Title: Prenatal mercury exposure, neurodevelopment and apolipoprotein E genetic polymorphism

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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; ;

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.

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 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 sores 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.

Keywords: mercury, selenium, lead, cord blood, neurodevelopment, apolipoprotein E, genetic polymorphism

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 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 protect or increase) 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 modify 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), levels of Pb in cord blood were considered as a confounder in the statistical assessment of Hg-neurodevelopment association, as they 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 also collected for total Hg and creatinine determination. Maternal hair (1-3 cm closest to scalp) and blood samples were collected by hospital personnel. Urine was collected by the mothers at

home. 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. Hair samples were stored in transparent mercury-free plastic bags in the dark and periodically sent to the Jožef Stefan Institute (JSI), Ljubljana for analysis. 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 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 and the study group (period of sampling). All measurements were made under strict quality control procedures and gave comparable results. In addition, the JSI laboratory participated in

a series of inter-laboratory comparisons 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; Miklavčič et al., 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) Scale 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 attended 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 levels and outcome (Bayley 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 population.

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 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).

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 %)

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

3.2 Exposure assessment, *Apoe* genotypes and neurodevelopmental performance

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

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 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.

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)*	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)*	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)*	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

*Stratification was done by maternal 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 3. Bayley-III scores for children of 18 months of age, stratified by *Apoe* genotype (presence of ε4 allele). n – sample number, mean ± 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

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. 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* x Hg) was observed for this domain in the fully adjusted model (Model 3) (Table 4 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* x 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* x 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).

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.

			β (CI 95%), p-value, dependant variable ***model R^2, model p-value***				
Model	**Subjects**	**n**	**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* x 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* x 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* x 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.

			β (CI 95%), p-value ***model R^2, model p-value***				
Model	**Subjects**	**n**	**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* x 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* x 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* x 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.

Regression models additionally adjusted for Se and Pb levels revealed a marginally significant positive influence of serum Se levels on 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 influence was insignificant in ε4 carriers (cord blood model β=15.0, p=0.316; hair model β=11.3, p=0.416). A negative influence of cord blood Pb levels was observed for the motor domain 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 effect was insignificant in all subjects (cord blood model β=-2.17, p=0.111; hair model β=-1.54, p=0.141). The negative effect of cord blood Pb levels was observed for the fine motor scale in all subjects (cord blood model β =-0.64, p=0.020; hair model β =-0.65, p=0.019), in ε2 and ε3 carriers (cord blood model β=-0.82, p=0.008; hair model β=-0.82, p=0.008), but not in ε4 carriers (cord blood model β=0.73, p=0.294; hair model β=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.

Mercury exposure

Cord blood total Hg levels observed in the present study were 0.16 to 9.95 ng/g (GM 1.58 ng/g) in the Slovenian and 0.79 to 32.3 ng/g (GM 3.40 ng/g) in the Croatian population, while in maternal hair the total Hg levels were 24 to 2075 ng/g (GM 273 ng/g) in Slovenian and 19 to 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 (± 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).

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 sub-clinical 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 also with higher Hg exposure levels 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).

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, 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 4Table 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. (*submitted*).

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 only 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).

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.

Limitations and strengths of the study

The main limitation of the present study was the limited sample size, with relatively low number of ε4 and ε2 carriers compared to the wildtype (ε3/ε3). Furthermore, the number of children with elevated Hg levels, that is above 5.8 ng/g, was relatively low: 11 among ε4 carriers and37 among ε4 non-carriers. Two different birth cohorts, one from Slovenia and one from Croatia, were merged for the purpose of this study, but both followed the same study design and implementation providing comparable datasets with same variables. 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 models introduced additional disadvantage into our study. For example, data on duration of breastfeeding was lacking for a significant number of the mothers and inclusion of this confounder into our models yielded insignificant and illogical correlations and was therefore omitted from the regression analysis.

Although Hg exposure in the present study was characterized mainly by fish consumption, there is a certain part of Hg exposure arising from the amalgam fillings, which is expected to contribute more to Hg exposure at lower levels. Unfortunately, our database lacked information on the number of amalgam fillings for most of the Croatian women, therefore this information 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 we could be able to quantify the 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 indeed 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) were in good correlation with cord blood levels ($r=0.92$), which was confirmed by a good agreement between the regression models (Table 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 1strd trimester, therefore it would also be worth measuring Hg levels in hair segments corresponding to this period of vulnerability.

Adjusting for beneficial/neurotoxic chemicals in relation to neurodevelopment is of crucial importance in such studies, since adjustment for Se and Pb levels in cord blood resulted in more negative β estimates on cognitive and in less significant Hg influence on motor score (Model 3 compared to Model 2, Table 4Table 5). Determination of PUFA levels in the blood of children, representing a beneficial nutritional cofounder in assessing Hg-related effects (Nisevic et al., *submitted*), 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 also 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 are planned for the follow-up.

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Declaration of interest

The authors report no declaration of interest.

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