Prenatal exposure to low-level methylmercury alters the child's fine motor skills at the age of 18 months

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ABSTRACT

Objectives: To compare motor, cognitive and language characteristics in children aged 18 months who were prenatally exposed to low-level methyl-mercury (MeHg), and to analyze the eventual differences in these characteristics in relation to cord blood THg concentration.

Patients and methods: The total number of 205 child-mother pairs was included in the study, and total cord blood mercury was measured in 198 of them. Out of the 198 already measured samples, 47 of them have also been tested for metyl-mercury in cord blood. Data regarding the 47 samples of MeHg levels has been used for calculating the correlation between cord blood THg and cord blood MeHg. MeHg and THg showed a significant correlation ($r=0.95$, $p<0.05$). One month after the delivery, mothers were asked to complete the questionnaire regarding socioeconomic factors, breastfeeding of their infants, and dietary habits during pregnancy. Neurodevelopmental assessment of motor, cognitive and language skills were conducted on 168 children using The Bayley Scales of Infant and Toddler Development, Third Edition (BSID-III). Regarding the cord blood THg concentration, 135 children were divided in 4 quartile groups. Their neurodevelopmental characteristics have been compared.

Results: The cord blood THg concentration median and inter-quartile range was 2.98 ng/g (1.41-5.61 ng/g). There was a negative correlation between cord blood THg concentration and fine motor skills (rho= -0.22**,** p=0.01). It is evident that children grouped in 2nd ,3rd and 4th quartile had statistically significant lower fine motor skills assessment related to those grouped in 1st quartile (2nd quartile - 1.24, p=0.03; 3rd quartile - 1.28, p=0.03; 4th quartile - 1.45, p=0.01). The differences in fine motor skills assessments between children in 2nd and 3rd and 3rd and 4th quartile were not statistically significant.

Conclusion: Intrauterine exposure to low-level THg (MeHg) is associated with alterations in fine motor skills at the age of 18 months.

Key words: cord blood, methyl-mercury, motor skills, neurodevelopment, prenatal exposure.

The work described in this manuscript has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

The research protocol has been reviewed and approved by the two ethics committee; Ethics Committee of the University of Rijeka and Ethics Committee of the University Hospital Centre Rijeka, Rijeka, Croatia.

INTRODUCTION

Neurotoxic effects of mercury (Hg) on human central nervous system (CNS) are well known (Myers et al. 2000a). Human fetuses are particularly affected when exposed to a high dosage (Harada 1995). Even though severe Hg poisoning is extremely rare, chronic low-level exposure (< 100 ppm in maternal hair) mainly by fish and sea food consumption is very common (Karagas et al. 2012). Thus, methyl-mercury (MeHg), neurotoxic form of Hg, which is produced from inorganic Hg by biomethylation, accumulates in fish. Despite numerous findings regarding neurodevelopmental effects of chronic low and moderate prenatal MeHg exposure were still inconsistent (Karagas et al. 2012; Bose-O'Reilly et al. 2010). Most cited are studies from Seychelles Island and Faroe Islands with completely opposite results regarding the effect of prenatal Hg exposure on child neurodevelopment (Grandjean et al. 1997; Myers et al. 2003b). While Seychelles study found no negative association, Faroe study found negative association between Hg and child neurodevelopment. Schoeman et al. proposed several reasons in their review which explain why different results might be obtained despite overall similar Hg exposures (Schoeman et al. 2009). The most comprehensible causes include differences in study populations, confounders, outcome measures, Hg concentrations, biological samples and eventually other possible contaminants in fish.

Therefore, a prospective study to investigate the effects of prenatal low level Hg exposure from maternal consumption of fish and seafood on child neurodevelopment was conducted (http://phime.oikon.hr). Here we present the results regarding the impact of the intrauterine exposure to mercury on the development of children at the age of 18 months from Croatian cohort.

STUDY POPULATION AND METHODES

Two-hundred-five (205) pregnant women and their children (evaluated in the newborn's age and at the age of 18 months) were included in the study. The pregnant women were permanent residents of the study area for at least 2 years- the coastal city of Rijeka, Croatia and its county (Primorsko-goranska). The basic characteristics of study sample were shown on table 1. The detailed description of the study protocol, inclusion and exclusion criteria have been described and published elsewhere (Valent et al. 2013a). The research protocol was approved by the Ethics Committee of the University of Rijeka and by the Ethics Committee of the University Clinical Centre Rijeka, Croatia

Cord blood total Hg (THg) concentration has been measured in 198 of examinees, and MeHg measurement on 47 examinees out of this same group (47 subjects have been tested for both THg and MeHg levels). These data was used to calculate the correlation between cord blood THg and MeHg in our study group.

THg determination: THg in blood was determined by thermal combustion at 650 °C, amalgamation and atomic absorption spectrometry using a Direct Mercury Analyzer (Milestone, USA). The procedure has been described in detail elsewhere (EPA Method 7473, 1998). About 0.200 g of blood was weighed in a sample boat. The reference material (RM) Seronorm Trace Elements in Whole Blood L-1 (LOT No: MR4206) was used to check the accuracy of the results for THg in blood and the value found (2.2 ± 0.18 ng/ml) was in good agreement with the reference value (2.2 ± 0.2 ng/ml). The limit of detection (LOD) of the method calculated as three times the standard deviation of the blanks was 0.02 ng/g blood, while the limit of quantification (LOQ) calculated as ten times the standard deviation of the blanks was 0.07 ng/g blood. The estimated uncertainty for THg in blood samples at levels higher or equal to 1 ng/g was 7 % (k=2).

MeHg determination: About 200 mg of blood sample was weighed directly in a 30 ml screw capped Teflon vial in which acid leaching using mixture of 5% H_2SO_4, (p.a.), 18% KBr (p.a.) and 1 M solutions of $CuSO_4$ (p.a.) performed. MeHgBr was extracted into CH_2Cl_2 and back extracted into aqueous phase. MeHg was then ethylated, and purged as methyl-ethyl-mercury (MeHgEt) onto a Tenax trap followed by thermal desorbed at 180°C. MeHgEt was then separated on an isothermal GC column. Hg species were converted to Hg(0) by pyrolysis at 600°C and measured by a cold vapour atomic fluorescence detector (CV AFS). The procedure has been described in detail elsewhere (Horvat et al. 1993; Liang et al. 1994).

The accuracy of the results for MeHg in mother's blood or cord blood and MeHg was checked by analysing RM lyophilised whole human blood PT-WB1 obtained from a non-exposed population which was used a quality control material. MeHg in PT-WB1 was determined by the laboratories participating in the PHIME interlaboratory comparison. The determined value (6.2±0.3 ng/g) was in good agreement with the assigned value (6.3±0.5 ng/g). The estimated uncertainty of MeHg values in blood samples was 12% (k=2). The LOD of the method for MeHg determination in blood calculated on the basis of three times the standard deviation of the blanks was 0.02 ng/g blood, while the LOQ calculated as ten times the standard deviation of the blanks was 0.07 ng/g blood.

After the delivery, mothers were asked to complete the questionnaire regarding socioeconomic factors, breastfeeding of their infants, dietary habits during pregnancy including detailed food frequency assessment. The assessment was based on their consumption of 138 food items adapted from a validated food frequency questionnaire which includes over 22 fish species commonly fished or marketed in the study area.

Neurodevelopmental assessment of motor, cognitive and language skills was conducted on 168 children using the Bayley Scales of Infant and Toddler Development, Third Edition (BSID-III) at their age of 18 months (± 2 months) (Bayley 2006). There were 37 children in which BSID-III was not performed because they did not respond to the invitation for psychological testing at 18 months. The BSID-III test was performed by two trained child psychologists. Table 2 shows results of BSID-III in study subjects – children at the age of 18 months shown as arithmetic mean with standard deviation, median and minimum and maximum value. Out of all the children with performed BSID-III, the complete data including all questionnaires regarding socioeconomic factors, breastfeeding habits, and dietary habits during pregnancy was available in 135 child-mother pairs, which were used for performing multivariate and other statistical analyses.

The normality of distribution was assessed by Kolmogorov-Smirnov test, so mainly non-parametric tests were used. Distributions of BSID-III scores were calculated by arithmetic mean and standard deviation, median, quartiles and minimum-maximum range. The associations of neurodevelopment with concentration of cord blood THg were investigated by means of Spearman correlations. Multivariate linear regression was used to assess the association between THg and neurodevelopment, after adjustment for potential confounding factors such as: maternal intake of fresh, frozen and canned fish during pregnancy, maternal age, breastfeeding, alcohol intake and cigarette smoking during pregnancy, number of siblings, parental educational level and parental working status.

Regarding the cord blood THg concentration and BSID-III assessment, children were divided in 4 quartile groups: 1^{st} quartile ($<25^{th}$ centile), N=29: THg values: ≤ 41 ng/g; 2^{nd} quartile (26^{th} -50^{th} centile), N=36: THg values: > 1,41 ng/g ≤ 2,97 ng/g; 3^{rd} quartile (51^{st}-75^{th} centile), N=31: THg values, >2,97 ng/g ≤ 5,61 ng/g: 4^{th} quartile (> 75^{th} centile), N=39: THg values > 5,61 ng/g. Differences between groups were assessed by the Kruskal-Wallis test. Post-hoc analyzes and linear regression were used to assess differences within 4 quartile groups. To assess the post-hoc significance of differences between pairs of groups we have used linear regression, each quartile above the lowest has an average decrease of more than one point (1.2-1.4), significant, compared to the lowest THg quartile.

Statistical significance was consider if $p<0.05$.

RESULTS AND DISCUSSION

The median value of cord blood THg (N=198) in our cohort was 2.98ng/g, with interquartile range (25^{th}-75^{th} centile) from 1.41ng/g to 5.61ng/g. Even these measurements do not reflect the exact level of Hg in target organ, such as the fetal brain, but they represent the only possible method at the moment to analyze the effect of Hg on human health. Furthermore, cord blood Hg level is considered the best biomarker of prenatal MeHg exposure, better than maternal hair or blood, and much better than dietary assessment of fish consumption during pregnancy (Murata et al. 2007).

Most of the published studies report measures of THg rather than MeHg. Our study was based on THg in 198 subject and MeHg measurement in 47 subjects within 198 of them. However, linear regression equation showed excellent correlation between cord blood THg and MeHg levels, Figure 1. Linear regression line clearly showed that measured concentrations of MeHg are in absolute correlation with measured concentrations of THg. Since the 95% confidence interval for intercept include value zero, and 95% confidence interval for slope includes value one it may be considered that y=x, i.e indicating excellent correlation (Bilic-Zulle, 2011). THg and MeHg concentrations have 0.95 ($p<0.05$) correlation.

Therefore our results may be considered and commented as the human epidemiological study that used MeHg as a biomarker.

Figure 1. Correlation between MeHg cord blood and THg cord blood concentration

The question which is still open is the exact definition of “low level exposure” since it has not been defined as an absolute value (Schoeman et al. 2009; US Environmental Protection Agency 2004; Goldman and Shannon 2001). Our results of MeHg levels would classify Adriatic coast of Croatia area with low level of Hg exposure. The maternal fish consumption during pregnancy in our cohort was moderate, on average 1.5 servings/week, which is comparable with Italian cohort and US cohort of pregnant women (Valent et al. 2013; Oken et al. 2008). The overall consumption is very low and there is no danger of toxic or over limited Hg exposure (Bošnir et al 1999). Moreover, the research has shown that in the population of Croatia, the daily intake of THg and MeHg throughout seafood is lower than the Provisional Tolerable Weekly Intake (Blanuša and Jureša 2001). We based our definition of low level exposure on an assessment of the literature and major studies with moderate Hg exposure and fish consumption. There are several investigations which clearly showed that sediments, soils, and fish from Croatia are generally not contaminated by Hg (Mikac et al 2006; Vedrina-Dragojević 2004.).

The main interest of our study was neurodevelopmental outcome of children prenatally exposed to low level of THg. So, BSID-III composite cognitive score, composite and sub-scale of language and motor scores were analyzed regrading THg level. The median BSID-III score for composite cognitive development was 105, for composite language development 109 and for composite motor development 107, table 2.

The correlation between concentrations of cord blood THg and BSID-III scores is shown in table 3. The only statistically significant correlation was negative correlation between BSDI-III sub-scale of fine motor skill and level of cord blood THg. In fact, regardless of relatively low concentrations that we measured there was an evidence that prenatal THg exposure had an adverse effect on child neurodevelopment in the area of fine motor skills. The increase of cord blood THg level significantly decreases fine motor skills score in children at the age of 18 months prenatally exposed to low-levels of THg.

Table 3. Correlation between cord blood THg and scores of BSID-III

We have gotten the same results when we compared 4 groups of children divided by quartile cord blood THg concentration and BSID-III scores. The sole statistically significant difference was found in BSID-III sub-scale of fine motor skills (p=0.04), table 4.

Table 4. Comparison of cord blood THg level by inter-quartile range and fine motor skills BSID-III sub scale

To detect whether some differences exist within the inter-quartile ranges of THg concentrations and fine motor skills BSID-III sub scale, post-hoc analyses was performed. The results are shown in table 5 and table 6.

Table 5. Post-hoc analyses of cord blood THg level of 1st quartile versus 2nd, 3rd, and 4th quartile regarding fine motor skills BSID-III sub scale

It was evident that children within the 2nd, 3rd, and 4th quartile had statistically significant lower scores on the fine motor scale measured by BSID-III than those within the 1st quartile. In relation to the 1st quartile, every following quartile showed median score decrease by more than one point, - 1.24, 1.28, 1.45, respectively. That leads us to the conclusion that the higher the THg concentration, the lower the median fine motor skills score.

Even so, additional post-hoc analyze of cord blood THg level of 1st quartile versus 2nd, 2nd versus 3rd, and 3rd versus 4th quartile regarding fine motor skills BSID-III sub scale showed statistically significant differences between the children within the 1st and 2nd

quartile but not between children within the 2nd and 3rd and 3rd and 4th quartile, table 6.

Table 6. Post-hoc analyses of cord blood THg level of 1st quartile versus 2nd, 2nd versus 3rd, and 3rd versus 4th quartile regarding fine motor skills BSID-III sub scale

The obtained results could be explained by some possible "plateau" effects of THg concentration at the marginal level between 1st and 2nd quartile. Regarding our results, even prenatal exposure to low level of Hg cause changes in fine motor skills. An open question remains; what would be the exact "low-level" which causes changes. We speculated that the Hg level could be between 1st and 2nd quartile which can, at some point of prenatal brain development, cause damage to the brain tissue. The more negative effects should be expected parallel with the higher Hg levels, but only after exposure of Hg toxic levels. Therefore we speculate that there are "plateau" effects between "low-level" and "toxic level" of Hg. Levels of Hg in between those margins would actually represent prenatal negative effects of "low-level Hg exposure" on brain tissue. We could not have tested this due to small sample numbers. On the other hand, in the 1st quartile there were 9 children with THg concentration less or equal 0.71ng/g, level which is certainly proven to have no negative effect on child's neurodevelopment. One can assume that solely these children had high scores on BSID-III fine-motor scale and contributed to statistical significance which is not found comparing children within the 2nd vs. 3rd and 3rd vs. 4th quartile.

When performing any exposure human epidemiological study with the effect on health, confounding factors are generally numerous. In our study, confounding factors included maternal dietary habits during pregnancy and socio-economic factors.

Multivariate linear regression analyses adjusted for selected potentially confounding factors showed statistically significant negative effect of maternal employment (**β= -3.04, p=0.02)** and consummation canned fish - tuna, mackerel, sardine- during pregnancy (**β= -0.71, p=0.05)** on fine motor skills BSID-III sub scale in children prenatally exposed to low-level of THg, table 7. In this model there were no statistically significant correlation between cord blood THg concentrations as a continuous variable effect and fine motor skills in children (**β= -0.06, p=0.28)**.

Table 7. Multivariate linear regression analyses adjusted for selected potentially confounding factors.

The logical explanation is that mothers who are unemployed spent more time with their child resulting in their better motor skills. Dietary habits, especially fish consumption during pregnancy, is a well-known factor which influences THg level concentration in mother's blood as well as cord blood and consequently child's neurodevelopmental outcome (Koren and Bend 2010; Miklavčić et al. 2011; Jedrychowski et al. 2007). Our previous research revealed that the mean THg value in fish from that particularly part of Croatia was 143ng/g (min 8ng/g, max 586ng/g) (Miklavčić et al 2013). Anyhow, we did not include that data in our study. Correlation of THg or MeHg level and fish consumption does not depend only on servings of fish by week but on the type of the fish, the concentrations of THg/MeHg in the fish, possible other toxic elements in the fish or even some protective ingredients like polyunsaturated fatty acids as well. That could be potential the reason why in this model we did not have significance regarding THg concentration in cord blood and fine motor skills assessed by BSID-III. In the similar model from the cohort originated from Mediterranean coastal area of northern Italy results revealed that child neurodevelopment was associated with maternal intelligence quotient (IQ) and child intake of fresh fish rather than with Hg exposure (Valent et al. 2013b). At this point we did not test results obtained by the supplementary questionnaire which was administrated to assess changes in residence, maternal marital and occupational status, child intake of the fish, diseases and daycare attendance at the time when BSID-III was performed.

There are a few possible limitations of the study. First of all, the number of participants could be considered rather low. Anyhow, 205 pairs of mother child represent 7.3% to 8.2% of new-borns born yearly at that particular part of Mediterranean coastal region of Croatia. The study sample is very homogenous, prospectively followed and the inclusion/exclusion criteria were strictly followed. We consider that the sample of 8% of homogeneous participants have sufficient statistical power for presenting the relation with the whole population. Thereafter, one might argue that the exposure to polychlorinated biphenyl (PCB) through fish intake might affect the BSID-III score and that PCB is a significant confounding factor. Anyhow, several previous prospective studies have clearly shown that in that exact part of The Adriatic Sea PCB levels never exceeded the maximal allowed quantity of this substance in fish (Calić et al. 2007). Therefore, the exclusion of PCB in multivariate analyse might only present a minor limitation of the study. The fact that two experienced and trained child psychologists performed BSID-III on all children can be perceived as a form of limitation but as well as a strength of the study. Both of them work together, have great experience in working with children, and all tests were performed in close communication between the psychologists. Ultimately, we believe there is very little chance of intervariability regarding the tests due to different psychologists performing it. In spite of these, the importance of the limitation factors is minored by the bare idea of only presenting new interesting revelations regarding the exposure to mercury and its prenatal effects.

In conclusion, our result revealed that prenatal exposure to low-level of THg has a negative effect on fine motor skills in children at 18 months. Moreover, in our previous study with the same cohort but different Hg biomarker we confirmed that the size of the cerebellum is significantly smaller in those with higher Hg concentration (Cace et al. 2011). That is concordant with the experimental work which proved that continuous exposure to low concentration of Hg impairs cerebellar granule cell migration (Mancini et al 2009). Since the cerebellum is directly responsible for fine motor skills, we can conclude that these results confirm our previous findings, even though different methodology was used to a certain extent. Thus, morphological changes in cerebellum caused by higher concentrations of Hg correspond with functional changes in cerebellum reflected as impaired fine motor skills. These results need to be confirmed in a large number of children but it is more important to find whether these results have clinical significance. Nevertheless, we showed that the increase of THg or MeHg level in cord blood, to a certain level, was in direct correlation with the decrease of fine motor skills score.

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