. M., Colditz, P. B., & Björkman, S. T. (2017). Review: The blood-brain barrier; protecting the developing fetal brain. *Placenta*, *54*, 111–116. https://doi.org/10.1016/J.PLACENTA.2016.12.005

Gochfeld, M. (2007). Framework for gender differences in human and animal toxicology. *Environmental Research*, *104*(1), 4–21. https://doi.org/10.1016/J.ENVRES.2005.12.005

Godfrey, M. E., Wojcik, D. P., & Krone, C. A. (2003). Apolipoprotein E genotyping as potential biomarker for mercury neurotoxicity. *Journal of Alzheimer's Disease*, *5*(3), 189–195. https://doi.org/10.3233/JAD-2003-5303

Golding, J., Gregory, S., Iles-caven, Y., Hibbeln, J., Emond, A., & Taylor, C. M. (2016). Associations between prenatal mercury exposure and early child development in the ALSPAC study. *Neurotoxicology*, *53*, 215–222. https://doi.org/10.1016/j.neuro.2016.02.006

Goodrich, J. M., Wang, Y., Gillespie, B., Werner, R., Franzblau, A., & Basu, N. (2011). Glutathione enzyme and selenoprotein polymorphisms associate with mercury biomarker levels in Michigan dental professionals. *Toxicology and Applied Pharmacology*, *257*(2), 301–308. https://doi.org/10.1016/j.taap.2011.09.014

Grandjean, P., & Budtz-Jørgensen, E. (2007). Total imprecision of exposure biomarkers: implications for calculating exposure limits. *American Journal of Industrial Medicine*, *50*(10), 712–719. https://doi.org/10.1002/ajim.20474.Total

Grandjean, P., & Herz, K. T. (2011). Brain development and methylmercury: Underestimation of neurotoxicity. *Mount Sinai Journal of Medicine*, *78*(1), 107–118. https://doi.org/10.1002/msj.20228.BRAIN

Grandjean, P., Satoh, H., Murata, K., & Eto, K. (2010). Adverse effects of methylmercury: Environmental health research implications. *Environmental Health Perspectives*, *118*(8), 1137–1145. https://doi.org/10.1289/ehp.0901757

Grandjean, P., Weihe, P. A. L., White, R. F., Debes, F., Araki, S., Yokoyama, K., … Jørgensen, P. J. (1997). Cognitive Deficit in 7-Year-Old Children with Prenatal Exposure to Methylmercury. *Neurotoxicology and Teratology*, *19*(6), 417–428. https://doi.org/10.1016/S0892-0362(97)00097-4

Grandjean, P., Weihe, P., Jørgensen, P. J., Clarkson, T., Cernichiari, E., & Viderø, T. (1992). Impact of Maternal Seafood Diet on Fetal Exposure to Mercury, Selenium, and Lead. *Archives of Environmental Health: An International Journal*, *47*(3), 185–195. https://doi.org/10.1080/00039896.1992.9938348

Gundacker, C., Fröhlich, S., Graf-Rohrmeister, K., Eibenberger, B., Jessenig, V., Gicic, D., … Husslein, P. (2010). Perinatal lead and mercury exposure in Austria. *Science of The Total Environment*, *408*(23), 5744–5749. https://doi.org/10.1016/j.scitotenv.2010.07.079

Gundacker, C., Komarnicki, G., Jagiello, P., Gencikova, A., Dahmen, N., Wittmann, K. J., & Gencik, M. (2007). Glutathione- S -transferase polymorphism , metallothionein expression , and mercury levels among students in Austria. *Science of The Total*

Environment, *385*, 37–47. https://doi.org/10.1016/j.scitotenv.2007.07.033

Gundacker, C., Neesen, J., Straka, E., Ellinger, I., & Dolzing, H. Hengestschläger, M. (2000). Genetics of the human placenta: implications for toxicokinetics. *Archives of Toxicology*, *90*, 2563–2581. https://doi.org/10.1006/s00204-016-1816-6

Harada, M. (1995). Minamata Disease: Methylmercury poisoning in Japan caused by environmental pollution. *Critical Reviews in Toxicology*, *25*(1), 1–24. https://doi.org/org/10.3109/10408449509089885

Hernández, A. F., Gil, F., Leno, E., López, O., Rodrigo, L., & Pla, A. (2009). Interaction between human serum esterases and environmental metal compounds. *NeuroToxicology*, *30*(4), 628–635. https://doi.org/10.1016/j.neuro.2009.04.003

Hill, K. E., & Burk, R. F. (2001). Selenoprotein P. In D. L. Hatfield (Ed.), *Selenium Its Molecular biology and Role in Human Health*. Massachusetts, USA: Kluwer academic publishers.

Hong, Y.-S., Kim, Y.-M., & Lee, K.-E. (2012). Methylmercury exposure and health effects. *Journal of Preventive Medicine & Public Health*, *45*(6), 353–363. https://doi.org/10.3961/jpmph.2012.45.6.353

Horvat, M. (1989). *Študije in razvoj analiznih metod za določanje nizkih koncentracij živega srebra in njihova uporaba pri analizi bioloških vzorcev in vzorcev iz okolja. Doktorska disertacija.* Univerza E. Kardelja v Ljubljani.

Horvat, M., Snoj Tratnik, J., & Miklavčič, A. (2012). Mercury: Biomarkers of exposure and Human Biomonitoring. In L. E. Knudsen & D. F. Merlo (Eds.), *Biomarkers and Human Biomonitoring. Volume 1: Ongoing Programs and Exposures.* (pp. 381–417). Cambridge, UK: The Royal Society of Chemistry.

Huang, Y., & Mahley, R. W. (2014a). Apolipoprotein E: Structure and function in lipid metabolism, neurobiology, and Alzheimer's diseases. *Neurobiology of Disease*, *72*, 3–12. https://doi.org/10.1016/j.nbd.2014.08.025

Huang, Y., & Mahley, R. W. (2014b). Apolipoprotein E: Structure and function in lipid metabolism, neurobiology, and Alzheimer's diseases. *Neurobiology of Disease*, *72*, 3–12. https://doi.org/10.1016/j.nbd.2014.08.025

Huebbe, P., Nebel, A., Siegert, S., Moehring, J., Boesch-Saadatmandi, C., Most, E., … Rimbach, G. (2011). APOE 4 is associated with higher vitamin D levels in targeted replacement mice and humans. *FASEB J*, *25*(9), 3262–3270. https://doi.org/10.1096/fj.11-180935

Jasienska, G., Ellison, P. T., Galbarczyk, A., Jasienski, M., Kalemba-Drozdz, M., Nenko, I., … Ziomkiewicz. (2015). Apolipoprotein R (ApoE) polymorphism is related to differences in potential fertility in women: a case of antagonistic pleotropy? *A Proc R Soc*, *282*. https://doi.org/20142395

Jiang, C., Hsi, H., Fan, C., & Chien, L. (2014). Fetal exposure to environmental neurotoxins in Taiwan. *Plos One*, *9*(10). https://doi.org/10.1371/journal.pone.0109984

Julvez, J., Smith, D., Golding, J., Ring, S., Pourcain, S., Gonzalez, J. R., & Grandjean, P. (2013). Prenatal methylmercury exposure and genetic predisposition to cognitive deficit at age 8 years. *Epidemiology*, *24*(5). https://doi.org/10.1097/EDE.0b013e31829d5c93

Julvez, J., Smith, G. D., Ring, S., & Grandjean, P. (2019). A birth cohort study about the genetic modification of prenatal methylmercury association with child cognitive development. *American Journal of Epidemiology*, *188*(10), 1784–1793. https://doi.org/10.1093/aje/kwz156

Kajiwara, A., Yasutake, Y., Adachi, K., & Hirayama, T. . (1996). Methylmercury transport across the placenta via neutral amino acid carrier. *Archives of Toxicology*, *70*(5), 310–311. https://doi.org/10.1007/s002040050279

Karagas, M. R., Choi, A. L., Oken, E., Horvat, M., Schoeny, R., Kamai, E., … Korrick, S. (2012). Evidence on the human health effects of low-level methylmercury exposure. *Environmental Health Perspectives*, *120*(6), 799–806. https://doi.org/10.1289/ehp.1104494

Khan, M. A. K., & Wang, F. (2009). Mercury-selenium compounds and their toxicological significance: toward a molecular understanding of the mercury-selenium antagonism. *Environmental Toxicology and Chemistry*, *28*(8), 1567–1577. https://doi.org/10.1897/08-375.1

Khan, M. A. K., & Wang, F. (2010). Chemical demethylation of methylmercury by Selenoamino acids. *Chemical Research in Toxicology*, *23*, 1202–1206. https://doi.org/10.1021/tx100080s

Kim, B.-M., Choi, A. L., Ha, E.-H., Pedersen, L., Nielsen, F., Weihe, P., … Grandjean, P. (2014). Effect of hemoglobin adjustment on the precision of mercury concentrations in maternal and cord blood. *Environmental Research*, *132*, 407–412. https://doi.org/10.1016/j.envres.2014.04.030

Kjellström, T., Kennedy, P., Wallis, S., & Mantell, C. (1986). Physical and mental development of children with prenatal exposure to mercury from fish. Stage 1; Preliminary tests at age 4; Report 3080. In *National Swedish Environmental Protection Board*. Solna, Sweden:

Kjellström, T., Kennedy, P., Wallis, S., Stewart, A., Friberg, L., Lind, B., … Mantell, C. (1989). Physical and mental development of children with prenatal exposure to mercury from fish: stage 2, interviews and psychological tests at age 6. In *Report 3642 (National Swedish Environmental Board)*. Retrieved from National Swedish Environmental Protection Board, Research Dept website: http://agris.fao.org/agris-search/search.do?recordID=SE19910039355

Knezović, Z., Trgo, M., & Sultović, D. (2016). Monitoring mercury environment pollution through bioaccumulation in meconium. *Process Safety and Environmental Protection*, *101*, 2–8. https://doi.org/10.1016/j.psep.2016.01.013

Kozikowska, I., Binkowski, J. Ł., Szczepanska, K., Sławska, H., Miszczuk, K., Sliwinska, M., … Stawarz, R. (2013). Mercury concentrations in human placenta, umbilical cord, cord blood and amniotic fluid and their relations with body parameters of newborns. *Environmental Pollution*, *182*, 256–262. https://doi.org/10.1016/j.envpol.2013.07.030

LaKind, J. S., Brent, R. L., Dourson, M. L., Kacew, S., Koren, G., Sonawane, B., … Uhl, K. (2005). Human Milk Biomonitoring Data: Interpretation and Risk Assessment Issues. *Journal of Toxicology and Environmental Health, Part A*, *68*(20), 1713–1769. https://doi.org/10.1080/15287390500225724

Liang, L., Bloom, N. S., & Horvat, M. (1994). Simultaneous determination of mercury

speciation in biological materials by GC/CVAFS after ethylation and room-temperature precollection. *Clin. Chem.*, *40*(4), 602–607.

Liang, L., Horvat, M., & Bloom, N. S. (1994). An improved speciation method for mercury by GC/CVAFS after aqueous phase ethylation and room temperature precollection. *Talanta*, *3*, 371–379. https://doi.org/10.1016/0039-9140(94)80141-X

Lindow, S. W., Knight, R., Batty, J., & Haswell, S. J. (2003). Maternal and neonatal hair mercury concentrations: the effect of dental amalgam. *BJOG : An International Journal of Obstetrics and Gynaecology*, *110*(3), 287–291. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12628269

Liu, M., Kuhel, D. G., Shen, L., Hui, D. Y., & Woods, S. C. (2012). Apolipoprotein E does not cross the blood-cerebrospinal fluid barrier, as revealed by an improved technique for sampling CSF from mice. *AJP: Regulatory, Integrative and Comparative Physiology*, *303*(9), R903–R908. https://doi.org/10.1152/ajpregu.00219.2012

Llop, S., Ballester, F., & Broberg, K. (2015). Effect of gene-mercury interactions on mercury toxicokinetics and neurotoxicity. *Current Environmental Health Reports*, *2*(2), 179–194. https://doi.org/10.1007/s40572-015-0047-y

Llop, S., Ballester, F., Murcia, M., Forns, J., Tardon, A., Andiarena, A., … Lopez-Espinosa, M.-J. (2016). Prenatal exposure to mercury and neuropsychological development in young children: the role of fish consumption. *International Journal of Epidemiology*, *46*(3), dyw259. https://doi.org/10.1093/ije/dyw259

Llop, S., Engström, K., Ballester, F., Franforte, E., Alhamdow, A., Pisa, F., … Broberg, K. (2014). Polymorphisms in ABC transporter genes and concentrations of mercury in newborns – evidence from two mediterranean birth cohorts. *Plos One*, *9*(5). https://doi.org/10.1371/journal.pone.0097172

Llop, S., Guxens, M., Murcia, M., Lertxundi, A., Ramon, R., Riano, I., … INMA Project. (2012). Prenatal exposure to mercury and infant neurodevelopment in a multicenter cohort in Spain: Study of potential modifiers. *American Journal of Epidemiology*, *175*(5), 451–465. https://doi.org/10.1093/aje/kwr328

Llop, S., Lopez-Espinosa, M.-J., Rebagliato, M., & Ballester, F. (2013). Gender differences in the neurotoxicity of metals in children. *Toxicology*, *311*(1–2), 3–12. https://doi.org/10.1016/j.tox.2013.04.015

Magos, L., Halbach, S., & Clarkson, T. W. (1978). Role of catalase in the oxidation of mercury vapor. *Biochemical Pharmacology*, *27*(9), 1373–1377.

Mahaffey, K. R., Clickner, R. P., & Bodurow, C. C. (2004). Blood organic mercury and dietary mercury intake: National Health and Nutrition Examination Survey, 1999 and 2000. *Environmental Health Perspectives*, *112*(5), 562–570. https://doi.org/10.1289/ehp.6587

Mahaffey, K. R., Clickner, R. P., & Jeffries, R. A. (2009). Adult women's blood mercury concentrations cary regionally in the United States : Association with patterns of fish consumption. *Environmental Health Perspectives*, *117*(1), 47–53. https://doi.org/10.1289/ehp.11674

Martin, C. R., Ling, P. R., & Blackburn, G. L. (2016). Review of infant feeding: Key features of breast milk and infant formula. *Nutrients*, *8*(279), 1–11. https://doi.org/10.3390/nu8050279

Meplan, C., Crosley, L. K., Nicol, F., Beckett, G. J., Howie, A. F., Hill, K. E., … Hesketh, J. E. (2007). Genetic polymorphisms in the human selenoprotein P gene determine the response of selenoprotein markers to selenium supplementation in a gender-specific manner (the SELGEN study). *The FASEB Journal*, *21*, 3063–3074. https://doi.org/10.1096/fj.07-8166com

Miklavčič, A., Cuderman, P., Mazej, D., Snoj Tratnik, J., Krsnik, M., Planinšek, P., … Horvat, M. (2011). Biomarkers of low-level mercury exposure through fish consumption in pregnant and lactating Slovenian women. *Environmental Research*, *111*(8), 1201–1207. https://doi.org/10.1016/j.envres.2011.07.006

Miyata, M., & Smith, J. D. (1996). Apolipoprotein E allele-specific antioxidant activity and effects on cytotoxicity by oxidative insults and beta-amyloid peptides. *Nature Genetics*, *14*(1), 55–61. https://doi.org/10.1038/ng0996-55

Morin, A. M., Gatev, E., McEwen, L. M., MacIsaac, J. L., Lin, D. T. S., Koen, N., … Jones, M. J. (2017). Maternal blood contamination of collected cord blood can be identified using DNA methylation at three CpGs. *Clinical Epigenetics*, *9*(1), 75. https://doi.org/10.1186/s13148-017-0370-2

Murcia, M., Ballester, F., Enning, A. M., Iñiguez, C., Valvi, D., Basterrechea, M., … Llop, S. (2016). Prenatal mercury exposure and birth outcomes. *Environmental Research*, *151*, 11–20. https://doi.org/10.1016/J.ENVRES.2016.07.003

Mutter, J., Naumann, J., Sadaghiani, C., Schneider, R., & Walach, H. (2004). Alzheimer disease: Mercury as pathogenetic factor and apolipoprotein E as a moderator. *Neuroendocrinology Letters*, *25*(5), 331–339.

Myers, G. J., Davidson, P. W., Cox, C., Shamlaye, C. F., Palumbo, D., Cernichiari, E., … Clarkson, T. W. (2003). Prenatal methylmercury exposure from ocean fish consumption in the Seychelles child development study. *Lancet*, *361*(9370), 1686–1692. https://doi.org/10.1016/S0140-6736(03)13371-5

Myers, G. J., Marsh, D. O., Davidson, P. W., Cox, C., Shamlaye, C. F., Tanner, M., … Clarkson, T. W. (1995). Main neurodevelopmental study of Seychellois children following in utero exposure to methylmercury from a maternal fish diet: outcome at six months. *Neurotoxicology*, *16*(4), 653–664.

Nagano, M., Yasutake, A., & Miura, K. (2010). Demethylation of methylmercury in human neuroblastoma, glioblastoma and liver cells. *Journal of Health Science*, *56*(3), 326–330.

Ng, S., Lin, C.-C., Hwang, Y.-H., Hsieh, W.-S., Liao, H.-F., & Chen, P.-C. (2013). Mercury, APOE, and children's neurodevelopment. *NeuroToxicology*, *37*, 85–92. https://doi.org/10.1016/j.neuro.2013.03.012

Ng, S., Lin, C.-C., Jeng, S.-F., Hwang, Y.-H., Hsieh, W.-S., & Chen, P.-C. (2015). Mercury, APOE, and child behavior. *Chemosphere*, *120*, 123–130. https://doi.org/10.1016/j.chemosphere.2014.06.003

NRC. (2000a). Comparison of studies for use in risk assessment. In *Toxicological effects of methylmercury* (pp. 250–270). Washington, D.C.: National Academies Press.

NRC. (2000b). *Scientific Frontiers in Developmental Toxicology and Risk Assessment*. https://doi.org/10.17226/9871

NRC. (2000c). *Toxicological Effects of Methylmercury*. Washington, D.C.: National

Academy Press.

Nyholt, D. R., Yu, C.-E., & Visscher, P. M. (2009). On Jim Watson's APOE status: genetic information is hard to hide. *European Journal of Human Genetics*, *17*(2), 147–149. https://doi.org/10.1038/ejhg.2008.198

Oriá, R. B., Patrick, P. D., Zhang, H., Lorntz, B., Maurício, C., Costa, D. E. C., … Guerrant, R. L. (2005). APOE4 Protects the Cognitive Development in Children with Heavy Diarrhea Burdens in Northeast Brazil. *Pediatric Research*, *57*(2), 310–316. https://doi.org/10.1203/01.PDR.0000148719.82468.CA

Ortega Garcia, J. S., Carrizo Gallardo, D., Ferris i Tortajada, J., Garcia, M. M. P., & Grimalt, J. O. (2006). Meconium and neurotoxicants: searching for a prenatal exposure timing. *Archives of Disease in Childhood*, *91*, 642–646. https://doi.org/10.1136/adc.2005.084129

Ostrea Jr., E. ., Bielawski, D. M., & Posecion Jr., N. C. (2006). Meconium analysis to detect fetal exposure to neurotoxicants. *Archives of Disease in Childhood*, *91*(8), 628–629. https://doi.org/10.1136/adc.2006.095059

Ostrea Jr., E. M., Morales, V., Ngoumgna, E., Prescilla, R., Tan, E., E., H., … Manlapaz, M. L. (2002). Prevalence of fetal exposure to environmental toxins as determined by meconium analysis. *NeuroToxicology*, *23*, 329–339. https://doi.org/10.1016/S0161-813X(02)00077-3

Ou, L., Chen, L., Chen, C., Yang, T., Wang, H., Tong, Y., … Wang, X. (2014). Associations of methylmercury and inorganic mercury between human cord blood and maternal blood: A meta-analysis and its application. *Environmental Pollution*, *191*, 25–30. https://doi.org/10.1016/j.envpol.2014.04.016

Park, B. Y., & Lee, B. K. (2014). Use of meconium in perinatal epidemiology: Potential benefits and pitfalls. *Annals of Epidemiology*, *24*(12), 878–881. https://doi.org/10.1016/j.annepidem.2014.09.009

Park, J. D., & Zheng, W. (2012). Human exposure and health effects of inorganic and elemental mercury. *Journal of Preventive Medicine and Public Health*, *45*, 344–352.

Peng, S., Liu, L., Zhang, X., Heinrich, J., Zhang, J., Schramm, K., … Shen, H. (2015). A nested case-control study indicating heavy metal residues in meconium associate with maternal gestational diabetes mellitus risk. *Environmental Health*, *14*(19), 1–10. https://doi.org/10.1186/s12940-015-0004-0

Petrovič, D., Zorc, M., & Peterlin, B. (2000). Effect of apolipoprotein E polymorphism and apolipoprotein A-1 gene promoter polymorphism on lipid parameters and premature coronary artery disease. *Folia Biologica*, *45*(5), 181–185.

Prior, T., Mullins, E., Bennet, E., & Kumar, S. (2014). Influence of parity on fetal hemodynamics and amniotic fluid volume at term. *Ultrasound in Obstetrics & Gynecology*, *44*, 688–692. https://doi.org/10.1002/uog.13332

Prpić, I., Milardović, A., Vlašić-Cicvarić, I., Špirić, Z., Radić Nišević, J., Vukelić, P., … Horvat, M. (2017). Prenatal exposure to low-level methylmercury alters the child's fine motor skills at the age of 18 months. *Environmental Research*, *152*, 369–374. https://doi.org/10.1016/j.envres.2016.10.011

Ramirez, G. B., Pagulayan, O., Stat, M. S., Akagi, H., Rivera, A. F., Eng, C., … Casintahan, D. (2003). Tagum Study II: Follow-up study at two years of age after

prenatal exposure to mercury. *Pediatrics*, *111*(3), 289–295. https://doi.org/10.1542/peds.111.3.e289

Rand, M. D., Vorojeikina, D., van Wijngaarden, E., Jackson, B. P., Scrimale, T., Zareba, G., … Watson, G. E. (2016). Methods for individualized determination of methylmercury elimination rate and de-methylation status in humans following fish consumption. *Toxicol. Sci.*, *149*(2), 385–395. https://doi.org/10.1093/toxsci/kfv241

Rayman, M. P. (2012). Selenium and human health. *The Lancet*, *379*(9822), 1256–1268. https://doi.org/10.1016/S0140-6736(11)61452-9

Rice, D. (2004). The US EPA reference dose for methylmercury: Sources of uncertainty. *Environmental Research*, *95*(3), 406–413. https://doi.org/10.1016/j.envres.2003.08.013

Rice, D., & Barone, S. (2000). Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. *Environmental Health Perspectives*, *108*(suppl 3), 511–533. https://doi.org/10.1289/ehp.00108s3511

Rothenberg, S. E., Keiser, S., Ajami, N. J., Wong, M. C., Gesell, J., Petrosino, J. F., & Johs, A. (2017). The role of gut microbiota in fetal methylmercury exposure: insights from a pilot study. *Toxicology Letters*, *3*(242), 60–67. https://doi.org/10.1016/j.toxlet.2015.11.022.THE

Rothenberg, S. E., Korrick, S. A., & Fayad, R. (2015). The influence of obesity on blood mercury levels for U.S. non-pregnant adults and children: NHANES 2007-2010. *Environmental Research*, *138*, 173–180. https://doi.org/10.1016/j.envres.2015.01.018.THE

Rothenberg, S. E., Wagner, C. L., Hamidi, B., Alekseyenko, A. V, & Azcarate-Peril, M. A. (2019). Longitudinal changes during pregnancy in gut microbiota and methylmercury biomarkers, and reversal of microbe-exposure correlations. *Environmental Research*, *172*, 700–712. https://doi.org/10.1016/j.envres.2019.01.014

Rowland, I. R., Davies, M. J., & Evans, J. G. (1980). Tissue content of mercury in rats given methylmercuric chloride orally: Influence of intestinal flora. *Archives of Environmental Health: An International Journal*, *35*(3), 155–160. https://doi.org/10.1080/00039896.1980.10667485

Ruggieri, F., Majorani, C., Domanico, F., & Alimonti, A. (2017). Mercury in children: Current state on exposure through human biomonitoring studies. *International Journal of Environmental Research and Public Health*, *14*(5). https://doi.org/10.3390/ijerph14050519

Sakamoto, M., Chan, H. M., Domingo, J. L., Oliveira, R. B., Kawakami, S., & Murata, K. (2015). Significance of fingernail and toenail mercury concentrations as biomarkers for prenatal methylmercury exposure in relation to segmental hair mercury concentrations. *Environmental Research*, *136*, 289–294. https://doi.org/10.1016/J.ENVRES.2014.09.034

Sakamoto, M., Murata, K., Domingo, J. L., Yamamoto, M., Oliveira, R. B., Kawakami, S., & Nakamura, M. (2016). Implications of mercury concentrations in umbilical cord tissue in relation to maternal hair segments as biomarkers for prenatal exposure to methylmercury. *Environmental Research*, *149*, 282–287. https://doi.org/10.1016/j.envres.2016.04.023

Santonen, T., Aitio, A., Fowler, B. ., & Nordberg, M. (2015). Biological Monitoring and Biomarkers. In M. Nordberg, G.A., Fowler, B.A., Nordberg (Ed.), *Handbook on the toxicology of metals* (4th ed., pp. 155–171). London, UK: Elsevier.

Schoenmakers, S., Steegers-Theunissen, R., & Faas, M. (2019). The matter of the reproductive microbiome. *Obstetric Medicine*, *12*(3), 107–115. https://doi.org/10.1177/1753495X18775899

Shapiro, A. M., & Chan, H. M. (2008). Characterization of demethylation of methylmercury in cultured astrocytes. *Chemosphere*, *74*(1), 112–118. https://doi.org/10.1016/J.CHEMOSPHERE.2008.09.019

Sikorski, R., Paszkowski, T., & Szprengier-Juszkiewicz, T. (1986). Mercury in neonatal scalp hair. *The Science of the Total Environment*, *57*, 105–110. https://doi.org/10.1016/0048-9697(86)90015-x

Silbergeld, E. K. (2017). The Microbiome: Modulator of Pharmacological and Toxicological Exposures and Responses. *Toxicologic Pathology*, *45*(1), 190–194. https://doi.org/10.1177/0192623316672073

Smith, J. S., Ashford, J. W., & Perffeti, T. A. (2019). Putative survival advantageous in young apolipoprotein e4 carriers are associated with increased neuronal stress. *Journal of Alzheimer's Disease*, 885–923. https://doi.org/10.3233/JAD-181089

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

Snoj Tratnik, J., Falnoga, I., Trdin, A., Mazej, D., Fajon, V., Miklavčič, A., … Horvat, M. (2017). Prenatal mercury exposure, neurodevelopment and apolipoprotein E genetic polymorphism. *Environmental Research*, *152*, 375–385. https://doi.org/10.1016/j.envres.2016.08.035

Snoj Tratnik, J., Mazej, D., & Horvat, M. (2019). Analytical Quality Requirements in Human Biomonitoring Programs: Trace Elements in Human Blood. *International Journal of Environmental Research and Public Health*, *16*(13), 2287. https://doi.org/10.3390/ijerph16132287

Soma-Pillay, P., Nelson-Piercy, C., Tolppanen, H., & Mebazaa, A. (2016). Physiological changes in pregnancy. *Cardiovascular Journal of Africa*, *27*(2), 89–94. https://doi.org/10.5830/CVJA-2016-021

Spiller, H. A. (2018). Rethinking mercury: the role of selenium in the pathophysiology of mercury toxicity. *Clinical Toxicology*, *56*(5), 313–326. https://doi.org/10.1080/15563650.2017.1400555

Stern, A. H., & Smith, A. E. (2003). An assessment of the cord blood: Maternal blood methylmercury ratio: Implications for risk assessment. *Environmental Health Perspectives*, *111*(12), 1465–1470. https://doi.org/10.1289/ehp.6187

Suzuki, T., Shishido, S., & Urushiyama, K. (1976). Mercury in Cigarettes. *The Tohoku Journal of Experimental Medicine*, *119*(4), 353–356. https://doi.org/10.1620/tjem.119.353

Tatsuta, N., Kurokawa, N., Nakai, K., Suzuki, K., Iwai-Shimada, M., Murata, K., & Satoh,

H. (2017). Effects of intrauterine exposures to polychlorinated biphenyls, methylmercury, and lead on birth weight in Japanese male and female newborns. *Environmental Health and Preventive Medicine*, *22*(1), 39. https://doi.org/10.1186/s12199-017-0635-6

Taylor, C. M., Golding, J., & Emond, A. M. (2016). Blood mercury levels and fish consumption in pregnancy: Risks and benefits for birth outcomes in a prospective observational birth cohort. *International Journal of Hygiene and Environmental Health*, *219*(6), 513–520. https://doi.org/10.1016/j.ijheh.2016.05.004

Templeton, D. M., Ariese, F., Cornelis, R., Danielsson, L.-G.-, Muntau, H., van Leeuwen, H. P., & R., L. (2000). Guidelines for terms related to chemical speciation and fractionation of elements. Definitions, structural aspects, and methodological approaches. IUPAC Recommendations 2000. *Pure and Applied Chemistry*, *72*(8), 1453–1470.

Trdin, A., Falnoga, I., Fajon, V., Živković, I., Snoj Tratnik, J., Prpić, I., … Horvat, M. (2019). Mercury speciation in meconium and associated factors. *Environmental Research*, *179*. https://doi.org/10.1016/j.envres.2019.108724

Trdin, A., Snoj Tratnik, J., Mazej, D., Fajon, V., Krsnik, M., Osredkar, J., … Horvat, M. (2019). Mercury speciation in prenatal exposure in Slovenian and Croatian population - PHIME study. *Environmental Research*, *177*. https://doi.org/10.1016/j.envres.2019.108627

Trdin, A., Snoj Tratnik, J., Stajnko, A., Marc, J., Mazej, D., Sešek Briški, A., … Falnoga, I. (2020). Trace elements and APOE polymorphisms in pregnant women and their newborns. *Environment International*.

Tuminello, E. R., & Han, S. D. (2011). The apolipoprotein E antagonistic pleiotropy hypothesis: Review and recommendations. *International Journal of Alzheimer's Disease*, *2011*, 1–12. https://doi.org/10.4061/2011/726197

UNEP/AMAP. (2013). *Technical Background Report for the Global Mercury Assessment 2013. Arctic Monitoring and Assessment Programme.* Oslo, Norway/UNEP Chemicals Branch, Geneva, Switzerland.

Unuvar, E., Ahmadov, H., Riza Kiziler, A., Aydemir, B., Toprak, S., Ulker, V., & Ark, C. (2007). Mercury levels in cord blood and meconium of healthy newborns and venous blood of their mothers: Clinical, prospective cohort study. *Science of The Total Environment*, *374*, 60–70. https://doi.org/10.1016/j.scitotenv.2006.11.043

US EPA. (1997). *Mercury Study. Report to Congress. Volume I: Executive Summary.*

Vahter, M., Akesson, A., Lind, B., Bjors, U., Schutz, A., & Berglund, M. (2000). Longitudinal study of methylmercury and inorganic mercury in blood and urine of pregnant and lactating women, as well as in umbilical cord blood. *Environmental Research*, *84*(2), 186–194. https://doi.org/10.1006/enrs.2000.4098

Vahter, M., Mottet, N. K., Friberg, L. T., Lind, S. B., Charleston, J. S., & Burbacher, T. M. (1995). Demethylation of methyl mercury in different brain sites of Macaca-fascicularis monkeys during long-term subclinical methyl mercury exposure. *Toxicology and Applied Pharmacology*, *134*(2), 273–284. https://doi.org/10.1006/TAAP.1995.1193

Valent, F., Horvat, M., Sofianou-Katsoulis, A., Spiric, Z., Mazej, D., Little, D., … Barbone,

F. (2013). Neurodevelopmental effects of low-level prenatal mercury exposure from maternal fish consumption in a mediterranean cohort: Study rationale and design. *International Journal of Epidemiology*, *23*(2), 146–152. https://doi.org/10.2188/jea.JE20120030

van Gelder, B. M., Tijhuis, M., Kalmijn, S., & Kromhout, D. (2007). Fish consumption, n−3 fatty acids, and subsequent 5-y cognitive decline in elderly men: the Zutphen Elderly Study. *The American Journal of Clinical Nutrition*, *85*(4), 1142–1147. https://doi.org/10.1093/ajcn/85.4.1142

van Wijngaarden, E., Thurston, S. W., Myers, G. J., Harrington, D., Cory-Slechta, D. A., Strain, J., … Davidson, P. W. (2017). Methyl mercury exposure and neurodevelopmental outcomes in the Seychelles Child Development Study Main cohort at age 22 and 24years. *Neurotoxicology and Teratology*, *59*, 35–42. https://doi.org/10.1016/j.ntt.2016.10.011

Vitek, M. P., Brown, C. M., & Colton, C. a. (2009). APOE genotype-specific differences in the innate immune response. *Neurobiology of Aging*, *30*(9), 1350–1360. https://doi.org/10.1016/j.neurobiolaging.2007.11.014

Walker, C.-D. (2005). Nutritional aspects modulating brain development and the responses to stress in early neonatal life. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *29*(8), 1249–1263. https://doi.org/10.1016/j.pnpbp.2005.08.010

Watson, A. L., Skepper, J. N., Jauniaux, E., & Burton, G. J. (1998). Changes in concentration, localization and activity of catalase within the human placenta during early gestation. *Placenta*, *19*(1), 27–34. https://doi.org/10.1016/S0143-4004(98)90095-9

Wells, E. M. (2019). Dietary correlates of methylmercury in seafood consumers and non-seafood soncumers. Retrieved 9 October 2019, from ICMGP Abstract Volume website: https://mercury2019krakow.com/public/files/ICMGP2019-abstract-volume.pdf

Wells, E. M., Herbstman, J. B., Lin, Y. H., Hibbeln, J., Halden, R. U., Witter, F. R., & Goldman, L. R. (2017). Methyl mercury, but not inorganic mercury, associated with higher blood pressure during pregnancy. *Environmental Research*, *154*, 247–252. https://doi.org/10.1016/j.envres.2017.01.013.Methyl

Wells, E. M., Herbstman, J. B., Lin, Y. H., Jarrett, J., Verdon, C. P., Ward, C., … Goldman, L. R. (2016). Cord blood methylmercury and fetal growth outcomes in Baltimore newborns: Potential confounding and effect modification by omega-3 fatty acids, selenium, and sex. *Environmental Health Perspectives*, *124*(3), 373–379. https://doi.org/10.1289/ehp.1408596

WHO. (2016). Mercury and health. Fact sheet. Retrieved 1 July 2019, from http://www.who.int/mediacentre/factsheets/fs361/en/

Wright, R. O., Hu, H., Silverman, E. K., Tsaih, S. W., Schwartz, J., Bellinger, D., … Hernandez-Avila, M. (2003). Apolipoprotein E genotype predicts 24-month Bayley Scales Infant Development Score. *Pediatric Research*, *54*(6), 819–825. https://doi.org/10.1203/01.PDR.0000090927.53818.DE

Yin, Z., Jiang, H., Syversen, T., Rocha, J. B. T., Farina, M., & Aschner, M. (2009). The methylmercury-L-cysteine conjugate is a substrate for the L- type large neutral amino acid transporter, LAT1. *Journal of Neurochemistry*, *107*(4), 1083–1090.

https://doi.org/10.1111/j.1471-4159.2008.05683.x.The

Yoshida, M. (2002). Placental to fetal transfer of mercury and fetotoxicity. *The Tohoku Journal of Experimental Medicine*, *196*(2), 79–88.

Zoidis, E., Seremelis, I., Kontopoulos, N., & Danezis, G. P. (2018). Selenium-dependent antioxidant enzymes: Actions and properties of Selenoproteins. *Antioxidants (Basel, Switzerland)*, *7*(5). https://doi.org/10.3390/antiox7050066

Bibliography

Journal Articles Related to the Dissertation

Trdin, A., Snoj Tratnik, J., Mazej, D., Fajon, V., Krsnik, M., Osredkar, J., Prpić, I., Špirić, Z., Petrović, O., Marc, J., Neubauer, D., Kodrič, J., Kobal, A.B., Barbone, F., Falnoga, I., Horvat, M. (2019). Mercury speciation in prenatal exposure in Slovenian and Croatian population – PHIME study. *Environmental Research*, 177. https://doi.org/10.1016/j.envres.2019.108627

Trdin, A., Falnoga, I., Fajon, V., Živković, I., Snoj Tratnik, J., Prpić, I., Špirić, Z., Horvat, M. (2019). Mercury speciation in meconium and associated factors. *Environmental Research*. https://doi.org/10.1016/j.envres.2019.108724

Trdin, A., Snoj Tratnik, J., Stajnko, A., Mazej, D., Sešek Briški, A., Kastelec, D., Prpić, I., Petrović, O., Špirić, Z., Marc, J., Horvat, M., Falnoga, I. (2019). Trace elements and APOE polymorphisms in pregnant women and their newborns. *Environment International*, accepted for publication.

Snoj Tratnik, J., Falnoga, I., Trdin, A., Mazej, D., Fajon, V., Miklavčič, A., Kobal, A.B., Osredkar, J., Sešek Briški, A., Krsnik, M., Neubauer, D., Kodrič, J., Stropnik S., Gosar, D., Lešnik Musek, P., Marc, J., Jurkovič Mlakar, S., Petrović, O., Vlašić-Cicvarić, I., Prpič, I., Milardović, A., Radić-Nišević, J., Vuković, D., Fišić, E., Špirić, Z., Horvat, M. (2017). Prenatal mercury exposure, neurodevelopment and apolipoprotein E genetic polymorphism. *Environmental research*, 152, 375-385. https://doi.org/10.1016/j.envres.2016.08.035

Other Journal Articles

Snoj Tratnik, J., Falnoga, I., Mazej, D., Kocman, D., Fajon, V., Jagodic, M., Stajnko, A., Trdin, A., Šlejkovec, Z., Jeran, Z., Osredkar, J., Sešek-Briški, A., Krsnik, M., Kobal, A.B., Kononenko, L., Horvat, M. (2019). Results of the first national human biomonitoring in Slovenia: Trace elements in men and lactating women, predictors of exposure and reference values. *International Journal of Hygiene and Environmental Health*, 22(3), 563-582. https://doi.org/10.1016/j.ijheh.2019.02.008

Published scientific conference contribution abstract

Falnoga, I., Trdin, A., Marc, J., Snoj Tratnik, J., Mazej, D., Stajnko, A., Špirić, Z., Horvat, M. (2016). Apolipoprotein E (ApoE) genotypes in relation with Hg, As, Pb and Se levels in mothers and their newborns. In: *Human biomonitoring: science and policy for a healthy future*. Berlin, Germany: 2nd International Conference on Human Biomonitoring.

Snoj Tratnik, J., Falnoga, I., Trdin, A., Mazej, D., Sešek-Briški, A., Osredkar, J., Krsnik, M., Kobal, A.B., Neubauer, D., Kodrič, J., Stropnik, S., Gosar, D., Marc, J., Prpić, I., Špirić, Z., Horvat, M. (2016). Neurodevelopment, low level mercury exposure and

genetic polymorphisms in a birth cohort from Slovenia and Croatia. In: *Abstracts, conference proceedings*. Barcelona, Spain: 8th International Conference on Children's Health and the Environment.

Snoj Tratnik, J., Falnoga, I., Trdin, A., Mazej, D., Sešek-Briški, A., Osredkar, J., Krsnik, M., Kobal, A. B.,, Neubauer, D., Kodrič, J., Stropnik, S., Gosar, D., Marc, J., Prpić, I., Špirić, Z., Horvat, M. (2016). Prenatal mercury exposure, neurodevelopment and APOE genetic polymorphism. In: *10th Young researchers' day, 31 March, 2016, Ljubljana: program and abstract book*. Ljubljana, Slovenia: Jožef Stefan Institute.

Trdin, A., Falnoga, I., Snoj Tratnik, J., Stajnko, A., Mazej, D., Marc, J., Krsnik, M., Osredkar, J., Prpić, I., Špirić, Z., Horvat, M. (2017). Apolipoprotein E genotypes in relation with Hg (MeHg) concentrations in pregnant females. In: *9th Jožef Stefan International Postgraduate School Students' Conference and 11th Young researchers' Day*. Ljubljana, Slovenia: Jožef Stefan International Postgraduate School.

Trdin, A., Falnoga, I., Snoj Tratnik, J., Mazej, D., Stajnko, A., Marc, J., Krsnik, M., Osredkar, J., Prpić, I., Špirić, I., Horvat, M.. (2017). Apolipoprotein E genotypes in relation with Hg, MeHg As and Se levels in mothers and their newborns. In: *Abstracts, 13th International Conference on Mercury as a Global Pollutant.* Providence, Rhode Island: ICMGP.

Trdin, A., Falnoga, I., Snoj Tratnik, J., Stajnko, A., Mazej, D., Marc, J., Krsnik, M., Osredkar, J., Prpić, I., Petrović, O., Špirić, Z., Horvat, Milena. (2017). Associations between apolipoprotein E genotypes and Hg concentrations in cord blood. In: *Biomonitoring for chemical risk assessment and control: book of abstracts*. Naples, Italy: 10th International Symposium on Biological Monitoring in Occupational and Environmental Health.

Snoj Tratnik, J., Falnoga, I., Trdin, A., Stajnko, A., Mazej, D., Horvat, M. (2017). Low level mercury exposure, neurodevelopment and genetic polymorphisms in Slovenian and Croatian birth cohorts. In: *Journal of trace elements in medicine and biology.* Saint Petersburg, Russia: joint 16th International Symposium on Trace Elements in Man and Animals (TEMA-16), 12th Conference of the International Society for Trace Element Research in Humans (ISTERH 2017) and 13th Conference of the Nordic Trace Element Society (NTES 2017).

Stajnko, A., Snoj Tratnik, J., Trdin, A., Jagodic, M., Mazej, D., France Štiglic, A., Horvat, M., Falnoga, I. (2017). Metallothionein polymorphisms and trace elements in Slovenian mother-child pairs (CROME - LIFE + and HEALS study). In: 9th Jožef Stefan International Postgraduate School Students' Conference and 11th Young researchers' Day. Ljubljana, Slovenia: Jožef Stefan International Postgraduate School.

Trdin, A., Snoj Tratnik, J., Marc, J., Mazej, D., Prpić, I., Špirić, Z., Horvat, M., Falnoga, I. (2017). Positive association of apolipoprotein E allel $\varepsilon 4$ with plasma selenium in Croatian pregnant females. In: *Program and abstract book.* Stockholm, Sweden: 11th International symposium on selenium in biology and medicine and 5th International conference on selenium in the environment and human health.

Biography

The author of this dissertation Ajda Trdin was born on July 24, 1991 in Slovenj Gradec, Slovenia. She finished her primary school and high school diploma in Velenje, Slovenia. In 2010, she started pursuing her bachelor's degree of Laboratory Biomedicine (BSc) at the Faculty of Pharmacy, University of Ljubljana, Slovenia. In 2013, she continued her master's study (MSc) of Laboratory Biomedicine at the Faculty of Pharmacy, University of Ljubljana. Ajda received an award for outstanding achievements during her master's study at the Faculty of Pharmacy and she was at the top 5% in her class. For the defense of the master's thesis entitled "Connection between mutations in the gene for apolipoprotein E with concentrations of mercury in the mothers and newborns" she was awarded a master of laboratory biomedicine degree in September 2015.

In 2015, she started her PhD study at the Jožef Stefan International Postgraduate School, Ljubljana, Slovenia, under the supervision of Prof. Dr. Milena Horvat. All of the experimental and research work was conducted at the Department of Environmental Sciences, Jožef Stefan Institute. Her research focuses on methods to assess prenatal mercury exposure and relation between mercury exposure and child's neurodevelopment. During PhD studies, she attended workshops on the topic of human biomonitoring and human mercury exposure. In addition, she presented her work at several international conferences related to toxicology, environmental health, and mercury as a global contaminant.