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Exposure to mercury among 9-year-old children and neurobehavioural function

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ABSTRACT

Mercury (Hg) is an environmental neurotoxicant whose main route of exposure in humans is the consumption of seafood. The aim of this study was to explore the relationship between Hg exposure at 9 years old and behaviour assessed at 9 and 11 years old.

Study subjects were mother–child pairs participating in the INMA (Environment and Childhood) Project in Valencia (Spain). Total Hg (THg) was measured in hair samples from the children at 9 years old. Behaviour and emotions were assessed at 9 (n = 472) years and 11 (n = 385) years of age using the Child Behaviour Checklist test (CBCL) and the Conners Parents Rating Scale-Revised: Short Form (CPRS-R:S). Furthermore, the attention function was assessed by the Attention Network Test at 11 years old. Socio-demographic, lifestyle and dietary information was collected through questionnaires during pregnancy and childhood. Polymorphism in *BDNF*, *APOE* and *GSTP1* were genotyped in cord blood DNA. Multivariable negative binomial regression models were built in order to study the association between THg concentrations and the scores obtained by the children at 9 and 11 years old. Effect modification by sex and genetic polymorphisms was assessed.

The association between Hg levels and CBCL scores was positive (worse neurobehavioural development) for the CBCL internalizing and total problem scales (Incidence Rate Ratio [95% confidence interval] = 1.07 [1.01, 1.13] and 1.05 [0.99, 1.11], respectively). The association between Hg and the externalizing and total problems CBCL scales and the Attention Deficit Hyperactivity Disorder (ADHD) index of the CPRS-R:S was different according to sex, with boys obtaining worse scores with increasing Hg, compared to girls. Statistically significant interactions were also observed for genetic polymorphisms affecting the association between early exposure to Hg and both CBCL and CPRS-R:S scores. In conclusion, postnatal Hg exposure is associated with poorer neurobehavioural development in 9- and 11-year-old children. Sex and the presence of certain genetic polymorphisms modified this association.

1 Introduction

Mercury (Hg) is an environmental toxicant that is ubiquitously distributed and with its origins in both natural and anthropogenic sources (World Health Organization (WHO), 2007), the latter being the ones that have contributed the most to the current (environmental) levels (Outridge et al., 2018). The greater part of the inorganic Hg present in the atmosphere comes from point sources such as mining operations or industrial activities (Hintelmann, 2010). The atmospheric inorganic Hg is deposited in the soil surface and transformed into the

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organic form, mostly methylmercury (MeHg), by the action of some bacteria present in the aquatic sediments (Parks et al., 2013). MeHg is accumulated and biomagnified through the food chain, achieving the highest concentrations in the muscle tissues of long-lived predatory fish such as swordfish, red tuna, shark and pike. In fact, the intake of these big oily predatory fish and marine mammals is the main source of exposure to this compound in human beings (Hintelmann, 2010). However, other smaller fish also contain MeHg (Marín et al., 2018) and some of them such as lean fish and canned tuna, owing to their more frequent consumption, could contribute to a considerable extent to the Hg content in the human body in high fish-consuming populations (Soler-Blasco et al., 2019).

MeHg from diet is efficiently absorbed in the gastrointestinal tract (Siedlikowski et al., 2016). From the bloodstream, it can be transported across membranes and distributed to all tissues due to its capability to form a MeHg–cysteine complex (Clarkson et al., 2007). The central nervous system is the critical organ for MeHg exposure, the foetal and early postnatal stages being the most vulnerable with regard to its effects (Grandjean and Landrigan, 2014). The nervous system has a long development time that extends from the embryonic period to adolescence. Although most neurons have been formed by the time of birth, growth of glial cells and myelinization of axons continues for several years (Rice and Barone Jr, 2000).

The scientific literature on the association between early Hg exposure and child neurobehaviour is still scarce and controversial. While some birth cohort studies reported a deleterious effect of pre and early postnatal exposure to mercury on different aspects of children's behaviour in Canada (Boucher et al., 2012) and the Faroe Islands (Debes et al., 2006), other studies conducted in the Seychelles (Wijngaarden et al., 2013) and UK (Golding et al., 2016) failed to find any statistically significant association. Even a protective effect of early exposure to Hg on neurobehaviour was observed in recent studies conducted in USA (Xu et al., 2016), and Bangladesh (Gustin et al., 2017). The cause of this heterogeneity could be related to several factors with an influence on the relationship between Hg exposure and children's neuropsychological development, such as genetics. In fact, the association between pre- and postnatal exposure to MeHg and child neuropsychological development has been shown to be affected by polymorphisms in some genes across study populations (Julvez et al., 2019; Llop et al., 2017; Ng et al., 2014). Other causes of heterogeneity could be the beneficial nutrients of fish (Llop et al., 2016; Oken et al., 2008; Sagiv et al., 2012), or co-exposure with other pollutants present in fish such as polychlorinated biphenyls (PCBs) (Boucher et al., 2010; Stewart et al., 2003).

In previous studies, we reported relatively high levels of prenatal exposure to Hg (measured as cord blood total Hg [THg]) in the Spanish INMA (Environment and Childhood) birth cohort (Ramon et al., 2011). Thus, 64% and 24% of newborns had cord blood THg concentrations exceeding the equivalent to the current US Environmental Protection Agency reference dose (5.8 μg/L of MeHg in whole cord blood) and to the WHO Provisional Tolerable Weekly Intake (1.6 μg/kg of body weight per week), respectively. Despite these elevated levels we did not observe a clear association between cord blood THg levels and child neurodevelopment at 14 months (Llop et al., 2012) and at 4–5 years old (Llop et al., 2020, 2016). We also described a decreasing temporal trend in Hg concentrations from birth to 4 years old (Llop et al., 2014) and from 4 to 9 years old (Soler-Blasco et al., 2019) in the INMA-Valencia cohort. However, the levels observed at 9 years old were still high in comparison to those found in other European countries (Soler-Blasco et al., 2019). The aim of this study is to evaluate the relationship between Hg exposure at 9 years old and emotional, behavioural and attentional problems assessed at 9 and 11 years old, as well as considering the modifying effect of genetic polymorphisms and gender, in the INMA cohort located in Valencia.

2 Methods

2.1 Study population and design

The subjects in our sample were participants in the INMA Project, a multicentre birth cohort study that aims to investigate the effect of environmental exposures and diet during pregnancy and childhood on foetal and child development in different geographical areas of Spain (http://www.proyectoinma.org). The study protocol has been reported elsewhere (Guxens et al., 2012). Briefly, 855 pregnant women were recruited during their first antenatal visit (2003–2005) in the INMA cohort of Valencia (Spain). The inclusion criteria were: being at least 16 years of age, 10–13 weeks of gestation, singleton pregnancy, intention of undergoing follow-up and delivery in the corresponding centre of reference (La Fe Hospital), no impediment for communication, and no assisted conception. Excluding the women who withdrew from the study, were lost to follow-up, and had induced or spontaneous abortions or foetal deaths, we followed up a total sample of 787 women until delivery (2004–2006). Their children were enrolled at birth and were followed up until 9 (n = 472) and 11 (n = 385) years of age. The final study population was made up of 403 nine-year-old children (51.2% of total births) and 328 eleven-year-old children (41.7% of total births) who had available behavioural assessment data at each age, hair THg concentrations at 9, as well as the covariates included in the study. See the flowchart in Supplemental Figure S1.

Informed consent regarding the prenatal period was signed by the mother and in each phase of the postnatal period further consent was signed by one of the parents or a legal representative. The study protocol was approved by the Public Health Research Centre in Valencia (CSISP) and La Fe hospital ethics committees.

2.2 Mercury exposure

Hair samples were collected from the occipital scalp when the children were 9 years old. Hair samples were cut as close as possible to the root, a minimum length of 2 cm or at least 100 mg in weight being collected in each case, and were then stored in a plastic zip bag at room temperature until analysis. The analyses of THg were carried out in the Public Health Laboratory of Alava (Basque Country, Spain) using a direct mercury analyser. Hair samples were rinsed with 10 mL of Triton X-100 at 1% (Panreac, Barcelona). The samples were weighed in a weigh boat and analysed directly in AMA 254 and DMA-80 equipment by catalytic combustion, gold amalgamation, thermal desorption and atomic absorption spectrometry. Replicate analyses were performed for each sample. The limit of quantification (LOQ) of the method was 0.01 μg/g. No measurements of Hg in hair were below the LOQ. Hair sample batches were controlled with IAEA-086 (International Atomic Energy Agency, Austria) and NCS ZC 81002b (NCS Institute, Beijing, China) reference materials. Additionally, the accuracy of the method was also verified externally by participation in different inter-laboratory exercises organized by the Centre de toxicologie du Québec (Quebec Multielement External Quality Assessment Scheme, QMEQAS programme). In all cases satisfactory results were obtained (z-scores for Rounds 2013-3 and 2015-3: +0.42 and +0.13).

2.3 Behavioural assessment

The presence of emotional and behavioural problems in the children were assessed at 9 and 11 years old (mean [standard deviation (SD)] = 9.33 (0.23) and 11.00 (0.32) years, respectively) by using the Child Behaviour Checklist test (CBCL) (Achenbach and Ruffle, 2000) and the Conners Parent Rating Scales-Revised: Short Form (CPRS-R:S) (Kumar and Steer, 2003). Additionally, the Attention Network Test (ANT) (Fan et al., 2002) was administered to the 11-year-old children. All these tests have been validated for the Spanish population (Farré-Riba and Narbona, 1997; Forns et al., 2014; Rubio-Stipec et al., 1990). Higher scores on all the scales indicate greater behavioural problems.

The CBCL is a recognized tool for assessing the behaviour of children and includes several subscales, which allow identification of possible behavioural problems measured on three scales: (1) emotional problems

(internalizing scales: anxiety, depression, somatic complaints); (2) behavioural problems (externalizing scales: rule-breaking and aggressive behaviour); and (3) total problems (sum of internalizing, externalizing, and other scales: social, thought and attention problems). The CBCL is a one hundred and twelve-item parent-report questionnaire which refers to problems that might have occurred in the preceding two months and are rated on a three-point scale (0 = not true, 1 = somewhat or sometimes true and 2 = very or often true).

The CPRS-R:S consists of a list of 27 items to be rated by the mother at home and provides information on child behaviour, particularly in relation to inattention and hyperactivity. These items result in scores for an Attention Deficit Hyperactivity Disorder (ADHD) Index.

The ANT is a computational test to provide a measure of the efficiency of three different functions of attention: alerting (the ability to produce and maintain optimal vigilance and performance during tasks), orienting (involves shifting attention to sensory stimuli) and executive attention (involves detecting and resolving conflict among responses, error detection and response inhibition). The measure used in this test is the Hit Reaction Time Standard Error (HRT-SE) (expressed in milliseconds), considered a measure of response speed consistency throughout the test. A high HRT-SE indicates highly variable reactions and is considered a measure of inattentiveness.

2.4 Genetic analysis

DNA was extracted from cord blood by the Chemagen protocol (Baesweiler, Germany). This is a candidate gene approach where we have analysed single nucleotide polymorphisms (SNPs) in gens with a possible role in pathways associated with MeHg toxicity. We selected SNPs based on functional impact according to the literature, potential functional impact according to position and type of SNP (specifically, which may affect the protein structure/enzyme activity or SNPs at putative promoter sites that can affect gene expression); or tagSNPs that capture as much of the genetic variation within a gene segment as possible due to linkage disequilibrium (LD) with other SNPs. TagSNPs were selected according to Ensembl data for CEU (CEPH, Utah residents with ancestry from northern and western Europe). The SNPs and genes analysed were: rs662 (Paraoxonase 1 [*PON1]*), rs705381 (*PON1*), rs1695 (Glutathione S-Transferase Pi 1 [*GSTP1]*), rs1519480 (Brain Derived Neurotrophic Factor *[BDNF]*), rs7934165 (*BDNF*), rs6263 (*BDNF*), rs12273363 (*BDNF*), rs7103411 (*BDNF*), rs5110 (Apolipoprotein A4 [*APOA4]*), rs7412 (Apolipoprotein E *[APOE]*) (Supplemental Table S1).

Genotyping was performed using the HumanOmni1-Quad Beadchip (Illumina, San Diego, CA, USA) at CEGEN. Genotype calling was done using the GeneTrain2.0 algorithm based on HapMap clusters implemented in the GenomeStudio Illumina software. The following initial quality control thresholds were applied: sample call rate >98% and/or logRRatio SD < 0.3 (n = 4 were excluded). Then, sex, relatedness, heterozygosity and population stratification were checked. Principal component analysis (PCA) showed that there was no population stratification in the cohort. Genetic variants were filtered for single nucleotide polymorphism (SNP) call rate > 95%, and MAF > 1%.

2.5 Covariates and potential confounding variables

The recruited women completed two questionnaires during their pregnancy, one at the first trimester (mean(SD) = 12.2 (2.2) weeks of gestation) and the other at the third trimester (mean(SD) = 32.1 (2.2) weeks of gestation). The questionnaires were administered by trained interviewers and focused on dietary, socio-demographic, environmental and lifestyle information during pregnancy. The prenatal and perinatal covariates used in this study were maternal country of birth (Spain, other), parental age (years), child's sex, birth season, parental level of education (up to primary studies, secondary studies, university), area of residence (urban, semi-urban, rural), parental employment during pregnancy (non-worker, worker) and smoking during pregnancy (no, yes), as well as other exclusive maternal covariates such as body mass index (BMI, kg/m^2) before pregnancy, parity (0, 1, ≥ 2) and breastfeeding (yes/no). Family social class was defined from the maternal or paternal 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) (Class I + II: managerial jobs, senior technical staff and commercial managers; class III: skilled non-manual workers; and class IV + V: manual and unskilled workers) (Domingo-Salvany et al., 2013).

When children were 5 years old, the mother's intelligence was measured by the WAIS-III test (Wechsler, 1981) in order to assess associations with child's behavioural test outcomes. Information about the season of sampling (spring, summer, autumn, winter) and children's BMI (kg/m^2) at 9 years old was obtained. We calculated z-scores for BMI according to the WHO child growth standard (WHO Multicentre Growth Reference Study Group, 2006). Parental working status (non-working, working) and parental smoking habit (no, yes) were collected both at 9 and at 11 years of age.

Information on fish intake was collected at the age of 9 years by a semi-quantitative food frequency questionnaire previously validated in the same study population at the age of 7 years (Vioque et al., 2019). Portion sizes were adapted conveniently for the age of 9 years and several items were used to collect information for fish intake according to Spanish dietary habits: lean fish (including hake, sole and sea bream), swordfish, other big oily fish (including tuna, bonito, salmon), small oily fish (including mackerel, sardine, anchovy), canned tuna, and other fish (including shellfish, processed fish and other canned fish). The items had nine possible responses, ranging from 'never or less than once per month' to 'six or more per day'. For each participant, the responses for each serving of fish were converted into mean weekly intake to compute the total fish intake and the intake of specific fish groups.

2.6 Statistical analysis

We calculated the geometric mean (GM) and 95% confidence intervals (95%CI) of the THg concentrations according to socio-demographic, environmental, dietary and genetic characteristics of the study population. For further analyses, the variable THg at 9 years of age was log2-transformed due to its skewed distribution. The ANOVA F-test was applied to the logarithm to compare the geometric mean of THg concentrations across categories of the study population's characteristics.

In order to assess the relation between postnatal exposure to THg and the scores on the CBCL and CPRS-R:S behavioural tests, mixed multivariate negative binomial models were built considering the samples of both 9- and 11-year-olds (n = 731) through a two-step procedure. In the first step, a core model was built for each scale with parental and child socio-demographic variables as possible covariates. To do this, bivariate and multivariate negative binomial models were performed in order to study the relationship between each outcome and the covariates. The multivariate model was built using all the covariates associated with a p-value < 0.2 in the bivariate analysis. Following a backward elimination procedure, all the covariates associated with the outcome at a level of p < 0.1 were retained in the model. The Log2 THg variable was introduced into these adjusted models. In the second step, additional potential confounders were included if they changed the magnitude of the main effects by>10%. All covariates in the multivariate model for the THg concentration were considered to be potential confounders (Soler-Blasco et al., 2019). Children's age at assessment and sex were included in all models regardless of their statistical significance. The confounding effect of different fish intake variables was also tested by including them in the final models (big oily fish, total oily fish [sum of big and small oily fish], and total fish intake). A multivariable linear model was built for ANT only in the sample of 11-year-olds following the same procedure as explained above. The variables included in the multivariate models for

each behavioural test can be found in Supplemental Figure S2. Complementary figures with confidence interval ranges are shown in Supplemental Figures S3 to S7. The variables were considered statistically significant if the p-value < 0.05. Final multivariate models were mutually adjusted by both pre- and postnatal THg concentrations and coefficients are presented in Supplemental Table S3.

Generalized additive negative binomial models were fitted to evaluate non-linear patterns. Natural cubic splines with one or two internal knots were compared through Akaike (AIC) scores. Then, the lowest AIC non-linear model and linear model were compared using graphical examination and the Likelihood Ratio test (LRT).

Effect modifications by sex of the child, parental smoking, big oily fish consumption and the SNPs were assessed through inclusion of the interaction term in the main models. The effect modification was considered statistically significant if the p-value < 0.5, although marginally significant interactions (p-value < 0.1) were also considered.

Several sensitivity analyses were performed to evaluate the robustness of the multivariate models and these were repeated after eliminating certain population subgroups: preterm birth (n = 73), low birth weight (n = 75), and those in whom the quality test was uncertain (n = 86).

The validity of the regression models was tested by residual analyses. No influential data were identified by graphical representation. Collinearity diagnostics were conducted on the final models.

Statistical analysis was carried out using the R statistical package version 3.5.1 (R Core Team, 2017).

3 Results

Descriptive statistics of the study variables are displayed in Table 1. The mean age of the mothers included in the study at the time of conception was 30.5 years, around 94% of them were born in Spain, around 30% had a university degree, >40% belonged to the lowest social class and almost 50% lived in metropolitan areas. Around 80% worked during pregnancy and did not smoke in this period but both percentages were reduced to 70% at 9 years.

The THg GM was 0.89 μg/g (95%CI: 0.81–0.98). Around half of the children presented THg levels above the RfD proposed by the US EPA (i.e. 1 μg/g) and 13.1% of the children presented levels that were higher than the equivalent to the PTWI proposed by the WHO (i.e. 2.5 μg/g). THg concentrations were higher in girls, in children who were breastfed for more than 16 weeks, as well as in children whose mothers finished a university education and whose fathers had completed secondary school studies and were employed (Table 1). THg concentrations were inversely correlated with both maternal and child BMI and directly correlated with maternal intelligence and children's fish consumption. The category of fish that was consumed the most was lean fish (mean 1.4 servings/week; SD: 1.1), followed by the category of other fish (mean 1.2 servings/week; SD: 1.3). The mean consumption of big oily fish was 0.8 servings/week (SD: 0.7), for small oily fish it was 0.6 servings/week (SD: 0.2) and for swordfish it was 0.26 servings/week (SD: 0.3).

All the SNPs considered were in Hardy-Weinberg equilibrium. The minor allele frequencies (MAFs) for all SNPs were quite similar to those from other European populations (1000 genomes Europe project). The LD between rs6265 and rs7103411 was > 0.90. None of the SNPs genotypes showed significant differences in THg concentrations (Supplemental Table S1).

3.1 CBCL models

A descriptive of the children's scores obtained on each CBCL scale is shown in Supplemental Table S2. Scores were not correlated (Pearson's test p-value > 0.05) with hair THg concentrations. The association between children's THg concentrations and the scores on the CBCL test were linear and positive in the negative binomial mixed models for both the internalizing and the total behavioural problems scales (Incidence

Table 1
Hair total mercury concentrations at 9 years old according to socio-demographic and lifestyle characteristics and seafood consumption, INMA-Valencia cohort, Spain (2003–2014).

Covariates	N[a]	%	THg GM (GSD)	P-value[b]
Season of hair sample collection				
Winter	82	20.4	0.82 (2.64)	0.747
Spring	130	32.3	0.91 (2.60)	
Summer	103	25.6	0.96 (2.72)	
Autumn	87	21.6	0.88 (2.96)	
Child's sex				
Female	204	50.6	1.00 (2.36)	0.026
Male	199	49.4	0.80 (3.04)	
Parental social class[e]				
I + II (higher)	114	28.3	1.07 (2.28)	0.060
III	108	26.8	0.89 (2.60)	
IV + V (lower)	181	44.9	0.80 (3.01)	
Maternal country of birth[e]				
Spain	381	94.5	0.91 (2.71)	0.158
Other	22	5.5	0.67 (2.67)	
Maternal educational level[e]				
Up to primary	112	27.8	0.81 (3.10)	0.054
Secondary	172	42.7	0.84 (2.59)	
University	119	29.5	1.07 (2.46)	
Paternal educational level[e]				
Up to primary	173	43.1	0.73 (2.93)	0.001
Secondary	156	38.9	1.10 (2.46)	
University	72	18.0	0.93 (2.51)	
Parity				
0	227	56.3	0.95 (2.65)	0.373
1	151	37.5	0.83 (2.77)	
≥2	25	6.2	0.80 (2.82)	
Maternal working status during pregnancy[e]				
No	56	13.9	0.86 (2.39)	0.767
Yes	347	86.1	0.90 (2.76)	
Maternal smoking habit during pregnancy[e]				
No	322	79.9	0.93 (2.78)	0.130
Yes	81	20.1	0.77 (2.40)	
Breastfeeding (weeks)				
0	63	15.6	0.70 (3.12)	0.015
>0–16	90	22.3	0.87 (2.54)	
>16–24	65	16.1	1.22 (2.44)	
>24	185	45.9	0.89 (2.69)	
Maternal smoking habit at 9 years[f]				
No	270	67.3	0.97 (2.65)	0.025
Yes	131	32.7	0.77 (2.77)	
Paternal smoking habit at 9 years[f]				
No	276	68.8	0.89 (2.72)	0.780
Yes	125	31.2	0.92 (2.67)	
Maternal working status at 9 years[f]				
No	123	30.7	0.92 (2.72)	0.746
Yes	278	69.3	0.88 (2.71)	
Paternal working status at 9 years[f]				
No	67	17.0	0.70 (3.03)	0.024
Yes	327	83.0	0.95 (2.62)	
	N[a]	**Mean (SD)**	**Correlation vs. THg[c]**	**P-value[d]**
Maternal WAIS-III test (score)[g]	391	16.28 (4.47)	0.170	0.001
Maternal age (years)[e]	403	30.54 (4.14)	0.042	0.396
BMI before pregnancy (Kg/m²)	403	23.58 (4.30)	−0.107	0.032
BMI child at 9 years old (z-score)[f]	395	0.84 (1.34)	−0.116	0.021
Child's seafood consumption[f]				
Lean fish (servings/day)	402	1.36 (1.11)	0.279	<0.001

(continued on next page)

Table 1 (*continued*)

Covariates	N[a]	%	THg GM (GSD)	P-value[b]
Big oily fish (servings/day)	402	0.80 (0.70)	0.469	<0.001
Small oily fish (servings/day)	402	0.56 (0.72)	0.204	<0.001
Swordfish (servings/day)	401	0.26 (0.35)	0.460	<0.001
Canned tuna (servings/day)	402	0.46 (0.47)	0.311	<0.001
Other fish (servings/day)	401	1.23 (1.27)	0.019	0.707

THg was log2 transformed to carry out the correlation tests.
GM: Geometric Mean; GSD: Geometric Standard Deviation; SD: Standard Deviation; THg: Total Mercury.
[a] Missing values for some variables not included in percentages.
[b] p-value from ANOVA F-test.
[c] Pearson's test correlation coefficient.
[d] Pearson's test p-value.
[e] Information obtained during pregnancy.
[f] Information obtained at 9 years.
[g] information obtained at 5 years.

Rate Ratio [95% Confidence Intervals], p-value = 1.07 [1.01,1.13], 0.016 and 1.05 [0.99,1.11], 0.078, respectively) (Table 2). Subsequently, big oily fish consumption and the cord blood THg concentrations were also included in the model but the results obtained were similar. The coefficients for cord blood THg concentrations were not statistically significant (Supplemental Table S3).

The effect modification of several factors (sex, maternal smoking habit, children's fish intake and genetic characteristics) was also evaluated (Table 3). Statistically significant interaction p–values ($p < 0.05$) were observed for sex on both the CBCL externalizing and the total problems scales. The association between THg concentrations and the scores obtained for these scales were negative for females, although the coefficients were not statistically significant (0.94 [0.85, 1.03], 0.197 and 0.98 [0.90, 1.06], 0.615, respectively, in contrast to males (1.07 [0.99, 1.15], 0.091 and 1.09 [1.09 [1.02, 1.16], 0.008, respectively) (Fig. 1).

Some marginally statistically significant interactions ($p < 0.1$) were observed between THg concentrations and the SNPs *GSTP1* rs1695, *BDNF* rs1519480 and *BDNF* rs7934165. Children with the genotypes GA + GG for rs1695 obtained worse scores (1.18 [1.06, 1.32]) on the internalizing problems scale than the AA genotype (1.04 [0.93, 1.15]) with increasing THg concentrations (Fig. 2). Additionally, children with the genotypes CC for the rs1519480 and AA for rs7934165 also obtained worse scores on the externalizing problems scales (1.13 [1.01, 1.26] and 1.25 [1.05, 1.48], respectively) with increasing THg concentrations than the T and G allele carriers (0.98 [0.87, 1.09] and 1.05 [0.93, 1.17], respectively).

3.2 CPRS-R:S models

The scores for the ADHD index of the CPRS-R:S are not correlated with hair THg concentrations although a decrease in the score from 9 to 11 years has been found to be statistically significant (paired *t*-test p-value = 0.020) (Supplemental Table S2). The association between children's THg concentrations and the ADHD index was linear and positive, but not statistically significant (0.06 [-0.02,0.14], 0.146) (Table 2). The inclusion of the big oily fish consumption and the cord blood THg concentrations in the models did not affect the coefficients. The coefficients for cord blood THg concentrations were not statistically significant (Supplemental Table S3).

The results for the effect modification showed a statistically significant interaction between the THg concentrations and the ADHD index scores for sex, males obtaining worse scores (1.15 [1.04, 1.27]) than females (0.93 [0.83, 1.06]), with increasing THg (Table 3 and Fig. 1). The SNPs rs1519480, rs7934165 and rs7103411, all of which in the *BDNF* gene showed a significant effect modification of the association between THg concentrations and the index scores (Fig. 3). Children with CC genotypes for the rs1519480 (1.30 [1.12, 1.51]), CC genotype for rs7103411 (1.31 [1.13, 1.51]) and GG genotype for the rs7934165 (1.64

Table 2
Negative binomial mixed regression analyses between behavioural tests at 9–11 years old and THg exposure, INMA-Valencia cohort, Spain (2014–2016).

	All children (n = 385)								Only children with both THg measurements (n = 270)							
	Hair THg				Hair THg (adjusted for big oily fish)[†]				Hair THg				Hair THg + Cord blood THg			
	IRR	95%CI		P-value	IRR	95%CI		P-value	IRR	95%CI		P-value	IRR	95%CI		P-value
CBCL Internalizing	1.07	1.01	1.13	0.016	1.09	1.02	1.16	0.007	1.08	1.01	1.16	0.025	1.06	0.97	1.15	0.185
CBCL Externalizing	1.02	0.96	1.08	0.596	1.02	0.95	1.09	0.571	1.04	0.97	1.13	0.238	1.02	0.92	1.12	0.743
CBCL Total problems	1.05	0.99	1.11	0.078	1.05	0.99	1.12	0.105	1.05	0.98	1.14	0.169	1.05	0.98	1.14	0.169
CPRS-R:S (ADHD index)	1.06	0.98	1.15	0.146	1.06	0.96	1.16	0.234	1.11	1.01	1.22	0.034	1.11	0.98	1.25	0.098
ANT[‡]	2.87 *	−2.87	8.62	0.327	3.07 *	−3.29	9.44	0.345	5.24 *	−1.64	12.14	0.137	12.19 *	3.65	20.73	0.006

ANT: Attention Network Test; CBCL: Child Behaviour Checklist; CI: confidence interval; CPRS-R:S: Conners Parental Rating Scale; IRR: Incidence Rate Ratio.
Children's hair and cord blood THg concentrations were log2 transformed.
All models adjusted for children's sex and age at evaluation.
Internalizing problems scale model additionally adjusted for maternal country of birth, educational level, parity, prenatal BMI and smoking habit at 9 years, paternal working status at 9 years and child's BMI at 9 years.
Externalizing problems scale model additionally adjusted for maternal prenatal BMI and smoking habit at 9 years, paternal education level and working status at 9 years and child's BMI at 9 years.
Total behavioural problems model additionally adjusted for maternal country of birth, educational level, parity, prenatal BMI and smoking habit at 9 years, paternal educational level and working status at 9 years, and child's BMI at 9 years.
Conners Parental Rating Scale model additionally adjusted for maternal country of birth, educational level, parity, prenatal BMI and smoking habit at 9 years, paternal educational level and season of sampling.
Attention Network Test model additionally adjusted for maternal smoking during pregnancy and mother's intelligence was measured by the WAIS-III test at 5 years.
[*] β values from multivariable linear models.
[†] Big oily fish includes swordfish, tuna, bonito, salmon.
[‡] ANT models were carried out only in children at 11 years of age.

Table 3
Negative binomial mixed regression models between behavioural tests at 9–11 years old and THg exposure, as well as effect modifications by sex, maternal smoking habit at 9 years old, children's big oily fish consumption and genetic polymorphisms, INMA-Valencia cohort, Spain (2014–2016).

			CBCL Internalizing				CBCL Externalizing				CBCL Total problems				CPRS-R:S (ADHD index)				ANT (HRT-SE)			
		n	IRR	95%CI		P-value	IRR	95%CI		P-value	IRR	95%CI		P-value	IRR	95%CI		P-value	β*	95%CI		P-value
Sex (ref 'female')		204	1.02	0.93	1.12	0.668	(0.94)‡	0.85	1.03	0.197	(0.98)‡	0.90	1.06	0.615	(0.93)‡	0.83	1.06	0.313	−1.32	−9.84	7.20	0.761
Sex (ref 'male')		199	1.10	1.03	1.18	0.005	(1.07)‡	0.99	1.15	0.091	(1.09)‡	1.02	1.16	0.008	(1.15)‡	1.04	1.27	0.008	6.14	−1.40	13.69	0.112
Smoking mother (ref 'No')		270	1.06	0.99	1.14	0.081	1.00	0.93	1.08	0.939	1.03	0.96	1.09	0.416	1.05	0.95	1.15	0.355	4.18	−2.95	11.30	0.251
Smoking mother (ref 'Yes')		131	1.08	0.99	1.19	0.082	1.04	0.94	1.15	0.939	1.09	1.00	1.19	0.052	1.09	0.95	1.26	0.213	2.33	−7.22	11.88	0.633
Big oily fish intake (ref < median)		203	1.13	1.01	1.25	0.027	1.00	0.91	1.08	0.417	1.05	0.99	1.13	0.112	1.05	0.99	1.13	0.103	2.73	−4.68	10.15	0.470
Big oily fish intake (ref > median)		200	1.13	1.01	1.25	0.028	1.00	0.91	1.08	0.917	1.08	0.98	1.19	0.144	1.04	0.89	1.21	0.643	8.42	−2.45	19.29	0.130
SNP	Gene																					
rs662 (ref CC)	*PON1*	107	1.11	0.98	1.25	0.099	1.09	0.97	1.25	0.153	1.11	0.99	1.23	0.064	1.15	0.97	1.36	0.100	−0.37	−11.75	11.00	0.949
rs662 (ref TC)		90	1.10	0.99	1.22	0.089	1.02	0.91	1.15	0.667	1.06	0.97	1.17	0.212	1.17	1.01	1.36	0.037	5.83	−6.36	18.02	0.350
rs662 (ref TT)		26	1.19	0.93	1.52	0.156	1.17	0.86	1.46	0.388	1.19	0.96	1.48	0.119	1.05	0.76	1.46	0.755	4.02	−7.45	14.67	0.456
rs705381 (ref TT)	*PON1*	159	1.06	0.97	1.16	0.174	1.02	0.93	1.12	0.685	1.06	0.98	1.15	0.136	1.13	1.00	1.28	0.058	1.74	−7.00	10.49	0.696
rs705381 (ref TC + CC) **		62	1.22	1.05	1.42	0.009	1.16	0.99	1.35	0.063	1.15	1.01	1.31	0.036	1.22	0.98	1.51	0.072	2.05	−14.17	18.27	0.805
rs1695 (ref AA)	*GSTP1*	106	(1.04)†	0.93	1.15	0.474	1.06	0.95	1.20	0.263	1.07	0.97	1.19	0.162	1.14	0.98	1.32	0.085	0.21	−10.37	10.79	0.969
rs1695 (ref GA + AA) **		117	(1.18)†	1.06	1.32	0.002	1.05	0.93	1.17	0.419	1.11	1.01	1.22	0.035	1.18	1.01	1.38	0.032	3.53	−7.50	14.55	0.532
rs1519480 (ref CC)	*BDNF*	120	1.13	1.01	1.25	0.028	(1.13)†	1.01	1.26	0.036	1.13	1.03	1.25	0.010	(1.30)‡	1.12	1.51	0.001	4.19	−5.82	14.21	0.413
rs1519480 (ref TC + TT) **		103	1.07	0.96	1.20	0.205	(0.98)†	0.87	1.09	0.696	1.03	0.93	1.14	0.517	(1.00)‡	0.86	1.17	0.981	−1.32	−13.11	10.48	0.827
rs7934165 (ref AA)	*BDNF*	59	1.05	0.92	1.20	0.444	(1.25)†	1.05	1.48	0.011	1.05	0.93	1.17	0.452	(0.96)‡	0.81	1.15	0.657	−2.99	−16.04	10.05	0.653
rs7934165 (ref AG)		112	1.12	0.99	1.26	0.065	(0.99)†	0.88	1.13	0.933	1.07	0.97	1.20	0.183	(1.10)‡	0.94	1.30	0.227	4.17	−7.80	16.13	0.496
rs7934165 (ref GG)		52	1.15	0.98	1.35	0.084	(1.02)†	0.89	1.16	0.819	1.17	1.01	1.35	0.036	(1.64)‡	1.30	2.05	0.000	4.79	−10.73	20.31	0.546
rs6265 (ref TT)	*BDNF*	136	1.08	0.98	1.20	0.116	1.04	0.93	1.15	0.507	1.06	0.97	1.16	0.202	1.23	1.06	1.42	0.004	3.73	−6.48	13.95	0.474
rs6265 (ref TC + CC) **		86	1.13	1.00	1.27	0.042	1.08	0.96	1.22	0.206	1.12	1.01	1.25	0.031	1.06	0.90	1.25	0.492	−0.58	−12.34	11.18	0.923
rs12273363 (ref CC)	*BDNF*	150	1.08	0.99	1.19	0.095	1.07	0.97	1.17	0.181	1.08	1.00	1.17	0.065	1.17	1.03	1.34	0.013	4.30	−4.90	13.49	0.361
rs12273363 (ref TC + TT) **		73	1.14	1.00	1.31	0.048	1.02	0.89	1.17	0.772	1.09	0.97	1.23	0.149	1.10	0.90	1.32	0.378	−2.66	−16.33	11.01	0.703
rs7103411 (ref CC)	*BDNF*	128	1.09	0.99	1.22	0.084	1.06	0.95	1.19	0.288	1.08	0.99	1.19	0.098	(1.31)‡	1.13	1.51	0.001	6.21	−4.48	16.89	0.257
rs7103411 (ref TC + TT) **		95	1.11	0.99	1.25	0.064	1.05	0.93	1.19	0.411	1.09	0.99	1.21	0.087	(1.01)‡	0.86	1.17	0.948	−2.81	−13.84	8.22	0.618
rs5110 (ref TT)	*APOA4*	186	1.08	1.00	1.17	0.045	1.05	0.97	1.15	0.229	1.07	1.00	1.16	0.051	1.12	0.99	1.25	0.073	0.75	−7.82	9.33	0.864
rs5110 (ref GT + GG) **		37	1.01	0.84	1.21	0.920	0.96	0.79	1.15	0.637	1.02	0.86	1.20	0.836	1.20	0.92	1.55	0.175	10.43	−7.57	28.43	0.258
rs7412 (ref CC)	*APOE*	192	1.12	1.03	1.21	0.010	1.07	0.98	1.16	0.126	1.09	1.02	1.19	0.011	(1.19)†	1.06	1.34	0.003	2.67	−5.41	10.75	0.518
rs7412 (ref CT + TT) **		31	1.11	1.03	1.21	0.010	1.07	0.98	1.16	0.126	1.10	1.02	1.19	0.011	(0.88)†	0.59	1.16	0.294	−1.58	−28.67	25.51	0.909

Children's hair THg concentrations were log2 transformed.
ANT: Attention Network Test; CBCL: Child Behaviour Checklist; CI: confidence interval; CSRS: Conners Parental Rating Scale; HRT-SE: hit reaction time standard error; IRR: Incidence Rate Ratio; SNP: Single Nucleotide Polymorphisms.
All models adjusted for children's sex and age at evaluation.
Internalizing problems scale model additionally adjusted for maternal country of birth, educational level, parity, prenatal BMI and smoking habit at 9 years, paternal working status at 9 years and child's BMI at 9 years.
Externalizing problems scale model additionally adjusted for maternal prenatal BMI and smoking habit at 9 years, paternal education level and working status at 9 years and child's BMI at 9 years.
Total behavioural problems model additionally adjusted for maternal country of birth, educational level, parity, prenatal BMI and smoking habit at 9 years, paternal educational level and working status at 9 years, and child's BMI at 9 years.
Conners Parental Rating Scale model additionally adjusted for maternal country of birth, educational level, parity, prenatal BMI and smoking habit at 9 years, paternal educational level and season of sampling.
Attention Network Test model additionally adjusted for maternal smoking during pregnancy and mother's intelligence measured by the WAIS-III test at 5 years.
()‡ interaction term p-value < 0.05.
()† interaction term p-value < 0.1.
* β values from multivariable linear models.
** Homozygots genotype with frequency <10% were pooled with the heterozygotes.

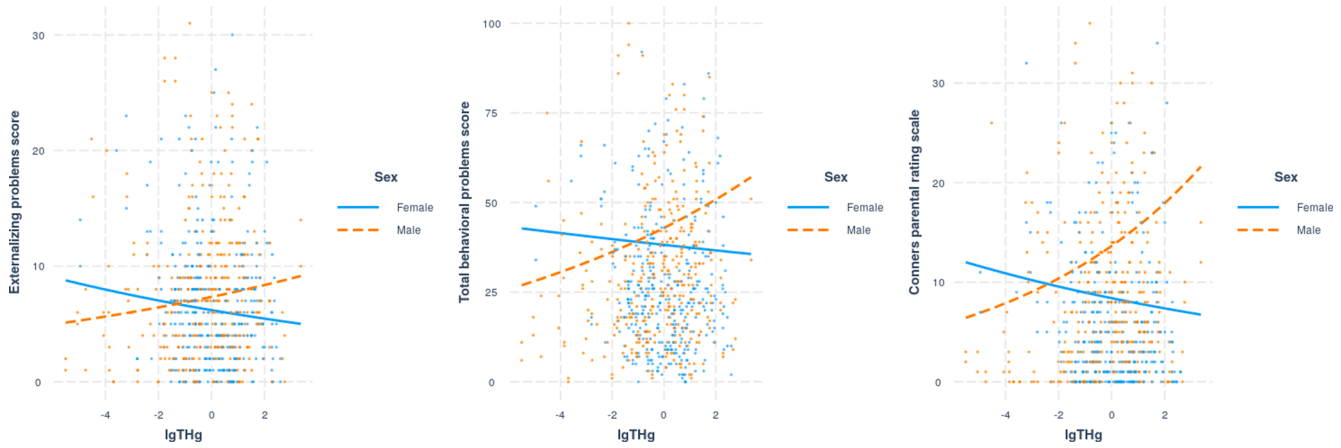

Fig. 1. Significant interactions between hair THg concentrations and children's scores for the CBCL externalizing and total problems scales and the CPRS-R:S ADHD index according to sex, INMA-Valencia cohort, Spain (2014–2016).

Fig. 2. Association between hair THg concentrations and children's scores for the CBCL internalizing problems scale according to GSTP1 rs1695 SNP, the CBCL externalizing problems scale according to BDNF rs1519480 and rs7934165 SNPs, INMA-Valencia cohort, Spain (2014–2016).

[1.30, 2.05]) obtained worse scores with increasing THg. The interaction between THg and the *APOE* rs7412 was also close to significance ($p < 0.1$), the children with the CC genotype being the ones who obtained worse scores with increasing THg (1.19 [1.06, 1.34]).

3.3 ANT model

The association between THg and the HRT-SE of the ANT was positive but not statistically significant (Table 2). This association remained similar when big oily fish consumption was included in the model but it became positive and statistically significant when the cord blood THg concentrations were included (12.19 [3.65, 20.73]). Neither was any statistically significant effect modification observed (Table 3). In the sensitivity analysis (Supplemental Table S4), when the low birth weight children were removed from the model the main effect of THg on the ANT scores became positive and marginally statistically significant. The coefficients for cord blood THg concentrations were not statistically significant (Supplemental Table S3).

4 Discussion

In this Spanish birth cohort study, we assessed the relationship between hair THg concentrations in 9-year-olds and behavioural function evaluated at 9 and 11 years of age. Children with increased THg concentrations obtained worse scores on the CBCL internalizing and total problems scales. Factors such as sex and genetics seem to play a role in this relationship. Overall, boys obtained worse scores than girls on the CBCL externalizing and total problems scales and in the ADHD index of the CPRS-R:S with increasing THg concentrations. Regarding genetics, statistically significant interactions were observed for polymorphisms in the *GSTP1*, *BDNF* and *APOE* genes. Fish intake adjustment did not affect the observed associations.

Previous studies about the influence of Hg exposure during early development on children's behaviour function are scarce and their results are inconclusive. Therefore, results derived from the Faroe birth cohort study, conducted on a population with high Hg concentrations, evaluated the association between cord blood Hg (geometric mean [GM = 22.5 μg/L), hair mercury at 7 (GM = 2.99 μg/g) and at 14 years old (GM = 0.96 μg/g) with children's behaviour at 7 and 14 years old (Debes et al., 2006). Prenatal Hg concentrations were adversely associated with attention at 7 years old, although the results for postnatal exposure were not statistically significant. However, results from the Seychelles cohort, also conducted on a population with high Hg concentrations, showed that both pre (maternal hair, mean (standard deviation [SD]) = 6.8 [4.5] ppm) and postnatal hair Hg concentrations (66 months, mean[SD] = 6.5 [3.3] ppm and 19 years, mean[SD] = 10.29 [6.06] ppm) did not associate with different domains of behaviour assessed at 66 months (Davidson et al., 1998) and 19 years of age (Wijngaarden et al., 2013). In another cohort conducted on children highly exposed to Hg, specifically Inuit children from Canada, neither cord blood Hg (mean (SD) = 22.2 (18.4) μg/L) nor blood Hg at 5 years (mean(SD) = 9.6 (8.9) μg/L) were associated with different aspects of behaviour assessed at 5 years old

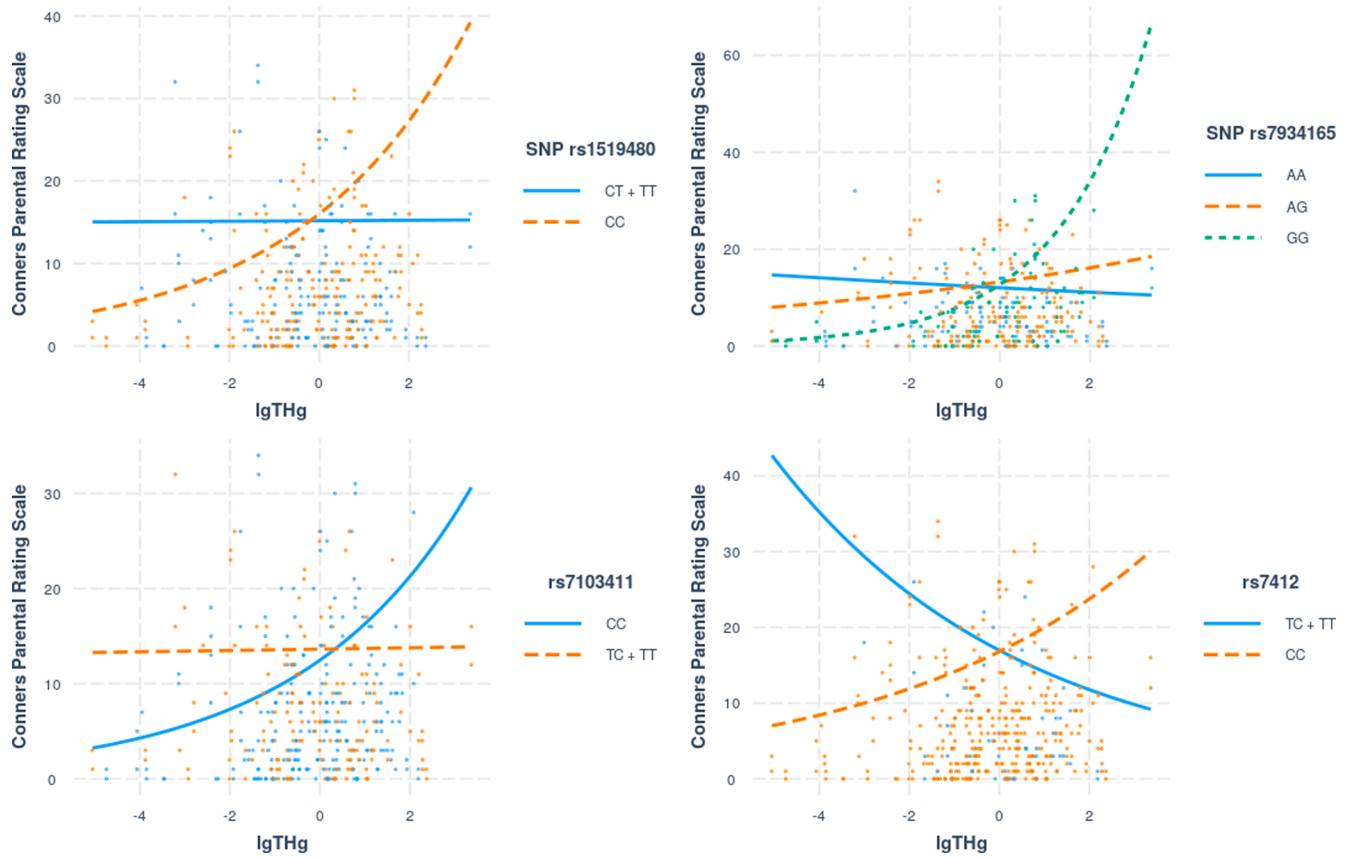

Fig. 3. Association between hair THg concentrations and the CPRS-R:S ADHD index according to the SNPs BDNF rs1519480, rs7934165, rs7103411 SNPs and the APOE rs7412 SNP, INMA-Valencia cohort, Spain (2014–2016).

(Plusquellec et al., 2010). Subsequently, the association between pre and postnatal exposure to Hg and children's behaviour at 11 years old was also evaluated in the same cohort (Boucher et al., 2012). Cord blood Hg concentrations were adversely associated with attention problems and with ADHD inattentive and hyperactive-impulsive types (association that is statistically significant for Hg concentrations above 11 μg/L). No statically significant associations were observed for blood Hg concentrations measured at 11 years old (mean [SD] = 4.6 (3.0) μg/L).

Other studies conducted on populations with lower exposure to Hg examined the association between early Hg exposure and children's behaviour but the results were also heterogeneous. Thus, maternal blood Hg concentrations measured during the first trimester of pregnancy (median = 0.68 μg/L) were associated with increasing anxiety (Patel et al., 2019), and maternal peripartum hair Hg (median = 0.45 μg/g) was also associated with inattention and impulsivity/hyperactivity (Sagiv et al., 2012), both studies being conducted on 8-year-old children from USA. Behavioural outcomes were assessed in children between 4 and 17 years old who were participants in the ALSPAC cohort (UK) (Golding et al., 2016). Prenatal exposure to Hg was measured in maternal blood samples collected during the first half of pregnancy (median = 1.86 μg/L). Overall, no significant association was found between prenatal exposure to Hg and children's behaviour, although some positive association (the greater the level of Hg, the fewer the problems the child had with his/her peers) was observed. The results were similar for the children of mothers who ate fish during pregnancy and those who did not eat it. Another study conducted in USA, the HOME study, also found a marginally positive association between behaviour (better attention and the need for less special handling) and increasing maternal (GM = 0.64 μg/L) and cord (GM = 0.72 μg/L) blood Hg. Additionally, higher prenatal Hg exposure was associated with other developmental outcomes, such as more frequent asymmetric reflexes in girls; however, these associations were attenuated when the authors adjusted the models for fish intake (Xu et al., 2016). In Bangladesh, Hg at 10 years old (median hair-Hg was 0.674 μg/g) did not associate with cognitive development, but children in the highest tertile of hair-Hg had a lower prevalence of hyperactivity and peer relationship problems, compared to children in the lowest tertile (Gustin et al., 2017).

In summary, these results derived from previous studies suggest that the prenatal period could be the most vulnerable in relation to Hg exposure and its association with neurobehavioural effects during childhood. However, the literature regarding postnatal studies is too scarce to draw any definite conclusions and the results were heterogeneous. One cause of this heterogeneity could be related to the genetic background. Although the number of studies is limited, polymorphisms in some genes have been identified as possible effect modifiers of the association between early exposure to Hg and child neuropsychological development (Andreoli and Sprovieri, 2017; Llop et al., 2015). Thus, Woods et al. (2014) observed that children who were carriers of the variant allele for the rs6265 SNP in the *BDNF* gene obtained impaired scores in the memory and learning domains at 2 and 7 years old with increasing urinary mercury (Woods et al., 2014). Additionally, the SNP rs2049046, also in *BDNF*, was also found to modify the association between Hg exposure and the performance scores in 8-year-old children who were AA carriers (Julvez et al., 2013). BDNF, encoded by the *BDNF* gene, is a neurotrophin closely linked to synaptic plasticity throughout the central nervous system. Reports have demonstrated that different BDNF genotypes moderate the impact of environmental conditions on child neurodevelopment (Miguel et al., 2019). In our study, we found statistically significant interactions for other *BDNF* SNPs (rs1519480, rs7934165 and rs7103411). Children with the CC genotype for rs1519480, GG genotype for rs7934165 and CC genotype for rs7103411 obtained more scores in the ADHD index of the CPRS-R:S with increasing

Hg. Although these SNPs have not been extensively examined yet in relation to neurodevelopment, the CC genotype for rs7103411 has been related to impaired memory performance in a 70-year-old population (Laing et al., 2012), and the CC genotype for rs1519480 has been associated with bipolar disorder (Liu et al., 2008; Sears et al., 2011).

We found other statistically significant interactions between hair Hg concentrations and other SNPs, specifically for rs1695 in the *GSTP1* gene and rs7412 in the *APOE* gene. Children who were carriers of the G allele for rs1695 obtained a higher incidence rate ratio in the CBCL internalizing problems with increasing Hg than the AA. This same genotype was related to impaired mental development associated with prenatal Hg exposure among children participating in the Seychelles cohort (Wahlberg et al., 2018). This gene codes for the enzyme glutathione S–transferase pi 1 isoform, which has been involved in the elimination of Hg through its conjugation with glutathione (GSH) (Custodio et al., 2004). In our study, however, this polymorphism was not associated with hair Hg concentrations, suggesting that the association of this SNP with neurobehavioural development may be mediated by mechanisms other than Hg kinetics. Ng et al. (2014) investigated the role of the *APOE* variants in the relationship between Hg exposure and behaviour in 2-year-old children from Taiwan. Children who were e4 carriers with an elevated cord blood Hg concentration (>12 μg/L) obtained significantly higher scores in the syndrome categories of general internalizing, emotionally reactive and anxiety/depression as well as CBCL total scores (Ng et al., 2014), although the authors did not report the interaction p-values. Conversely, the *APOE* e4 variant was associated with a neurodevelopmental advantage in children exposed to lead, suggesting possible protection against lead exposure among e4 carriers (Wright et al., 2003). Additionally, a cross-sectional study conducted on elderly people showed that a moderate weekly fish consumption correlated with less Alzheimer disease neuropathology among *APOE* e4 allele carriers (Morris et al., 2016). This interesting finding could explain, in part, the association observed in our study; children with the CC genotype (e4) in the rs7412 SNP had reduced scores on the ADHD index of the CPRS-R:S with increasing Hg exposure. This association with Hg exposure could really be showing the protective effect of fish intake. APOE is a crucial factor involved in cholesterol metabolism, neurite growth and neuron repair in the central nervous system (Weisgraber et al., 1994). The metabolism of cholesterol is believed to play a major role in neurite outgrowth and synaptogenesis (Dietschy, 2009). However, whether carriers of the APOE e4 variant are more susceptible to neurological disorders or if this variant confers an advantage against unfavourable exposures remains unclear and deserves further research.

Children's sex was also found to be a modifier of the association between Hg exposure and children's behaviour; specifically, boys obtained higher scores (impaired behaviour) than girls on the CBCL externalizing and total problems scales, and on the ADHD index of the CPRS-R:S with increasing Hg. These differences do not seem to be related to Hg exposure, but to susceptibility, since we observed statistically higher Hg concentrations in girls than in boys. Sex differences in the susceptibility to mercury toxicity have been reported previously but without any consistent pattern (Llop et al., 2013). In relation to the behaviour domain, previous studies on children showed heterogeneous results depending on the test used for the behaviour assessment; thus, no influence of sex on Hg toxicity was observed in the Seychelles cohort using the CBCL and the Profile of Mood States (assesses moods and feelings) (Davidson et al., 1998; Wijngaarden et al., 2013). Contrary to our results, Patel et al. (2019) observed a statistically significant interaction between maternal Hg concentrations at delivery and increased anxiety symptoms among girls, this association not being significant for boys (Patel et al., 2019). Sagiv et al. (2012) found sex-related differences for the association between Hg and children's behaviour but with different patterns as a function of the test applied. They did not observe any sex differences in the association between Hg and the results for the Conners Rating Scale (evaluates problem behaviours). Nevertheless, boys obtained worse scores on the Wechsler Intelligence Scale for Children (evaluates processing speed and distractibility) than girls, and a protective effect was observed for girls with hair Hg concentrations < 1 μg/g in the reaction time of the NES2 Continuous Performance Test (evaluates response time and variability) (Sagiv et al., 2012). Finally, in the HOME study, the association between prenatal Hg and some of the scales of the NICU Network Neurobehavioural Scale (asymmetric reflexes, attention and less need for handling) was positive (protective) for girls (Xu et al., 2016). Sex differences in the susceptibility to Hg could be related to the development of particular areas of the brain under the influence of certain hormones, such as sex and thyroid hormones. Generally, sex hormones have protective effects against oxidative stress insults in the central nervous system via the estrogenic receptor (Barron and Pike, 2012; Zárate et al., 2017). However, recent findings indicate that this protecting role could disappear in an oxidative stress environment where sex hormones could have negative effects on cell viability by exacerbating oxidative stress-induced cell loss (Duong et al., 2020). Disruption of thyroid homeostasis and sex differential regulation of TH-dependent gene expression have been reported as other potential mechanisms of sex-specific toxicity of perinatal exposure to metals in the developing brain (Khan et al., 2012). Further epidemiological studies should explore these possible action mechanisms of the association between early exposure to Hg and children's neurodevelopment in greater depth in order to elucidate the influence of sex on Hg neurotoxicity.

Another factor that could be the origin of the heterogeneity in the results on the association between Hg and children's behaviour may be the synergistic or antagonistic effect of the co-exposure to other neurotoxicants also present in fish, such as PCBs. Previous studies taking into the account co-exposure to Hg and PCBs in relationship to children's behaviour observed that PCB concentrations were negatively associated with externalizing and internalizing problems assessed with the CBCL test (Kim et al., 2018; Tatsuta et al., 2012).

We included the variable cord blood THg concentrations in the models since it has been associated with children's neuropsychological development in previous studies. The results obtained were virtually the same except for the HRT-SE (a measure of inattentiveness) of the ANT, where the children obtained higher scores (better performance) with increasing postnatal Hg concentrations. This association is difficult to interpret and, to date, studies relating children's attention with mercury exposure are scarce. However, a plausible explanation could be linked to the beneficial effect of fish consumption. In fact, a positive association between prenatal mercury concentrations and children's attention was observed in some other previous studies (Xu et al., 2016; Davidson et al., 2018). Additionally, in previous INMA studies we reported a positive association between cord blood THg concentrations and children's cognitive development assessed at 5 years of age (Llop et al., 2016). This association became negative among children whose mothers consumed fewer than 3 weekly servings of fish during pregnancy. Moreover, a recent study with the same population showed an association between high fish consumption during pregnancy and improvements in some attention outcomes in 8-year-olds by using the same ANT test (Julvez et al., 2020). We have also calculated the coefficients for the association between cord blood Hg concentrations and the scores for the behavioural tests but we did not observe any statistically significant association. All these results suggest a possible role of the beneficial nutrients found in fish in the complex relationship between mercury concentrations and children's behavioural and cognitive outcomes that requires further reflection.

One advantage of this study is its prospective design, which made it possible to obtain detailed information concerning maternal and child characteristics that may affect neuropsychological development. This prospective follow-up will also enable us to study the possible adverse effects of Hg exposure on neuropsychological development in the future. In addition, we have used diverse tests that assess different aspects of children's behaviour at two ages, at 9 and 11 years old, which has allowed us to apply a longitudinal statistical analysis. In addition, the study of the influence of genetics and other factors on the effect of Hg

concentrations on children's behaviour could contribute to the identification of high risk subpopulations and improve public interventions aimed at preventing the neurobehavioral impacts of mercury exposure. As a weakness, 18.6% of the children who reached the 11-year-old assessment did not participate in the study, but we did not observe any noteworthy differences between the included and excluded study population (Supplemental Table S5). In addition, around 60% of the children who were included in the cohort at birth did not meet the 11-year-old assessment. The loss to follow-up population is an inherent characteristic in most longitudinal studies that is usually related to the socioeconomic status (loss to follow-up tends to be more pronounced among the less advantaged social classes and population with lower levels of education). This situation could bias the observed associations; however, it has been observed that even when more than half of the cohort was lost to follow-up, qualitative conclusions about the direction and approximate magnitude of inequalities did not change dramatically (Howe et al., 2013). Additionally, the sensitivity analyses performed confirm the stability of our results. Other study limitations were: (1) the lack of assessment of other neurotoxic chemicals present in fish such as other heavy metals or PCBs; (2) both the CBCL and the CPRS–R are parent reports to measure children's behaviour, which may have resulted in additional measurement bias; (3) the CPRS-R:S test is a screening tool, which implies that the diagnosis of ADHD needs further evaluation; (4) effect modifications related to other genetic factors were not examined; and (5) n-3 PUFA concentrations beneficial to child neurodevelopment were not measured, but the intake of all groups of seafood was employed instead, showing no significant changes.

In conclusion, we observed an association between hair THg concentrations and children's behaviour assessed at 9 and 11 years old. Specifically, children with increased hair THg concentrations obtained worse scores on the CBCL internalizing and total problems scales. Factors such as sex and genetics seem to play a role in this relationship. Overall, boys obtained worse scores than girls in the CBCL externalizing and total problems and in the ADHD index of the CPRS-R:S with increasing THg concentrations. Regarding genetics, statistically significant interactions were observed for polymorphisms in the *GSTP1*, *BDNF* and *APOE* genes. At present, there is insufficient evidence of the possible neurotoxic effects of postnatal Hg exposure at moderate doses; therefore, more research on this topic is needed. As the neurotoxicity of Hg could appear at older ages it is advisable to continue evaluating children neuropsychologically throughout childhood and adolescence in our cohort.

CRediT authorship contribution statement

Manuel Lozano: Formal analysis, Methodology, Writing - original draft. **Mario Murcia:** Data curation, Validation, Visualization. **Raquel Soler-Blasco:** Validation, Writing - review & editing. **Llúcia González:** Investigation. **Gorka Iriarte:** Writing - review & editing. **Marisa Rebagliato:** Writing - review & editing. **Maria-Jose Lopez-Espinosa:** Writing - review & editing. **Ana Esplugues:** Writing - review & editing. **Ferran Ballester:** Supervision. **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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.106173.

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