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



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### 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;  $\text{CH}_3\text{Hg}^+$ , MeHg) mainly through seafood consumption and to  $\text{Hg}^0$  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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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<sup>0</sup>; 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  $-24^{\circ}\text{C}$ .

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 <sup>a</sup>
Body mass index (BMI; kg/m <sup>2</sup> )	23.8 (17.1–44.5), N = 582	23.0 (16.8–41.1), N = 232	23.6 (16.8–44.5), N = 814	<b>0.012<sup>a</sup></b>
Parity (% of nulliparous)	52, N = 562	45, N = 233	50, N = 795	0.080 <sup>b</sup>
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	<b>0.000<sup>c</sup></b>
Ever-smoking (% of smokers)	31, N = 584	42, N = 234	35, N = 818	<b>0.002<sup>b</sup></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 <sup>c</sup>
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	<b>0.037<sup>c</sup></b>
Child sex (% boys/girls)	49/51, N = 373	50/50, N = 208	49/51, N = 582	0.728 <sup>b</sup>

Bolded are statistically significant differences between both populations. Statistical tests used to test the difference between Slovenian and Croatian population:

<sup>a</sup> ANOVA.

<sup>b</sup>  $\chi^2$ .

<sup>c</sup> 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<sub>3</sub> 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<sub>4</sub> 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,  $\chi^2$ , 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<sup>2</sup>) 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				MeHg (ng/g) median range			% MeHg (%) median range			IHg (ng/g) median range		
	Slo	Cro	All	All <sup>a</sup>	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	/	/	/	1.73 0.03–19.6	/	/	85.9 4.3–100	/	/	0.36 0.01–12.6	/
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.

<sup>a</sup> 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  $\epsilon 4$  in children and/or mothers (genotypes  $\epsilon 4$  vs.  $\epsilon 2$  and  $\epsilon 3$ ). 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 fetuses' 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 new-borns 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  $1 \mu\text{g/g}$  ( $N = 75$ ). 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  $346 \text{ ng/g}$ . Methylmercury in hair presented  $\sim 100\%$  of THg. In maternal blood, which was available only for the Croatian population, the median THg value was  $2.04 \text{ ng/g}$ . Methylmercury on average presented 85.9% of THg in maternal peripheral blood. The median value of IHg concentrations in maternal blood was  $0.36 \text{ ng/g}$ . The median value of THg in cord blood was  $1.84 \text{ ng/g}$ . Methylmercury presented 98.4% of THg in cord blood on average. The median value of IHg concentrations in cord blood was  $0.04 \text{ ng/g}$ . 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,  $\sim 10\%$  of women exceeded the threshold of  $1 \mu\text{g/g}$  for total mercury in hair, which was established by NRC (National Research Council, 2000). Based on the updated hair Hg limit of  $0.58 \mu\text{g/g}$  (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  $5.8 \mu\text{g/L}$  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  $\geq 3.5 \mu\text{g/L}$ , 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,  $\sim 12\%$  of women exceeded limit value (HBM-I) for THg defined at  $5 \mu\text{g/L}$  in maternal blood, and two women exceeded the action level (HBM-II) of  $15 \mu\text{g/L}$  (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  $1.73 \text{ ng/g}$ , MeHg presented only 62.4% (range: 4%–100%, CV: 42%) of THg on average; if we included only those lower than  $0.72 \text{ ng/g}$  (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  $1.69 \text{ ng/g}$ , MeHg presented 92.4% (range: 8%–100%, CV: 27%) of THg on average; if we included only those lower than  $0.76 \text{ ng/g}$  (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  $\sim 13 \text{ ng/g}$ , %MeHg  $\sim 5\%$ ), 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		IHg	Seafood intake	BMI	Age	Parity
	THg	MeHg	MeHg	MeHg					
MH	THg	0.892*** (225)	0.887*** (225)	0.903*** (611)	-0.060 (611)	0.509*** (554)	-0.221*** (798)	0.140*** (784)	-0.082* (776)
MB	THg	0.960*** (225)	0.960*** (225)	0.913*** (208)	-0.304*** (208)	0.440*** (198)	-0.199** (223)	0.174* (212)	-0.128 (224)
	MeHg			0.943*** (208)	-0.338*** (208)	0.480*** (198)	-0.217** (223)	0.199** (212)	-0.143* (224)
CB	IHg			-0.312** (208)	0.198** (208)	-0.181* (198)	0.144* (223)	-0.085 (212)	0.087 (224)
	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)
Seafood intake	IHg					-0.009 (527)	-0.046 (619)	-0.026 (612)	0.045 (613)
BMI							-0.126** (565)	0.035 (567)	-0.005 (563)
Age								0.087* (794)	0.099* (786)
									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 ( $R_s$ ) 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 assess 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	In THg MB	In MeHg MB
	$\beta$ ; p (95% CI)	$\beta$ ; p (95% CI)
	p < 0.001, R <sup>2</sup> = 0.2465; N = 150	p < 0.001, R <sup>2</sup> = 0.3029; N = 150
Seafood intake (meals per day)	<b>0.80; 0.000 (0.47; 1.13)</b>	<b>1.27; 0.000 (0.80; 1.74)</b>
Smoking (smokers vs. non-smokers)	-0.02; 0.853 (-0.23; 0.19)	-0.03; 0.831 (-0.34; 0.28)
Pre-pregnancy BMI <sup>a</sup> (kg/m <sup>2</sup> )	-0.02; 0.100 (-0.04; 0.004)	<b>-0.04; 0.020 (-0.07; -0.001)</b>
Mother's age (years)	<b>0.02; 0.090 (-0.003; 0.04)</b>	<b>0.04; 0.028 (0.004; 0.07)</b>
Parity (nulliparous vs. parous)	-0.17; 0.129 (-0.40; 0.05)	-0.23; 0.159 (-0.56; 0.09)
EGA <sup>b</sup> 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 In Pb (ng/g)	0.04; 0.680 (-0.16; 0.25)	-0.01; 0.959 (-0.31; 0.29)
Maternal plasma In Se (g/L)	<b>0.46; 0.081 (-0.06; 0.97)</b>	<b>0.92; 0.016 (0.17; 1.66)</b>
MODEL 2 CB	In THg CB	In MeHg CB
	$\beta$ ; p (95% CI)	$\beta$ ; p (95% CI)
	p < 0.001, R <sup>2</sup> = 0.3030; N = 388	p < 0.001, R <sup>2</sup> = 0.3016; N = 388
Seafood intake (meals per day)	<b>1.54; 0.000 (1.25; 1.82)</b>	<b>1.71; 0.000 (1.39; 2.04)</b>
Smoking (smokers vs. non-smokers)	0.02; 0.791 (-0.14; 0.19)	0.04; 0.683 (-0.15; 0.22)
Pre-pregnancy BMI <sup>a</sup> (kg/m <sup>2</sup> )	<b>-0.04; 0.000 (-0.06; -0.03)</b>	<b>-0.04; 0.000 (-0.06; -0.02)</b>
Mother's age (years)	<b>0.02; 0.014 (0.005; 0.04)</b>	<b>0.03; 0.007 (0.01; 0.05)</b>
Parity (nulliparous vs. parous)	<b>-0.14; 0.097 (-0.31; 0.03)</b>	<b>-0.19; 0.045 (-0.38; -0.004)</b>
EGA <sup>b</sup> (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 In Pb (ng/g)	0.13; 0.119 (-0.03; 0.30)	<b>0.17; 0.069 (-0.013; 0.36)</b>
Cord plasma In Se (g/L)	<b>0.32; 0.094 (-0.05; 0.69)</b>	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.

<sup>a</sup> BMI-body mass index.

<sup>b</sup> 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<sup>2</sup>) 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  $\epsilon 4$  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  $\epsilon 4$  carriers (Ng et al., 2015, 2013). Similarly, in our study (Snoj Tratnik et al., 2017), we found that children who were  $\epsilon 4$  carriers had higher cord blood THg levels in comparison with  $\epsilon 4$  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  $\epsilon 4$  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 In THg and child's ApoE genotype. Model B is the same as model A except that it is adjusted for cord blood In MeHg. Model C is adjusted for cord blood In THg and maternal ApoE genotype. Model D is adjusted for cord blood In 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  $\epsilon 4$  carrier) and cord blood In THg (Model E) or cord blood In 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<sup>2</sup> < 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  $\epsilon 4$  carriers. In the group of  $\epsilon 4$  carriers the  $\beta$ -estimate of the change in cognitive score is comparable for THg ( $\beta = -5.55$ ) and for MeHg ( $\beta = -5.33$ ). 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  $\epsilon 4$  carriers, a negative associations were observed between child's cognitive score and Hg levels, where  $\beta$ -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 In cord blood (CB) Hg using multiple linear regression models adjusted for ApoE genotype.  $\beta$ -estimate of change in neurodevelopment score. CI 95%-95% confidence interval.<sup>a</sup>  
 $\beta$  (CI 95%), p-value dependent variable model R<sup>2</sup>  
model p-value; N

Models	Cognitive	Language	Motor	Fine motor	Gross motor
<b>Child ApoE</b>					
In CB THg (Models <sup>A</sup> )	-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 <b>0.065, 0.008; 290</b>	-0.05 (-0.39, 0.26), 0.748 0.048, 0.126; 287
$\epsilon 2, \epsilon 3$ 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
$\epsilon 4$ 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
In CB MeHg (Models <sup>B</sup> )	-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	<b>-0.24 (-0.53, 0.04), 0.098</b> <b>0.061, 0.013; 290</b>	-0.04 (-0.31, 0.23), 0.767 0.048, 0.126; 287
$\epsilon 2, \epsilon 3$ 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
$\epsilon 4$ 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
<b>Maternal ApoE</b>					
In CB THg (Models <sup>C</sup> )	-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	<b>-0.29 (-0.63, 0.05), 0.090</b> <b>0.065, 0.026; 244</b>	0.18 (-0.14, 0.50), 0.279 0.044, 0.299; 243
$\epsilon 2, \epsilon 3$ 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
$\epsilon 4$ carriers	<b>-7.09 (-12.3, -1.88), 0.009</b> <b>0.407, 0.027; 36</b>	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), 0.129 0.145, 0.546; 37	0.92 (-0.29, 2.12), 0.129 0.299, 0.204; 37
Interaction $\epsilon 4$ 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), 0.186 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
In CB MeHg (Models <sup>D</sup> )	-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
$\epsilon 2, \epsilon 3$ 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
$\epsilon 4$ carriers	<b>-6.06 (-10.3, -1.87), 0.006</b> <b>0.422, 0.020; 36</b>	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 $\epsilon 4$ x MeHg	<b>-1.89 (-3.79, 0.02), 0.052</b> <b>0.326, 0.088; 36</b>	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
<b>Combined ApoE</b>					
In CB THg (Models <sup>E</sup> )	-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
$\epsilon 2, \epsilon 3$ 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
$\epsilon 4$ 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
In CB MeHg (Models <sup>F</sup> )	-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
$\epsilon 2, \epsilon 3$ 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
$\epsilon 4$ 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

<sup>a</sup> Models were designed in our previous work (Snjoi Tratinik et al., 2017). Models are adjusted for ApoE genotype, In 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). In cord blood plasma Se, and In cord blood Pb. Models A, C, and E are adjusted for In THg [ng/g] concentrations. Models B, D, and F are adjusted for In 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  $\epsilon 4$  carriers are mother-child pairs and at least one of them is an  $\epsilon 4$  carrier. Interaction  $\epsilon 4$  x THg (MeHg) model was adjusted the same as that in the group of  $\epsilon 4$  mothers, but instead of cord blood THg/MeHg levels and maternal ApoE  $\epsilon 4$  genotype we included maternal ApoE  $\epsilon 4$  x THg (MeHg). Bolded in italic are statistically significant associations; bolded in italic are marginally significant associations.



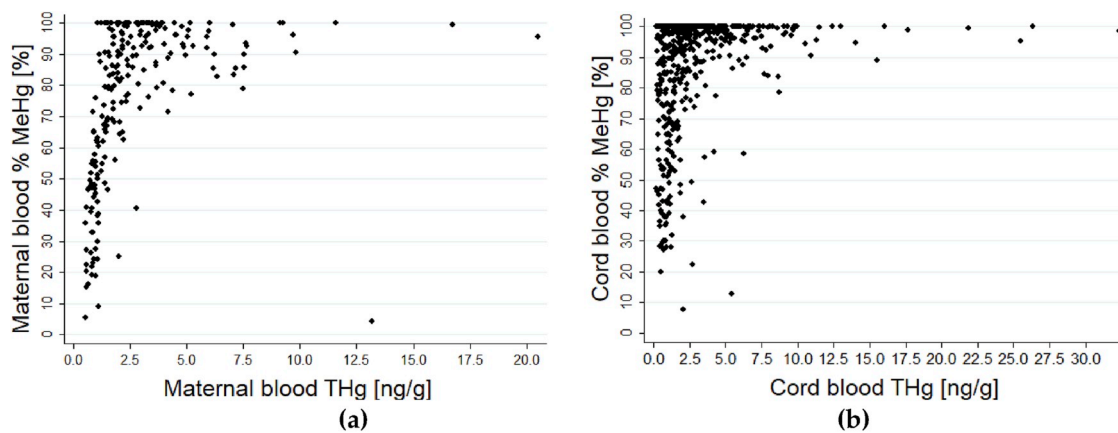


Fig. 1. Correlations between THg and % MeHg in (a) maternal blood (N = 225) and (b) cord blood (N = 633).

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  $\epsilon 4$  and cord blood Hg levels (ApoExHg). We observed marginally significant interaction between ApoE  $\epsilon 4$  and THg ( $p = 0.081$ ) on child's cognitive score and the model was not significant ( $p = 0.109$ ). However, interaction between ApoE  $\epsilon 4$  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  $\epsilon 4$  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  $\epsilon 4$  carrier), cord blood MeHg levels were significantly associated with decrease in cognitive score in the group of  $\epsilon 4$  carriers ( $p = 0.048$ ), compared with marginally significant association with THg ( $p = 0.074$ ). In this case,  $\beta$ -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  $\epsilon 4$  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 neuro-developmental 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.

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