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Pre and postnatal exposure to mercury and respiratory health in preschool children from the Spanish INMA Birth Cohort Study

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Abstract

Effects of mercury on maturing immune system have been reported, however the association with respiratory and allergy problems during infancy remains unclear. The aim of this study is to evaluate the association between pre and postnatal mercury exposure and respiratory and allergy problems among preschool children and to examine the role of potential modifying factors. Study subjects were children participant in Spanish Childhood and Environment Project (INMA, 2003-2008). We measured total mercury levels in cord blood (n=1868) and hair at 4 years of age (n=1347). Respiratory outcomes (wheezing, severe wheezing, chestiness, persistent cough, eczema and otitis) were obtained by questionnaires administered to parents. Associations were investigated by logistic regression adjusted for socio-demographic and lifestyle-related variables in each cohort and subsequent meta-analysis. We tested effect modification by factors related to individual susceptibility, diet and co-exposure with other pollutants. The geometric mean of cord blood and hair total mercury was 8.20 µg/L and 0.97 µg/g, respectively. No statistically significant association between pre or postnatal mercury exposure and respiratory and allergy outcomes was found. Notwithstanding, lower maternal intake of fruits and vegetables increased the risk of some respiratory outcomes due to the prenatal exposure to mercury (p_{int} <0.05). Moreover, an inverse association between prenatal mercury exposure and some respiratory outcomes was observed among children with higher maternal exposure to organocholorine compounds or smoking (p_{int} <0.05). Also, sex and postnatal smoking exposure modulated mercury postnatal effects on persistent cough (p_{int} <0.05). In conclusion, no association between pre and postnatal mercury exposure and respiratory and allergy problems among the whole population at study was found. However, diet and other toxicants could modulate this relation, especially during prenatal period. More research on this topic is warranted due to the limited evidence.

Keywords: mercury, respiratory, eczema, prenatal, children

1. Introduction

Respiratory and allergy problems represent a substantial burden of illness in children (GBD 2015 Child Mortality Collaborators, 2016). Asthma and allergy phenotypes are among the most frequent chronic disease in children and their prevalence and severity are increasing worldwide (Anto et al., 2017; Pearce et al., 2007). Likewise, ear infections are one of the major reasons for physicians consultations and taking antibiotics by children (Monasta et al., 2012).

Unique maturational events of respiratory and immune systems occurred during intrauterine and the early postnatal periods (Dietert, 2008; Kajekar, 2007). Exposure to environmental toxicants during these periods could lead to alterations in lung function (Korten et al., 2017) and persistent changes in immune function (Dietert, 2009). These effects could trigger an increased risk of asthma or allergic responses and an impaired capacity to fight to infections later in life (Dietert, 2009; Winans et al., 2011). Thus, identifying developmental toxicants that may predispose children to these common diseases is a priority from a public health perspective (Dietert, 2008; Prüss-Ustün et al., 2016).

There is a great deal of concern about effects of mercury (Hg) on human health (Sharma et al., 2019). Hg is a global pollutant and bioaccumulative, across the aquatic food chain, in its elemental and organic forms (i.e. methylmercury [MeHg]) (National Research Council, 2000). The contaminant can be transferred from the mother to the foetuses via placenta (Dietert, 2008; Grandjean et al., 2010) and the most common source of exposure *in utero* and after birth is maternal and children's fish consumption, respectively, being large oily fish the main contributor (Castaño et al., 2015; Llop et al., 2014; Ramon et al., 2011).

Major adverse effects of Hg, particularly MeHg, are neurological (Grandjean and Landrigan, 2014) but for several other health conditions the studies do not support conclusive associations and further epidemiological investigations to fully understand the health implications of Hg exposure have been recommended (Karagas et al., 2012; Sharma et al., 2019).

Extensive evidence based on experimental data supports immunotoxicity indicators of Hg exposure as inhibition of the immune response to infections, effects on immune-cell ratios, autoimmunity, immunosuppression, oxidative stress and pro-inflammatory response (Gardner et al., 2010; Silbergeld et al., 2000; Vas and Monestier, 2008). Data from epidemiological studies regarding immunotoxic effects of Hg on foetuses or infants have shown correlation between changes in blood immune cells proportion and inflammatory markers in prenatal (Belles-Isles et al., 2002; Grandjean et al., 2010; Nyland et al., 2011; Osuna et al., 2014; Oulhote et al., 2017) and postnatal exposure (Kim et al., 2015; Osuna et al., 2014). This immunomodulatory effects could be related with susceptibility and development of respiratory and allergy problems in childhood (Dietert, 2009; Winans et al., 2011) but regarding prior studies that have analysed the relationship between prenatal and/or postnatal Hg exposure and respiratory and allergic problems among children, a lack of consistency is observed (Croes et al., 2014; Emeny et al., 2019; Grandjean et al., 2010; Heinrich et al., 2017; Kim et al., 2015; Miyake et al., 2011; Razi et al., 2011; Shaheen et al., 2004).

Differences in methodology used between studies could explain heterogeneity in results. Additionally, findings concerning neurotoxicity of Hg have shown that Hg metabolism and toxicity may be modified by certain factors such as sex, diet or co-exposure to other pollutants (Castoldi et al., 2008; Chapman and Chan, 2000; Llop et al., 2013, 2012). However, information regarding the role of potential modifying factors on the association between Hg exposure and respiratory and allergy problems is still too scarce. Only Kim et al 2015 (Kim et al., 2015) explored sex differences on the association between postnatal Hg exposure and asthma in children at 11-12 years of age and, after stratifying by sex, the association remained only in boys.

In previous studies, we reported relatively elevated levels of prenatal and postnatal (at 4 years of age) Hg exposure in our cohort in comparison with other European countries and similar to other countries with high fish consumption (Llop et al., 2020, 2014; Ramon et al., 2011). Thus, 64% and 24% of newborns had cord blood total mercury (THg) concentrations exceeding the equivalent to the current United States Environmental Protection Agency (US

EPA) reference dose (6.4 µg/L of THg in whole cord blood) and to the WHO Provisional Tolerable Weekly Intake (PTWI) (1.6 µg/kg of body weight per week), respectively (Ramon et al., 2011; United Nations, 2020). We also described a decreasing temporal trend in Hg concentrations from birth to 4 years old, however, half of the children still had hair Hg concentrations above the reference dose proposed by the US EPA (i.e. 1.0 µg/g) and 11% above the equivalent for the PTWI proposed by the WHO at 4 years old (i.e. 2.5 µg/g) (Llop et al., 2020, 2014; United Nations, 2020).

Therefore, the purpose of the present study is to evaluate the association between pre and postnatal exposure to Hg with several respiratory and allergy problems (wheezing, severe wheezing, chestiness, persistent cough, eczema and otitis) in children 4 years old of the prospective INMA cohort study in Spain and to examine the role of potential modifying factors than may modulate these associations.

2. Methods

2.1 Study subjects

We included subjects from Asturias, Gipuzkoa, Sabadell and Valencia birth cohorts of the INMA (Environment and Childhood) project (http://www.proyectoinma.org). Details of this study have been fully described elsewhere (Guxens et al., 2012). Briefly, pregnant women (n=2644) were enrolled at the first trimester of pregnancy (November 2003- February 2008). The inclusion criteria were: at least 16 years of age, 10-13 weeks of gestation, singleton pregnancy, intention of undergoing follow-up and delivery in the corresponding center of reference, and no impediment for communication. Excluding the women who withdrew from the study, were lost to follow-up, and had induced or spontaneous abortions or fetal deaths, we followed up on a total sample of 2506 women until delivery. Their children were enrolled at birth and were followed up until they were 4 years of age (n=2005). The final study population was made up of participants with available information about prenatal (n=1868) and postnatal (n=1347) Hg levels and respiratory outcomes at 4 years of age. See flow chart describing the

selection process in Figure S1. Informed consent was obtained from all participants in each phase, and the study was approved by the regional ethical committees of each cohort.

2.2 Mercury exposure

Prenatal and postnatal Hg exposure were assessed from THg analysis of cord blood and hair at 4 years of age, respectively. Whole blood samples were collected from cord vessels using venipuncture before the placenta was delivered and then kept frozen at -80 ºC until analysis. Hair samples were collected from the occipital scalp when children were 4 years old, placed in a plastic bag and stored at room temperature until analysis. The analyses of THg were carried out in the Public Health Laboratory of Araba (Basque Country, Spain). Both sample types were analyzed in two different single-purpose Hg analyzers (AMA 254 Leco Corporation and DMA-80 Milestone). Blood samples were weighed in a boat and directly analyzed in the AMA 254 equipment by catalytic combustion, gold amalgamation, thermal desorption, and atomic absorption spectrometry. Hair samples were rinsed with 10 ml of Triton X-100 at 1% (PanreacR) (Oken et al., 2005) before performing the same procedure used with blood samples (in this case, AMA 254 and DMA-80 analyzers were used). In both cases, replicate analyses were performed for each sample. The limits of quantification of the method (LOQ) were 2.0 µg/L for blood samples and 0.01 µg/g for hair samples. Uncertainties (k=2) for blood samples were of 35%, 26% and 20% for 3.8, 12.0 and 37.0 µg/l concentration levels, whereas for hair samples were of 13% and 7% for 0.573 and 12.5 mg/kg concentration levels. For measurements in cord blood below the LOQ (n=86; 4,7 %) we used the LOQ/√2 approach as imputed level. No measurements of Hg in hair were below the LOQ.

Whole blood sample batches were internally controlled with Seronorm (Levels 2 and 3; SERO AS, Billingstad, Norway) accuracy-control materials (batches nº 0503109 and 0512627). Hair sample batches were controlled with IAEA-086 (batch nº 1995/617; International Atomic Energy Agency, Austria) and NCS ZC 81002b (NCS Institute, Beijing, China) reference materials. Additionally, the accuracy of the method was also externally verified by participation in different inter-laboratory exercises organized by the New York State Department of Health in

the Wadsworth Centre (Trace elements in whole blood PT program) and the Centre de toxicologie du Québec (Quebec Multielement External Quality Assessment Scheme, QMEQAS program). In all cases satisfactory results were obtained.

2.3 Respiratory outcomes

Respiratory symptoms were obtained by means of a questionnaire administered to parents at 4 years' follow-up. Outcomes studied were any episode of wheezing (defined as whistling sounds coming from the chest), severe wheezing (that causes children difficulty breathing and sleeping or interferes with their daily activities), chestiness (noises or phlegm on the chest), persistent cough (more than three consecutive weeks), eczema and otitis both defined through medical diagnostic reported by parents. All outcomes are referred to the child's last year of life period at the age of 4 years. Questions on respiratory symptoms and diagnosis were based on a structured questionnaire from the AMICS study (Polk et al., 2004; Sunyer et al., 2004).

2.4 Other variables

Information related to parental socio-demographic characteristics, maternal and child life styles and exposures was obtained by questionnaires administered to parents in different visits (during pregnancy, when children were between 11 and 21 months of age and when children were age four). Based on previous knowledge, we selected covariates regarding their potential effect on respiratory and allergy health. Variables obtained on prenatal period were: maternal education level, parental social class, maternal active and passive smoking, familiar allergic antecedents, fruit and vegetables intake, vitamin E intake, maternal level of circulating vitamin D, organocholorine compounds exposure during pregnancy.

Parental social class was defined from maternal or parental occupation during pregnancy with the highest social class, according to a widely used Spanish adaptation of the International Standard Classification of Occupations approved in 1988 (ISCO88) coding system (Class I + II: managerial workers, senior technical staff, and commercial managers; class III: skilled non-manual workers; class IV + V: manual workers) (Domingo-Salvany et al., 2000). The maternal level of circulating vitamin D was analyzed in maternal serum extracted in the

first trimester of pregnancy (Morales et al., 2012). Organocholorine compounds considered in the present study were Dichlorodiphenyldichloroethylene (DDE) and polychlorobiphenyl (PCB) congener 118, 138, 153 and 180 levels and they were analyzed in maternal blood samples taken at 12 week of gestation (Garí and Grimalt, 2010; Grimalt et al., 2010).

Variables obtained on postnatal period were: weeks of gestation, season of birth, preterm delivery (<37 weeks of gestation), small for gestational age (birth weight below the 10th percentile according to standard percentile charts for sex and gestational age in the Spanish population) (Carrascosa et al., 2004), breastfeeding weeks, attendance at nursery before 24 months. At 4 years' follow-up, child sex, child age, child length and BMI (standard scores by WHO (World Health Organization, 2006), paternal working situation, type of residence zone (no rural, rural), season of visit, people living at home, postnatal tobacco exposure, type of dwelling (house, apartment), redecoration or painting activities at home, exposure to gas cooking, presence of pets, dampness, mould stains at home, fruit and vegetables, vitamin E and vitamin D intakes.

We also selected potential confounders from those variables related with Hg exposure based on previous analyses (Llop et al., 2014; Ramon et al., 2011): maternal age at conception, maternal pre-pregnancy body mass index (BMI [Kg/m2]), maternal country of birth (Spain, other), maternal working situation, parity, and maternal and child fish intakes.

Dietary information was collected by using a semi-quantitative food frequency questionnaire (FFQ) of 110 food items among mothers (mean between first and third trimester of pregnancy) and a FFQ of 105 food items among children (available upon request at: http://epinut.edu.umh.es/bibliodieta). The FFQ were a modified versions from a previous FFQ based on the Harvard questionnaire (Willett et al., 1985) which was developed and validated among pregnant women (Vioque et al., 2013) and children of the INMA-Valencia cohort (Vioque et al., 2016). To adapt it for children, additional foods items and suitable portion sizes for children 4 years old were used in the questionnaire. In the present study, we calculated the weekly intake of fish (grams and servings per week) and n-3 polyunsaturated fatty acids (n-3 PUFA) (grams per week) and daily intake of total fruits and vegetables (grams per day) and

vitamin E (grams per day) in mothers during pregnancy and in children at 4 years old. We also assessed vitamin D intake (grams per day) among children at 4 years by means FFQ information. All dietary variables were adjusted for total energy intake using the residual method (Willett et al., 1985).

2.5 Statistical analysis

First, a descriptive study was conducted for maternal and child's characteristics according to each cohort. We also described percentages of outcomes and prenatal and postnatal Hg levels by means of geometric means and interquartile range. For further analysis, the variable THg was log-transformed scale (base 2) due to its skewed distribution.

The association between respiratory outcomes and prenatal and postnatal Hg concentration was assessed through cohort-specific multivariable logistic regression models combined using meta-analysis techniques. First, a core model was built for each outcome including those possible predictor covariates at the p value < 0.2 level in unadjusted analyses. Covariates remained in the model if related with the outcome at a level of p value <0.1 on the likelihood ratio test. Child's age, sex and type of residence zone were included in all models despite their statistical significance. Cord blood or hair THg were then incorporated and potential confounders were subsequently included if they confounded the associations of interest, i.e., they were related to Hg and to the outcomes at p value < 0.20, and changed the magnitude of the main effect by more than 10%.

To obtain the estimates of the association models were applied separately to each cohort and the resulting estimates were combined by means of meta-analysis. Heterogeneity among cohort estimates was quantified by means of the I-squared statistic (I^2) and, if detected (I^2 > 50%), the random-effect model was used. Estimates were expressed as odds ratio (OR) with corresponding 95% confidence intervals (95% CI). Moreover, generalized additive models (GAM) were fitted to assess the shape of the relations by means of natural cubic splines with one or two internal knots and comparing linear and nonlinear models using graphical examination and Akaike's Information Criterion (AIC).

We also performed a sensitivity analysis to evaluate the robustness of the meta-analysis multivariable models by excluding the participants being small in weight for gestational age (n=241), the preterm births (n=114) and not adjusting by total fish intake variables (during pregnancy and child 4-year intake). We also included n-3 PUFA consumption (during pregnancy and child 4-year intake) in the adjusted models as sensitivity analysis. Moreover, we tested effect modification by some individual factors: daily maternal and child fruit and vegetable consumption (≤ vs > median=525.6 g and ≤ vs > median=194.4 g respectively), daily intake of vitamin E during pregnancy and childhood (≤ vs > median=10.6 mg and ≤ vs > median=6.6 mg respectively), maternal level of circulating vitamin D (≤ vs > median=29.4 ng/ml) and child daily intake of vitamin D (≤ vs > median=2.7 mg), sex, prenatal and postnatal tobacco smoke (yes/no), weekly maternal and child fish intake (≤ vs > median 6.2 and 3.9 servings;≤ vs > median 78.6 g and 33.4 g, respectively), prenatal exposure to PCB congener 153 (≤ vs > median 0.28 ng/ml) and DDE (≤ vs > median 0.83 ng/ml), breastfeeding (< 6 months vs ≥ 6 months) and familiar allergic antecedents (yes/no). The median displayed was the global median for all cohorts. Effect modification was evaluated by including an interaction term in all the adjusted models. Statistically significant interaction terms were considered at $p<0.05$.

Analyses were carried out with Stata statistical package version 14 (StataCorp LP, College Station, Texas, USA) and R, version 3.4.3.

3. Results

Characteristics of the pregnant women and their children by geographic areas are presented in Table S1. Maternal mean ± SD age was 30.7 ± 4.3 years old, and the mean children's age at the time of the evaluation of respiratory problems was 52.5 ± 2.3 months. Most of the individual and family characteristics in pregnancy and postnatal period differed among cohorts. We found marked differences in educational level and social class among cohorts, especially with respect to Gipuzkoa where roughly half of the mothers have university studies and were classified in I+II social class. Maternal fish intake was higher in Asturias and Gipuzkoa and child fish intake in Sabadell. About 21.5% of mothers smoked during pregnancy

and the 60.1% were exposed to passive smoking. Over 67.1% of children were exposed to tobacco during postnatal period.

Of the total of children included in this study a 22.6% experienced wheezing, 10.1% severe wheezing, 38.7% chestiness, 15.9% persistent cough, 16.6% eczema and 27.8% otitis during the previous 12 months (Table 1). Statistically significant differences were found between percentages of respiratory outcomes in the study areas except for persistent cough. Higher percentages of children with eczema were found in Sabadell and wheezing, wheezing severity and chestiness in Asturias. Lower percentage of otitis was found in Gipuzkoa. The overall geometric mean of cord blood THg was 8.2 µg/L and varied from 6.3 µg/L in Sabadell to 10.8 µg/L in Asturias (Table 1). The overall geometric mean of THg in the 4-year old children's hair was 0.97 µg/g and varied from 0.83 µg/g in Sabadell to 1.10 µg/g in Valencia. The Pearson correlation coefficient between cord blood Hg and 4-year-olds' hair Hg concentrations, both log2 transformed, was 0.304 ($p < 0.001$).

The relation of respiratory and allergy outcomes with individual and family characteristics in pregnancy and postnatal period is shown in Table S2. Low parental social class was associated with a higher risk of wheezing, severe wheezing and chestiness in children. Smoking in pregnancy was a risk factor for wheezing (close to statistical significance), passive smoking exposure during pregnancy for sever wheezing and postnatal tobacco exposure for wheezing and chestiness. Children who were breastfed for a prolonged period showed a lower risk of wheezing. Male infants showed a higher risk of wheezing than female infants. Dietary variables showed no association with respiratory and allergy outcomes. Maternal DDE concentration in pregnancy was associated with increased risk of wheezing and chestiness, however no association was found with PCB 153.

In Figure 1 we showed the results of the relation between prenatal and postnatal Hg exposure and respiratory outcomes through combined cohort-specific logistic regression models using meta-analysis (see data in Table S3). All models were adjusted by child's age, sex and residence zone (residence zone was not included for Sabadell cohort). See variables included in cohort-specific models in Table S4. We found no statistically significant association between

prenatal and postnatal exposure to Hg and respiratory symptoms (wheezing, chestiness, persistent cough and severe wheeze), eczema and otitis at fourth year of life. All results by cohort showed no statistically significant heterogeneity ($I^2 < 50\%$) except when we evaluated the association between blood cord Hg and persistent cough. Based on the AIC, nonlinear models generally did not enhance the linear fit, except for relation between cord blood Hg and chestiness in Sabadell. Therefore, the relationship between prenatal and postnatal Hg levels and respiratory outcomes was assessed linearly (Figure S2 and Figure S3, respectively). After performing sensitivity analyses by eliminating children being small in weight for gestational age, preterm birth and by excluding the variable fish and by including n-3 PUFA consumption in the models, the results remained stable (Table S5).

Effect modification analysis for prenatal and postnatal exposure are presented in Figure 2 and 3 respectively (see data in Table S6 and Table S7). Among the potential effect modifiers evaluated, a statistically significant inverse association between postnatal Hg levels and persistent cough was found among girls (OR= 0.79, 95% CI: 0.65, 0.97), but this was not the case for boys that showed a higher risk but statistical significance was not reached (OR= 1.14, 95% CI: 0.95, 1.37) (p_{int} =0.010). A borderline interaction (p_{int} =0.097) was found between familiar allergy antecedents and postnatal Hg levels on otitis although associations within strata were not statistically significant. No statistical significant interactions between sex or familiar allergy antecedents and prenatal Hg levels were found for any outcome.

Regarding nutritional factors, we found a statistically significant interaction between maternal fruit and vegetable intake during pregnancy and prenatal exposure to Hg on chestiness (p_{int} =0.048) and otitis (p_{int} =0.015). When maternal intake was low, cord blood Hg levels were associated with an increased risk of otitis (OR= 1.23, 95% CI: 1.02, 1.48). A statistically significant inverse association between cord blood Hg levels and chestiness was found in the high intake strata (OR= 0.82, 95% CI: 0.69, 0.97). For postnatal analysis, no interactions with maternal and child fruit and vegetable intake were found.

On the other hand, no statistically significant interactions of prenatal and postnatal Hg exposure with maternal and child vitamins intake, maternal and child fish intake and breastfeeding were found for any outcome.

We further examined if associations between Hg levels and respiratory symptoms were modulated by other toxicants. We found statistically significant interactions between cord blood DDE and prenatal Hg levels (p_{int} =0.019) on persistent cough. In the high DDE exposure strata, cord Hg levels were borderline associated with lower risk of persistent cough (OR= 0.74, 95% CI: 0.54, 1.00). However, cord Hg levels did not have any effect on persistent cough when DDE exposure was low. We also detected an inverse association between cord Hg levels and eczema among infants with high maternal exposure to PCB 153 during pregnancy (OR = 0.76, 95% CI: 0.60, 0.92) (p_{int} =0.039).

Finally, we observed a statistically significant interaction between prenatal smoking and cord Hg levels in determining wheezing (p_{int} =0.035). Among those children whose mothers smoked during pregnancy, an inverse association between cord Hg levels and wheeze was found (OR = 0.61, 95% CI: 0.42, 0.87). On the other hand, we also detected a statistically significant interaction between postnatal smoking exposure and hair Hg levels on persistent cough (p_{int} =0.005). Hair Hg levels was associated with high persistent cough risk among children not exposed tobacco smoke (OR= 1.34, 95% CI: 1.02, 1.76). However, hair Hg level was borderline associated with lower persistent cough risk among children exposed tobacco smoke (OR= 0.87, 95% CI: 0.75, 1.02).

4. Discussion

In this longitudinal study, undertaken on preschool children from four geographic areas of Spain participants in the INMA birth cohort, with relatively high levels of prenatal and postnatal Hg exposure in comparison with other European countries (Llop et al., 2020, 2014; Ramon et al., 2011), no noteworthy association between cord blood and hair THg levels and respiratory and allergy symptoms at 4 years of age was found.

In agreement with our findings, in a birth cohort study conducted in Faroe Islands, cord blood MeHg concentrations were not related to asthma, allergy, atopic dermatitis and total IgE at 5 and 7 years of age, even after the adjustment for maternal fish consumption (Grandjean et al., 2010). However, they found an inverse association between cord blood MeHg and serum grass-specific IgE concentration at 7 years of age (Grandjean et al., 2010). In another study conducted on a Japanese population, Miyake et al. (2011) (Miyake et al., 2011) also showed no association between child hair total Hg concentration and risk of wheezing and eczema at 29-39 months of age, after adjustment for multiple variables. In the Avon Longitudinal Study of Parents and Children (ALSPAC) from UK no relevant associations between cord Hg and wheezing at 30-42 months and eczema at 18-30 months of age were found in 2044 children (Shaheen et al., 2004). But, although a large number of potential confounders were used in this study, nutritional variables were not included. In a case-control study conducted in Chinese children aged 9-12 years, blood THg concentration was not associated with eczema (Hon et al., 2012). Also, the results from a cross-sectional study conducted on German children aged 5-14 years did not support either an association between postnatal blood and urinary Hg concentration with asthma and wheezing (Heinrich et al., 2017).

By contrast, associations between early Hg exposure and respiratory or allergic outcomes have been reported in some other studies. In a case-control study with school beginners from Germany, urinary THg concentrations were associated with higher risk for acute eczematous lesions but not with a history of atopic eczema (Weidinger et al., 2004). Also, Razi et al, 2011 (Razi et al., 2011) observed higher serum Hg levels in the wheezy group than those acquired from the control group in a case-control study conducted in children aged 1-6 years from Turkey. An ecologic study found a negative association between hair Hg and asthma prevalence in adolescents from Belgium regions (Croes et al., 2014). Additionally, in a large prospective study conducted in 4,350 Korean children with no previous asthma diagnosis (Kim et al., 2015), blood THg concentrations at 7–8 years of age were associated with an increased risk of asthma and wheezing at ages up to 11–12 years, even though the analysis was controlled by children's fish consumption. Hg levels were also associated with immune cells percentages

and inflammatory markers. Finally, a recent study has shown among women who consumed fish, higher maternal toenail Hg concentrations were associated with an increased risk of lower respiratory infections and respiratory symptoms in infants at first year of life but no associations were found with upper respiratory infections, allergy or eczema (Emeny et al., 2019).

Among confounders, fish consumption plays an important role and most of the reviewed studies have shown incomplete adjustment for this variable. Fish intake is the main route for Hg exposure in general population but it is also a rich source of n-3 PUFA, selenium and vitamins. Several studies have suggested beneficial effects of eating fish during pregnancy and childhood on immune function, respiratory and allergy symptoms (Miles and Calder, 2017; Papamichael et al., 2018) although these protective associations remain unclear (Stratakis et al., 2017). In the present study, maternal and child fish consumption was elevated and the absence of association between Hg and the outcomes reported here could be related with these protective nutrients present in fish. However, when we controlled for maternal and child fish consumption, the results showed no essential changes. This could be owing to imprecision in estimating maternal and child fish intake by means of a questionnaire that may lead to an imprecise estimation of the fish-adjusted Hg effect (Grandjean and Budtz-Jørgensen, 2007). Also, associations adjusting by n-3 PUFA intake at pregnancy and 4 years, calculated from the FFQ, showed no substantial changes.

Other sources of discrepancy in the reviewed literature could be related to design, small sample size, Hg exposure assessment, parental-reported respiratory symptoms and problems, and variations in adjustment for other confounding variables. These differences hinder the comparison between studies, and as consequence, they make more difficult to draw conclusions about the impact of Hg exposure on respiratory and allergy outcomes in childhood. Moreover, to our knowledge, the present study is the first that has examined the association between prenatal and postnatal Hg exposure on chestiness, persistent cough and otitis.

In order to explore more in depth this issue, we assessed the influence of potential effect modifiers. In the current study, boys showed more risk of wheezing than girls and a significant sex interaction was found on the association between postnatal exposure to Hg and persistent

cough. Among the reviewed literature about respiratory or allergy outcomes, only Kim et al 2015 (Kim et al., 2015) explored sex differences, and in fact, after stratifying by sex, the association between Hg exposure and asthma remained only in boys but interaction was not statistically significant.

Our study provides some evidence that maternal fruit and vegetables consumption may interact with prenatal Hg exposure to modulate associations with respiratory symptoms and otitis. These problems involve systemic and airway inflammation responses, and oxidative stress could play a major role in these processes (Bowler, 2004; Garça et al., 2013; Ji and Li, 2016). Hg exposure has been associated with free radicals production and inactivation of antioxidant defences intensifying oxidative stress in cells (Vos and Monestier, 2008). Fruits and vegetables provide rich sources of antioxidants and vitamins that may protect from inflammatory response through tissue damage induction mediated by oxidants. By contrast, a low antioxidant diet may deplete the capacity to cope with oxidative stress (Hosseini et al., 2017). Fiber and phytochemical substances also present in fruit and vegetables may modify Hg bioaccessibility and metabolism (Chapman and Chan, 2000; Shim et al., 2009). Oxidative stress levels and diminished anti-inflammatory capacity induced by Hg along with low antioxidant, fiber and phytochemicals intake during pregnancy, could contribute to vulnerability to inflammation and/or infection of the structures of the middle ear (Garça et al., 2013; Wood and Gibson, 2009). Surprisingly, we observed an inverse association between prenatal Hg exposure and risk of chestiness, stronger in the high fruit and vegetable intake group. It is possible that unknown factors related to a healthy diet may explain these associations.

In addition, several in vitro studies have shown vitamin E may confer some protection against MeHg toxicity due to antioxidant properties (Chapman and Chan, 2000). However, no effect modification on pre and postnatal Hg exposure and respiratory outcomes association was found in this study.

There is limited evidence in the literature of associations between vitamin D and toxic metals. Some studies have shown higher vitamin D intake can be associated with lower maternal blood metals (Arbuckle et al., 2016) but others have suggested that excessive vitamin

D intake may intensify the absorption of them (Schwalfenberg and Genuis, 2015). In vitro studies have shown controversial results about the relation between vitamin D intake and Hg toxicokinetics (Schwalfenberg and Genuis, 2015). Our results have shown no statistically significant interaction with maternal vitamin D blood concentration and child vitamin D intake and pre and postnatal Hg exposure on respiratory and allergy outcomes.

Food could be also a source of exposure to organocholorine compounds, such as DDE and PCBs. A meta-analysis suggested that prenatal DDE exposure may be associated with some respiratory health symptoms including wheezing and tract infections, whereas for prenatal PCB 153 levels the association was not clear (Gascon et al., 2013). In our study, prenatal DDE exposure was associated with increased risk of wheezing and chestiness, however no association was found with PCB 153. Nevertheless, to our knowledge, interaction between Hg and organocholorine compounds on respiratory and allergy outcomes remains unexplored. Our results showed an inverse association between prenatal Hg levels and persistent cough when prenatal DDE exposure was above the median. A similar association with eczema was observed when prenatal PCB 153 exposure was also above the median. The interaction between Hg and PCBs has been more studied in relationship with neurodevelopment where synergistic effects have been suggested (Stewart et al., 2003). Conversely, in some studies, a competitive rather than a synergistic action have been observed (Castoldi et al., 2008). This effect cannot be considered a protective action related to toxicants co-exposure. Compensatory mechanisms may mask final outcome in young individuals but may be ensued later in life (Castoldi et al., 2008; Rice, 2004).

We found an effect modification of prenatal and postnatal smoke exposure on the association of Hg with wheezing and persistent cough. Noteworthy, similar to the interactions observed for PCBs and DDE, less risk of theses outcomes associated with Hg exposure were observed among children with maternal an infancy tobacco exposure. Among the many toxic substances present in tobacco smoke, cadmium may contribute to increased risk of respiratory problems (Razi et al., 2012). *In vitro* studies have shown that both cadmium and Hg were able to inhibit each other accumulation (Oliva Teles et al., 2005). Lead, other metal present in

tobacco smoke, also showed antagonistic effects with Hg on neurotoxicity in Faroe Island birth cohort (Yorifuji et al., 2011). Further research on the combined effects of several toxicants on respiratory illnesses are needed.

Although we did not observe an association between prenatal and postnatal Hg exposure and the respiratory and allergy outcomes, when modifier effects have been explored, we found that interactions occurred more frequently during prenatal than postnatal exposure. These results may suggest that the prenatal period could be a vulnerable development stage to modulation of Hg effects on respiratory health by diet and other toxicants.

A strength of the present study is prospective design which allows us obtain extensive information about covariates concerning parental and child characteristics related with respiratory outcomes as well as those may act as confounders of exposure. The considerable sample size in comparison with most epidemiological studies on this topic is another advantage. Also, THg levels in cord blood and hair are considered good indicators of prenatal and postnatal MeHg exposure (Bartell et al., 2004; Grandjean and Budtz-Jørgensen, 2007). Hair is a non-invasive method, preferable in children and so hair was chosen as the follow-up matrix. Moreover, a good correlation between Hg levels in hair and blood has been reported in populations where Hg comes mainly from fish (Branco et al., 2017; Croes et al., 2014). However, a potential source of bias in cohort studies is the loss of follow-up. In this study, 80% of children who participated at baseline take part in the fourth year survey. Compared with non-participants, study population was less likely to report a low maternal and social class level, low percentage of foreign and younger mothers and more active and passive smoking exposure during pregnancy. These differences could represent possible bias in estimating some exposure-outcomes associations although losses in cohort studies occurs more frequently in populations with unfavourable circumstances (Howe et al., 2013; Miyake et al., 2011). In addition, because several outcomes were examined, we cannot discard that some of the effect modification observed can be spurious. Another limitation includes the fact that information about some outcomes was determined based on questionnaire responses rather than medical records, increasing the likelihood of recall bias. This possible outcome misclassification would tend to

bias the estimation of effect toward the null. Nevertheless, the questionnaire used in the present study was adapted from the international AMICS study and it has been used in several studies since study AMICS began (1994-1998) (Polk et al., 2004; Sunyer et al., 2004). On the other hand, measurement of immune biomarkers could have allowed us to observe changes related to Hg exposure despite not having found an association with outcomes at this time, similar to Faroe cohort (Grandjean et al., 2010) but such information was not available at age 4 in our study. In this regard, several cohort studies show that allergic diseases peak at different ages in childhood (Just et al., 2017; van der Hulst et al., 2007). Atopic eczema has the highest incidence in the first 2 years of life and in later childhood decreases. Prevalence of doctor-diagnosed eczema by age 4 varies from 16.05% to 28.11% across 10 European cohorts (Uphoff et al., 2017). Recurrent wheezing and persistent coughing also are common respiratory symptoms in preschool children (Just et al., 2017). Prevalence rates of wheezing from birth until age 4 years varies from 9.82% to 55.37% across 10 European cohorts (Uphoff et al., 2017). These symptoms are typically associated with viral infections in this age group but may also be early allergic and asthma symptoms. Doctor-diagnosed asthma and allergic rhinitis outcomes at 4 years of age were not included in this study due to their low prevalence (3,4% and 3% respectively). Prevalence of asthma and rhinitis increase in later childhood and adolescence (Just et al., 2017; van der Hulst et al., 2007). So an effect of Hg on allergic symptoms later in life cannot be discarded, hence the importance of conducting longitudinal studies such as INMA.

In conclusion, in our study we found no association between prenatal and postnatal Hg exposure and respiratory and allergy problems although these associations could be modulated by diet and other pollutants especially during prenatal period. Our results have shown lower maternal intake of fruits and vegetables increased the risk of some respiratory outcomes due to the prenatal exposure to Hg. However, an inverse association between cord blood Hg levels and some respiratory outcomes was observed among children with higher maternal levels of organocholorine compounds or tobacco exposure. More research on this topic is needed due to the inconsistent and limited evidence. Moreover, since the association between early exposure

to Hg and respiratory and immunological outcomes could appear later in life, it would advisable to follow children until older ages. Longitudinal studies as INMA project, are crucial to determine if there are effects of prenatal and postnatal Hg exposure on respiratory and allergy symptoms at later ages.

Conflict of interest

The authors declare they have no actual or potential competing financial interests.

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Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (Hospital La Fe in Valencia, the Institut Municipal d'Assistència Sanitaria in Barcelona, Comité Ético de Investigación Clínica del Hospital Donostia, and Comité Ético de Investigación Clínica del Área Sanitaria de Gipuzkoa) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Abbreviations

Hg Mercury

THg Total mercury

MeHg Methylmercury

US EPA United States Environmental Protection Agency

PTWI Provisional Tolerable Weekly Intake

LOQ Limits of quantification of the method

BMI Body mass index

FFQ Food frequency questionnaire

PUFA Polyunsaturated fatty acids

DDE Dichlorodiphenyldichloroethylene

PCB Polychlorobiphenyl

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Figure captions:

Figure 1. Association between prenatal and postnatal mercury exposure and respiratory problems at the age of 4 years old in INMA birth cohort Spain. OR: Odds Ratio; CI: confidence intervals. OR (95%CI) from cohort-specific logistic regression models combined by meta-analysis. Prenatal mercury (Hg) exposure was measured through total Hg cord blood concentration (µg/L) whereas postnatal exposure was measured through hair Hg concentration at 4 years (µg/g) and were log2 transformed. Numerical values plotted here can be found in Table S3. All models were adjusted by child's age, sex and residence zone (residence zone was not included for Sabadell cohort). Additional adjusted variables included in cohort-specific models are shown in Table S4.

Figure 2. Estimates of effect modification analyses (adjusted OR and 95% confidence interval) of sex, familiar allergic antecedents, maternal dietary intakes during pregnancy, prenatal exposure to DDE and 153PCB and prenatal tobacco smoke exposure on the association between prenatal mercury concentration and respiratory outcomes. DDE: Dichlorodiphenyldichloroethylene; PCB: polychlorobiphenyl. OR (95%CI) from cohort-specific logistic regression models combined by meta-analysis. Prenatal mercury (Hg) exposure was measured through total Hg cord blood concentration (µg/L) and was log2 transformed. Effect

modification was evaluated by including an interaction term in all the adjusted models, pint: represent p-values of the interaction term. Numerical values plotted here can be found in Table S6

Figure 3. Estimates of effect modification analyses (adjusted OR and 95% confidence interval) of sex, familiar allergic antecedents, child dietary intakes at 4 years, breastfeeding and postnatal tobacco smoke exposure on the association between postnatal mercury concentration and respiratory outcomes. OR (95%CI) from cohort-specific logistic regression models combined by meta-analysis. Postnatal mercury (Hg) exposure was measured through hair Hg concentration at 4 years (µg/g) and was log2 transformed. Effect modification was evaluated by including an interaction term in all the adjusted models, pint: represent p-values of the interaction term. Numerical values plotted here can be found in Table S7

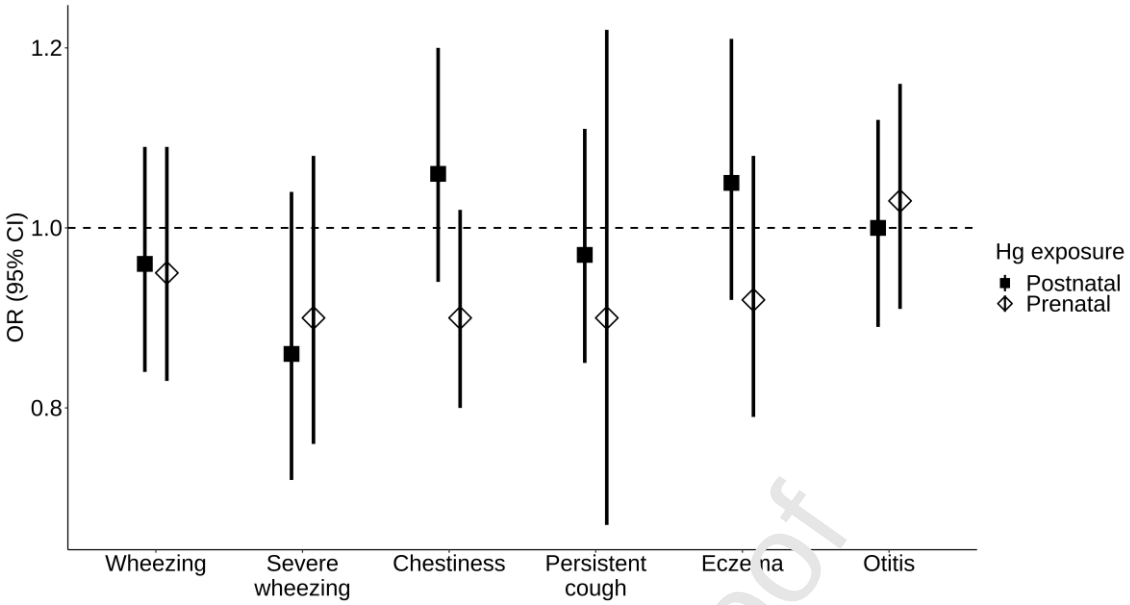


Fig. 1

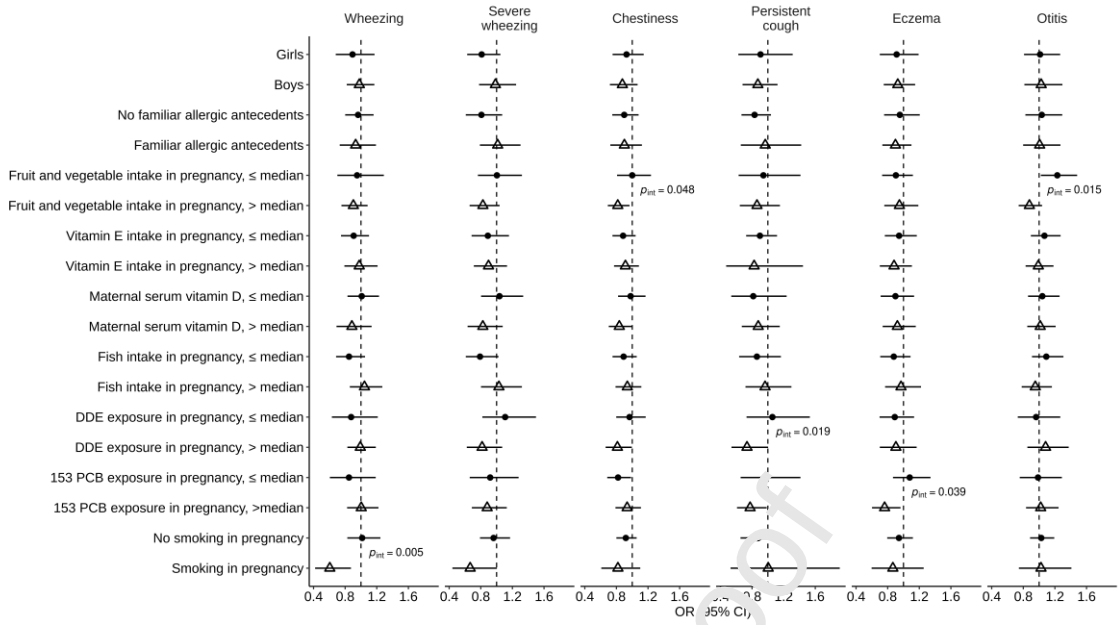


Fig. 2

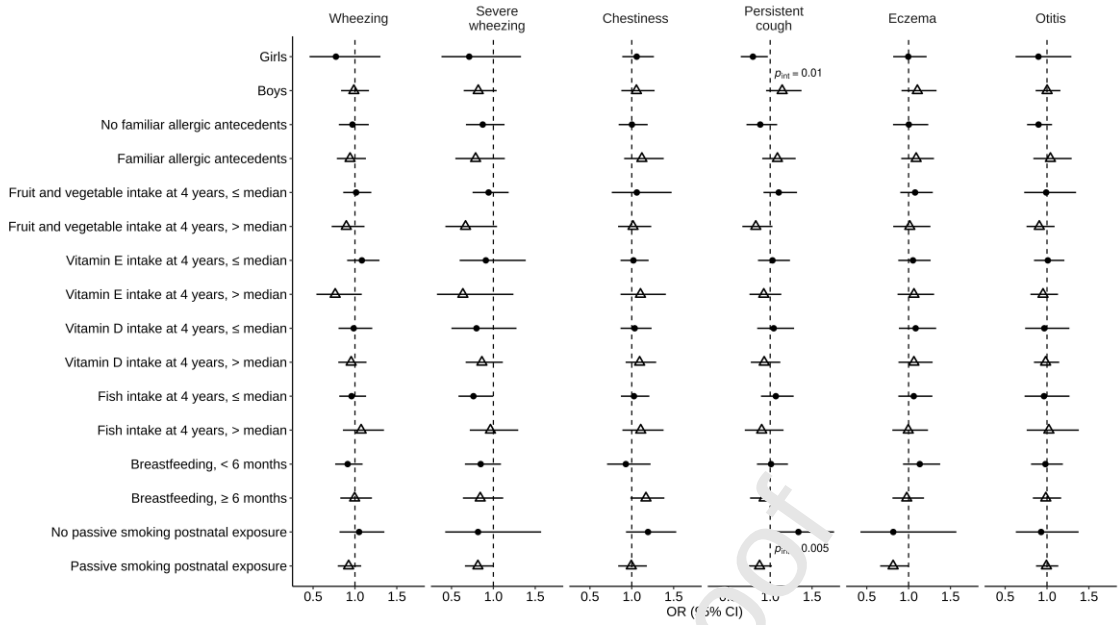


Fig. 3

Table 1. Prevalence of respiratory and allergy problems at 4 years old and total mercury concentration in cord blood and hair (4 years old) in all the population and by cohort.

	Overall	Asturias	Gipuzkoa	Sabadell	Valencia	*p*-value[a]
Wheezing, n (%)	427 (22.6)	149 (37.7)	57 (14.3)	110 (21.8)	111 (18.9)	<0.001
Severe wheezing, n (%)	190 (10.1)	56 (14.2)	41 (10.4)	42 (8.3)	51 (8.8)	0.018
Chestiness, n (%)	727 (38.7)	213 (53.9)	86 (21.8)	225 (44.6)	203 (34.8)	<0.001
Persistent cough, n (%)	299 (15.9)	70 (17.7)	63 (15.9)	79 (15.6)	87 (14.8)	0.678
Eczema, n (%)	311 (16.6)	32 (8.1)	68 (17.4)	115 (22.8)	96 (16.3)	<0.001
Otitis, n (%)	523 (27.8)	117 (29.6)	83 (21.1)	147 (29.2)	176 (30.0)	0.010
Hg cord blood, GM (IR) (µg/L)	8.23 (9.00)	10.81 (12.50)	7.60 (6.90)	6.29 (5.90)	9.43 (12.72)	<0.001
Hg hair (4 years old), GM (IR) (µg/g)	0.97 (1.04)	Na	0.98 (0.80)	0.83 (0.82)	1.10 (1.37)	<0.001

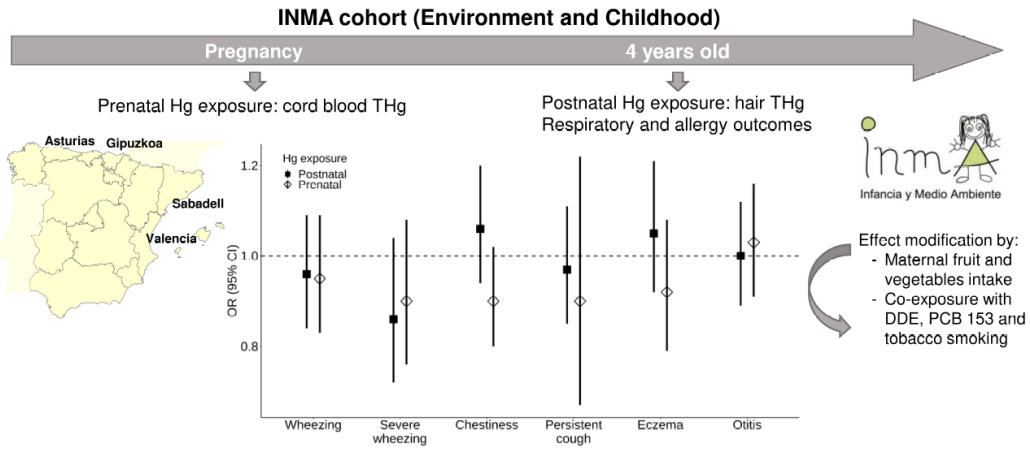
Note: Na: not available; n sample size; GM geometric mean; IR interquartile range; Hg: mercury;
[a] *p*-value when comparing outcomes prevalence between cohorts using Chi-Square test or when comparing mercury concentration (log2 transformed) between cohorts using ANOVA test.

CRediT author statement

Paula Carrasco: Methodology, Formal analysis, Writing - Original Draft, Writing - review & editing; **Marisa Estarlich;** Methodology, Formal analysis, Writing - review & editing; **Carmen Iñiguez;** Methodology, Validation, Writing - review & editing; **Amparo Ferrero:** Investigation, Methodology, Writing - review & editing; **Mario Murcia:** Data curation, Validation, Writing - review & editing; **Ana Esplugues:** Writing - review & editing; **Jesús Vioque:** Writing - review & editing, **Loreto Santa Marina:** Writing - review & editing, **Carlos Zabaleta:** Writing - review & editing, **Gorka Iriarte:** Writing - review & editing, **Ana Fernández-Somoano:** Writing - review & editing, **Adonina Tardon:** Writing - review & editing, **Martine Vrijheid:** Writing - review & editing **Jordi Sunyer:** Writing - review & editing; **Ferran Ballester:** Conceptualization, Supervision, Writing - review & editing **Sabrina Llop:** Conceptualization, Methodology, Writing - review & editing,

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.



Graphical abstract

Highlights

Cord blood THg concentrations were not associated with respiratory outcomes at 4 years of age

Hair THg concentrations at 4 years old were not associated with respiratory outcomes

The inclusion of fish and n-PUFA intakes in models did not change the results

Maternal fruit and vegetables intakes modulated prenatal Hg effects

Effect modification by co-exposure with other toxicants was observed