Semi-parametric longitudinal modeling to assess the association between mercury exposure and behaviour from 9 to 15 years of age in the INMA-Valencia cohort

Gabriel Riutort-Mayol†, Alejandro Oncina-Cánovas†, … , Antonio J. Signes-Pastor*

(†) Co-first authors

(*) Co-corresponding authors:

Abstract

Mercury (Hg), a toxic compound primarily accumulated through industrial processes and consumption of contaminated fish, poses risks to the central nervous system and development, particularly its methylmercury (MeHg) form. While prenatal exposure to MeHg has been linked to behavioural problems in childhood, its effects in adolescents remain controversial. We investigated this association among 870 children and adolescents aged 9, 11, and 15 from the "*INfancia y Medio Ambiente"* (INMA) cohort in Valencia. Hair samples were analysed for total Hg, and behavioural assessments were conducted using the validated Spanish version of the Child Behaviour Checklist (CBCL). We drew a causal graphical model including covariates such as breastfeeding, social class, children's sex, smoking during pregnancy, and total fish consumption. To analyse the association between Hg exposure and behaviour at different age visits, we employed a non-parametric longitudinal regression model based on additive Gaussian process and linear random effects. An increase in behavioural problems associated with Hg exposure was found at 9 years of age, with an 8.04% in the internalizing scale and a 7.41% in the total problems scale for each 1 μg/g of Hg exposure, which disappeared by 11 and 15 years. With regard to sex-specific differences, significant changes were observed, particularly at 11 and 15 years, with notable decreases in behavioural problems for boys, 11.77% on the internalizing scale and 17.10% on the externalizing scale, respectively, while girls exhibited no significant associations. Thus, the impact of Hg exposure on behavioural outcomes may be more pronounced in boys, potentially due to differences in mercury metabolism and hormonal protection. This study elucidated the nuanced dynamics of mercury exposure on behavioural outcomes in children and adolescents, revealing age-dependent effects and sex-specific

differences, which underscores the importance of tailored interventions and policies for mitigating Hg-related risks.

1. Introduction

Mercury (Hg), recognized as an environmental pollutant, poses a notable risk to human health owing to its capacity for bioaccumulation within the human body over prolonged periods (Bernhoft, 2012). Its primary dissemination into the environment stems from industrial activities, coal combustion, and natural occurrences, wherein Hg undergoes transformation into methylmercury (MeHg) within aquatic ecosystems (De Almeida Rodrigues et al., 2019). Consequently, the most notable source of human exposure arises from the consumption of contaminated fish, particularly those occupying upper trophic levels within aquatic food webs (Castaño et al., 2015; Sheehan et al., 2014).

MeHg is highly toxic due to its ability to easily cross biological membranes, including the blood-brain barrier and the placenta (Hong et al., 2012). The central nervous system is particularly vulnerable to MeHg toxicity as it accumulates in the brain, leading to neurotoxic effects (Branco et al., 2021). Early exposure to Hg can lead to developmental delays, cognitive impairments, and motor dysfunction (Al-Saleh et al., 2020). Historical incidents, such as Minamata in the 1950s and Iraq in the early 1970s, highlight the serious consequences of exposure to elevated levels of Hg. In Minamata, foetuses exposed to Hg showed central nervous alterations and physical deformities, with a hair Hg concentration 280 to 760 times higher than normal (Ekino et al., 2007). In Iraq, acute exposure caused dysesthesia, paralysis, and even death (Skerfving and Copplestone, 1976).

The risk factors associated with behavioural disorders have historically been linked to social and family environments or certain health pathologies. However, recent research has examined the impact of environmental contaminants, such as Hg, known for its neurotoxic effects. Studies conducted in New Zealand (Karatela et al., 2017), the Faroe Islands (Debes et al., 2006), and Korea (Ryu et al., 2017) reveal that behaviour disorders in children can occur even at lower concentrations of Hg, highlighting the importance of continued research on different exposure scenarios and Hg concentrations during vulnerable developmental stages. Fish, the primary source of Hg exposure in many populations (including ours (López-González et al., 2023)), is also a crucial source of essential nutrients such as selenium and omega-3 fatty acids, which are vital during pregnancy and childhood (Starling et al., 2015). Omega-3

supplementation has been shown in randomized controlled trials to improve various behavioural problems, including hyperactivity, impulsivity, aggression and Attention-Deficit/Hyperactivity Disorder (ADHD), possibly due to its role in modulating neurotransmitter function and reducing brain inflammation (Bos et al., 2015; Derbyshire, 2018; Raine et al., 2015). Consequently, fish consumption is the main confounder in the relationship between Hg exposure and behavioural disorders, as it influences both Hg and behaviour. Therefore, fish intake in childhood presents a dual challenge: it must be sufficient to ensure adequate omega-3 fatty acid levels while being carefully monitored to avoid excessive Hg exposure, which can negatively impact neurodevelopment and behaviour.

There is a lack of reliable studies in Spain that longitudinally evaluate the relationship between Hg exposure and behaviour in children and adolescents, or even cross-sectional studies in early adolescence. Recently, Lozano et al. (Lozano et al., 2021) and Sarzo et al. (Sarzo et al., 2024) conducted longitudinal studies within the same cohort as the present study, examining the relationship between prenatal and postnatal exposure to Hg (using total Hg (tHg) in hair) and behavioural changes (using the Child Behaviour Checklist (CBCL) scale) at ages 9 and 11 years. While they found no association between higher prenatal Hg exposure and children's behavioural functioning from early childhood to pre-adolescence (4 and 11 years) (Sarzo et al., 2024), elevated postnatal Hg exposure (measured by tHg in hair) was linked to poorer scores on internalizing and total scales at 9 years, with differences observed based on sex (Lozano et al., 2021). Further exploration is warranted to ascertain whether these associations persist over several years of follow-up. Unfortunately, the statistical methodology used in those studies may not be entirely appropriate for longitudinal designs, as they employed standard linear mixed-effects models with random effects defined only on individual-specific base level effects and not on their temporal trends. This limitation renders the results obtained as mere approximations.

Generalized linear mixed-effects models specially designed for longitudinal data have become the standard method for longitudinal studies due to their simplicity and interpretability (Gibbons et al., 2010; Stroup, 2016). However, they still suffer from a lack of rigor in the analysis of longitudinal data due to the difficulty in modelling non-linear effects and choosing appropriate covariance structures, among other reasons. Furthermore, linear models are sensitive to multicollinearity causing instability in parameter estimates (Gelman et al., 2020). Alternatively, Gaussian processes (GPs) are flexible probabilistic models for multivariate functions where nonlinearity and covariance structure are implemented naturally through covariance functions (Osborne,

2010; Rasmussen and Williams, 2006) which consider, implicitly, multiple interactions between covariates and regularization by collinearity and model complexity. GPs have become a popular non-parametric method for modelling, especially for time series data and longitudinal data, but also for spatial, spatio-temporal and multi-dimensional data (Banerjee et al., 2014; Diggle, 2013; Riutort-Mayol et al., 2020). However, GPs are sensitive to features with low signal-to-noise ratio, where linear effects might be preferred.

In our study, we hypothesized that higher levels of exposure to Hg have an adverse effect on child behaviour. Thus, our aim was to assess associations between Hg exposure using tHg on hair, as biomarker, and behaviour problems, measured by the Child Behaviour Checklist (CBCL), in a longitudinal study during childhood and adolescence, including 9-, 11- and 15-years-old participants from the *INfancia y Medio Ambiente* (INMA) birth cohort of Valencia using an innovative statistical approach based on the additive combination of latent Gaussian process functions and linear random effects.

2. Material and methods

2.1. Study population

We used data on participants in the INMA project from Valencia sub-cohort, in Spain. The main objective of the INMA project was to assess the impact of various environmental pollutants and exposures during pregnancy and early life, as well as their consequences on child growth and development (Guxens et al., 2012). Pregnant women were recruited during their initial antenatal visit from 2003 to 2005. Inclusion criteria encompassed: a) being at least 16-years old, b) 10–13 weeks into gestation, c) having a singleton pregnancy, d) intending to undergo follow-up and delivery at the designated centre (La Fe Hospital), e) no communication impediment, and f) no assisted conception. After excluding those who withdrew, were lost to follow-up, or experienced induced or spontaneous abortions or foetal deaths, the study followed 787 women until delivery (2004–2006) in Valencia. Their newborns were enrolled at birth and tracked until 9-years (n = 472), 11-years (n = 385) and 15-years (n = 279).

The final study population included in our analysis consisted of 368 9-years-old children (47% of total births), 297 11-years-old children (38% of total births) and 205 15-years-old children (26% of total births), having available data for behavioural

assessment at each age, as well as hair tHg concentrations and information available for all variables of interest throughout the study follow-up. No significant differences were found in the distribution of characteristics between the initial population in the INMA project and the actual sample population in this study (see **Table S1** in Supplemental). Supplemental **Figure S1** shows a flowchart that provides an overview.

Informed consent for the prenatal period was obtained from the mother, and additional consent was secured from one of the parents or a legal representative at each phase of the postnatal period. The study protocol received approval from the Public Health Research Centre in Valencia (CSISP) and La Fe Hospital ethics committees.

2.2. Mercury exposure

In our study, hair samples were used as a biomarker of exposure to tHg. Hair samples have been widely used in the literature because is a non-invasive method. Hair also accumulates Hg over longer periods than other biomarkers, such as blood, so it is a better biomarker of chronic exposure. Finally, while tHg in hair includes both organic and inorganic forms, it is greatly correlated with MeHg exposure (Nuttall, 2006), the most toxic form. The method for assessing tHg concentrations proceeded as outlined below.

Hair samples were collected from the occipital scalp at the ages of 9-, 11-, and 15-years. Each sample, obtained as close to the root as possible, met the criteria of a minimum length of 2 cm or a weight of at least 100 mg. Subsequently, these samples were carefully stored in plastic zip bags at room temperature until analysis. The assessment of tHg was conducted at the Public Health Laboratory of Alava in the Basque Country, Spain, utilizing a direct Hg analyser.

To prepare the samples for analysis, they were rinsed with 10 mL of 1% Triton X-100 (Panreac, Barcelona), then weighed in a weigh boat. The Hg analyses were performed directly using AMA 254 and DMA-80 equipment through catalytic combustion, gold amalgamation, thermal desorption, and atomic absorption spectrometry. Replicate analyses were carried out for each sample to ensure accuracy. The method's limit of quantification (LOQ) was determined to be 0.01 µg/g, and no measurements of Hg in hair fell below this threshold at any of the three time points.

The quality control of the hair sample batches involved using IAEA-086 (International Atomic Energy Agency, Austria) and NCS ZC 81002b (NCS Institute, Beijing, China) reference materials (Lozano et al., 2021). Furthermore, the method's accuracy was

externally validated by participating in various inter-laboratory exercises organized by the Centre de toxicologie du Québec (Quebec Multielement External Quality Assessment Scheme, QMEQAS program). In all instances, satisfactory results were achieved, as evidenced by z-scores for Rounds 2013-3 and 2015-3, which were +0.42 and +0.13, respectively.

Based on internationally recommended levels of Hg exposure and assuming a daily average intake of MeHg at 0.1 µg/kg body weight, it is estimated that a MeHg concentration of around 1 µg/g in hair corresponds to this intake (UNEP, WHO, 2008). Using this information, the study calculated the percentages of participants exceeding thresholds of 1.0 (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2012), 1.9 (Joint FAO/WHO Expert Committee on Food Additives, 2007), and 2.5 µg/g (*Toxicological Effects of Methylmercury*, 2000; U.S. Environmental Protection Agency (EPA), 2001) in hair for each year of the investigation.

2.3. Behavioural assessment

The parental version of the CBCL, a widely recognized tool for assessing behavioural changes developed by Achenbach and Ruffle (Achenbach and Ruffle, 2000), has been extensively tested across diverse cultures and provide information about ten specific areas. In our study, we used this tool to assess emotional and behavioural issues in children aged 9, 11, and 15 years. Specifically, we used a version validated for the Spanish population (Rubio-Stipec et al., 1990). It relies on reports from parents or caregivers, offering a comprehensive evaluation of children's behaviour and adaptive functioning within the age range of 6 to 18 years.

This widely recognized questionnaire comprises 112 items. Parents or caregivers assess the frequency or intensity of specific behaviours exhibited by the child, using a three-point response scale (0 = not at all true, 1 = somewhat or sometimes true, and 2 = very or often true) to evaluate issues that have occurred in the preceding two months. The CBCL includes various subscales that aid in identifying potential behavioural problems across three domains: (1) emotional issues (internalizing scales: anxiety, depression, somatic complaints); (2) conduct problems (externalizing scales: rule-breaking and aggressive behaviour); and (3) total problems (the sum of internalizing, externalizing, and other scales: social, cognitive, and attention problems). Elevated scores on the CBCL indicate an escalation in conduct issues. This comprehensive evaluation furnishes valuable insights into the behavioural well-being of children within the specified age groups.

2.4. Covariates

Questionnaires about on socio-demographic information during pregnancy/birth, childhood and adolescence (9-, 11- and 15-years old visit) of participants were administered by trained interviewers. The variables used in this study as adjustment variables were: breastfeeding (yes/no), social class (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), children's sex (male/female), smoking during pregnancy (yes/no) and total fish consumption (g/day). We considered also other relevant covariates about children and adolescents' lifestyles and exposures in our analysis: child's body mass index (BMI), passive smoking (Does anyone in the household smoke? yes/no). Other variables about the mother or the pregnancy period were also included: weeks of gestation (weeks), parity (0, 1, >2). It is the mother's intellectual quotient (IQ), the mother's and father's intelligence were measured at the 4–5-year visit with a sub-test of the WAIS-III (Wechsler, 2019), and it is standardised to mean 10 SD 3. The child's BMI was calculated using z-scores according to the WHO child growth standard (World Health Organization, 2006; World Health Organization, 2008). Total fish consumption was determined by a semi-quantitative food frequency questionnaire previously validated in the same study population at the age of 7 years (Vioque et al., 2019).

2.5. Directed Acyclic Graph (DAG)

In this study, we employed a Directed Acyclic Graph (DAG) (Textor et al., 2017) to identify and visualize the causal relationships between Hg exposure and behavioral outcomes, measured by the CBCL scales in children and adolescents (**Figure 1**). The DAG was constructed following a comprehensive review of the literature to ensure the validity of the hypothesized causal relationships. We initially included variables considered confounders due to their simultaneous association with both Hg exposure and CBCL scales, including children's sex, social class, total fish consumption, and smoking during pregnancy. Variables included as adjustment factors necessary to block indirect paths were feeding and weeks of gestation. Additionally, passive smoking, parity, and children's BMI were included due to their association with either the exposure or the outcome. The DAG enabled us to identify the minimum set of adjustment variables required to estimate the effect of Hg exposure on CBCL scales

without bias. This minimum adjustment set included feeding as an adjustment variable, while children's sex, social class, total fish consumption, and smoking during pregnancy were identified as confounders.

2.6. Statistical analysis

A descriptive analysis was performed to assess the distribution and variability within the data. Measures of spread including the range, mean, standard deviation (SD), median and interquartile range (IQR) were used to assess continuous variables. Frequency and proportion distributions were used to explore categorical variables and discrete numerical variables. For the later, the mean and variance were also reported. Histograms and boxplots were used to visualise the data and identify potential outliers.

The model formulated to assess the relation between exposure to Hg and CBCL scores consisted in an additive model composed of multivariate GP effects and linear random effects for longitudinal data. The CBCL scales are discrete score variables and Hg exposure concentrations are continuous variables, both longitudinally measured at 9-, 11- and 15-year visit. Some of the confounder and adjustment variables are time-varying covariates followed up at 9-, 11- and 15-year visit, while others were static covariates. The observational model for the outcome variable of CBCL scores (let us denote y as a CBCL score variable) was a negative binomial probability distribution of the mean μ and a parameter ϕ that accounts for the overdispersion of the observations in relation to their mean (μ),

$$y \sim \text{NegBinomial}(\mu, \phi),$$

were $E(y) = \mu$ is the expectation of y and $Var(y) = \mu + \mu^2/\phi$ is the variance of y.

A longitudinal predictor function was defined for the mean μ of the y scores, which is based on an additive function composed of GP function effects of time and confounder and adjustment covariates (i.e., the time variable and the minimum set of covariates returned by the DAG), and time-varying linear random effects of Hg exposure. The GP component allowed us to accurately model the join effects of time and confounder and adjustment covariates, exploiting the covariance structure of data and considering non-linear effects and high-order interactions. However, single feature effects are difficult to interpret using a multivariate GP as it is a multidimensional function and, furthermore, a GP is more sensitive to features with subtle effects with a low signal to noise ratio, as it is the case of the Hg exposure effects on CBCL, than linear models. For this reason, additive Hg exposure effects were defined as time-varying linear random effects. Thus,

the predictor function was defined over the logarithm of the mean μ of the scores y as follows:

$$log(\mu) = f(t,x) + b_t Hg,$$

where b_t are time-varying linear random effects of Hg exposure,

$$b_t \sim \text{Normal}\left(\mu_{b_t}, \sigma^2_{b_t}\right),$$

and $f(t,x)$ is a GP function of time t and confounder and adjustment covariates x,

$$f(t,x) \sim \text{GP}(0, k(t,x,t',x'),$$

with k being a squared exponential covariance function (Rasmussen and Williams, 2006). A more detailed description of the GP function and its covariance function can be briefly found in the **Appendix**.

In negative binomial regression, like Poisson regression, the value of a linear coefficient or random effect associated with a covariate in the predictor function (i.e., a continuous or categorical independent variable) represents a multiplicative increase in the outcome by 1 unit increase in the covariate (Gelman et al., 2020), such that $100 \cdot b_t$ represents the approximate % increase in CBCL score with 1 ug/g Hg.

The model was formulated from a Bayesian perspective and adjusted using sampling methods in the probabilistic programming software Stan (Carpenter et al., 2017; Stan Development Team, 2017). Stan software uses the Hamiltonian Monte Carlo sampling method (Neal, 1997) to estimate the marginal posterior probability distributions of every parameter of interest. Four simulation chains with different initial values have been launched. The convergence of the simulation chains was evaluated by the split-Rhat convergence diagnosis and the effective sample size of the chains (Gelman et al., 2013; Vehtari et al., 2019). In our case and for both models, a split-Rhat value lower than 1.05 has been obtained for all parameter simulation chains indicating good mixing of simulated chains. The convergence of the simulation chains indicates that the samples do come from the posterior distribution, and that the model is correctly specified without confusion or identifiability problems between parameters.

Magnitude and uncertainty of the parameters of interest, the random effects of Hg exposure, are given by their marginal posterior distributions. Significance of random effects can be determined by evaluating whether their accumulated probabilities of being less than or greater than zero are lower than a probability threshold. In this study we set the commonly used probability threshold of 0.05. From now to later we use that if the probability of being less than zero is 0.05 or less, the effect is positive and significant, and if it is 0.95 or greater, the effect is negative and significant.

The pre-processing of the data before sending it to Stan to perform model inference, as well as its post-processing, was carried out using R software for data analysis and processing (R Core Team, 2020).

3. Results

3.1. Population sample characteristics

Sociodemographic characteristics and lifestyle among mothers and their 9-, 11- and 15-years children are described in **Table 1**. In regard to mothers, most of them were employed (9-years= 69.6%; 11-years= 68.4%; 15-years=79.0%), belonged to the highest social class (9-years= 44.3%; 11-years= 37.7%; 15-years=36.6%), had only 1 child (9-years= 58.2%; 11-years= 59.3%; 15-years=57.6%) and did not smoke during pregnancy (9-years= 79.3%; 11-years= 78.1%; 15-years= 81.0%). The median age was about the same in the three periods (30-31-30 years) and the WAIIS score was also similar (9.8-10.5-10.5). Regarding children, the proportion of males and females were roughly similar throughout the period, with the biggest difference at the age of 11-year with 55% of females. The median (IQR) age of participants was of 9.1 (0.3); 10.9 (0.2); and 15.6 (0.6) at the ages of 9-, 11-, and 15- years, respectively. Passive exposure to tobacco smoke was greater at 11- and 15-years visits (50.3-55.9-59.5). When we examined differences in sociodemographic characteristics among mothers included and excluded in our study, they were similar in age, employment, and social class (**Table S1**). Children and adolescents during all three age visits in both groups also showed similarities in age and z-BMI. However, excluded participants throughout the study period were slightly more men (**Table S1**).

3.2. CBCL score scales description.

Histograms of the discrete scores of CBCL, internalizing, externalizing, and total problems scales by age visit are showed in **Figure 2.** The mean and variability of CBCL scale scores by follow-up visits for the study population are reported in **Table 2**.

3.3. Hg effects on CBCL scales

The mean and 95% credible intervals of the effects of Hg exposure on CBCL scales (internalizing, externalizing and total problems) by age visit, and significance of these

effects given by the accumulated probability of posterior distributions of effects to be less than zero, are shown in **Figure 3** and **Table 3**. Posterior distributions of effects with cumulative probabilities of being less than zero less than 0.05 (or even 0.10) can be considered as significant effects. The results are presented as the percent increase in CBCL score scales for each 1 μg/g of Hg. At the 9-year visit, after adjusting for the join effect of confounders, covariates, and age, we observed an 8.04% increase in the internalizing scale score and a 7.41% increase in the total problems scale score for each 1 μg/g of Hg exposure, with probability of being less than zero equal to 0.001 and 0.002, respectively. However, these associations were not shown at the 11- and 15-year visits. A slightly credible association of a 4.32% increase in the externalizing scale score with a probability of being less than zero equal to 0.07 was found at 9-year visit. However, no associations were found for the externalizing scale at the 11- and 15-year visits.

In the sex-stratified analysis of differences on CBCL scales, we observed different patterns among girls and boys during the follow up visits (**Figure 4** and **Table 4**). At the 9-year visit, boys exhibited an 10.92% increase in scores on the internalizing scale and a 9.87% increase in scores on the total problems scale for each 1 μg/g of Hg exposure, with probabilities of being less than zero equal to 0.02 and 0.01, respectively. During the 11-year visit, a 11.77% decrease in scores (an effect coefficient of -11.77%) was observed only for boys on the internalizing scale with a probability of being less than zero equal to 0.98. Finally, during the 15-year visit, a 17.10% decrease in scores (an effect coefficient of -17.10%) was observed only for boys on the externalizing scale. Girls did not show any significant association in the three age visits in any of the three scales.

4. Discussion

In this Spanish study, we observed that hair tHg levels at 9-years were associated with behaviour scales, particularly showing an increase in internalizing and total problems dimensions. However, this association was not sustained at the 11- and 15-year visits. Thus, we only observe adverse effects of exposure during childhood, not in adolescence. Furthermore, our exploration of sex differences revealed that hair tHg exposure is associated with behaviour sub-scales only in boys, with variations depending on the age of the visit.

In our study, an association was observed between tHg exposure at 9 years old and negative deviations in the internalizing and total problems areas of a widely used

behavioural scale, the CBCL. Previous research has explored this association between childhood Hg exposure and behavioural outcomes, yielding diverse findings. In contrast with our results, a study conducted in a well-known American cohort, such as the Health Outcomes and Measures of the Environment (HOME) study, examined this association but using prenatal exposure, showing that a higher concentration of Hg in the mother's blood was not consistently associated with changes in children's behaviour (Patel et al., 2019). Similarly, the Study on Child Development in Seychelles, a population cohort with high fish consumption (pregnant participants:106.8 g/d *vs* 67 g/d in our population), has been exploring this association for over two decades at different age periods, observing a lack of clear association (Myers et al., 2020). For example, a recent analysis conducted with 7-year-old children in this cohort observed that tHg measured in hair at that age was not associated with 17 neurodevelopmental parameters (Klus et al., 2023). Other studies, which have examined disorders with a significant behavioural component, such as autism spectrum disorder (ASD) or ADHD, have also not found a clear association. On one hand, some studies that have examined Hg concentrations in saliva, urine, and hair have not observed an association with an increased risk of ASD or ADHD (Barry et al., 2020; Gil-Hernández et al., 2020). In contrast, higher Hg exposure has been associated with an increased risk of ADHD in children aged 6 to 8 years (Tabatadze et al., 2018).

We did not observe associations between tHg exposure at 11- and 15-years and any of the areas of the behaviour scale used. To the best of our knowledge, this association has not been studied within these age ranges previously, and the few published studies yield contradictory results (Heng et al., 2022). Based on our findings, a hypothesis we might consider is that, while childhood represents a vulnerable period for the effects of Hg exposure, adolescence and the physiological changes it entails may offer some protective factors against such exposure.

This hypothesis aligns with emerging research suggesting that developmental stages influence the neurotoxic effects of Hg differently, with younger children displaying higher susceptibility due to their still-developing central nervous systems (Ruggieri et al., 2017). Additionally, hormonal and metabolic changes during adolescence may modulate toxicant absorption and metabolism, potentially providing a degree of resilience against certain environmental toxins, such as Hg (Heng et al., 2022; Ruggieri et al., 2017). However, further investigation is required to clarify these protective mechanisms and to resolve the inconsistent findings across studies.

Regarding sex differences, our study revealed a notable effect of Hg exposure on behaviour problems in boys but not in girls. Specifically, we observed at 9-years visit an

increment in internalizing and total problems sub-scales. This intriguing finding in unexplored and prompts further investigation into potential biological mechanisms underlying sex-specific susceptibility to Hg toxicity. One possible explanation is the differential metabolism of Hg between males and females, leading to variations in the accumulation and distribution of Hg in the body (Vahter et al., 2007). Additionally, hormonal differences between sexes may influence the neurotoxic effects of Hg on brain development, as oestrogen has been shown to exert protective effects against Hg-induced neurotoxicity in animal studies (Yamazaki et al., 2013). Further research integrating neurobiological and hormonal factors is warranted to elucidate the underlying mechanisms driving sex-specific responses to Hg exposure in children.

We must acknowledge that our study presents certain strengths but also some limitations. The most significant strength of this study may be related to the longitudinal statistical model used. The additive model based on GP and random effects has allowed, on the one hand, through the flexibility and potential of the GP effects, to estimate with precision and reliability the temporal trends of the behavioural variables and their relationship with the confounding and adjustment variables. On the other hand, this kind of analysis has allowed us to estimate plausible effects of low signal-to-noise ratio, such as the impact of Hg exposure on behaviour. Accurately detecting the temporal trends of the response variable and its multivariate relationship with the confounding and adjustment variables, considering multiple interactions, non-linear effects, and possible effects due to collinearity, is essential in longitudinal models to detect and evaluate rigorously the effects of the exposure variables of interest. These requirements that are necessary for a rigorous analysis in longitudinal model structures are not so easily met by using traditional linear mixed effects models, especially when considering multiple interactions and possible collinearity effects.

The first limitation concerns the study design. While follow-up studies are valuable for establishing causal relationships and mitigating certain biases like reverse causality, they are also susceptible to sample loss. In our investigation, we examined data across three different periods, with sample sizes progressively decreasing. We observed a decrease from 368 participants in the initial period (9 years) to 205 in the final period analysed (15 years), indicating a follow-up loss of approximately 45% from the original sample. This reduction in sample size could have compromised statistical power, which may partly explain why we only obtained statistically significant results for the 9-year-old visit.

With regard to the tool used to evaluate behavioural changes associated with Hg exposure, it also has some limitations that are worth mentioning. The CBCL scale

heavily relies on reports from parents and caregivers, thus allowing for the possibility of underestimation, overestimation, or misinterpretation of children's behaviour. Additionally, other characteristics of the parents and/or caregivers could also influence, such as their ability to observe and recall the child's behaviour or their honesty in responding faithfully. This tool may also exhibit cultural differences, often determined by socioeconomic status, as the interpretation of certain behaviours can vary, being considered normal in some contexts but problematic in others. This context-dependent interpretation could thus affect the validity of the tool. However, we decided to use this scale due to its versatility, wide usage in the literature for behaviour assessment, and ease of administration. In addition, it can be repeatedly used to monitor the child's progress over time, allowing for the evaluation of any changes that occur.

Another limitation concerns exposure, as we did not account for interactions between contaminants. While we focused our attention on a significant neurotoxin like Hg, other important neurotoxic compounds such as arsenic or polychlorinated biphenyls (PCBs) could potentially confound our results. Hair samples for Hg assessment may introduce potential sources of contamination, such as environmental exposure or hair treatments (Li et al., 2008). As mentioned in the methods section, although we did not directly measure MeHg, tHg in hair has been utilized as an adequate biomarker for this form (Nuttall, 2006).

5. Conclusions

In conclusion, our study explored the longitudinal relationship between tHg concentrations and behaviour, utilizing the CBCL score scales in children aged 9, 11, and 15 years old from Spain in an empirical study. With this aim, a modern and powerful non-parametric longitudinal model based on GP effects in an additive combination with linear random effects, has been used. The model has allowed us to accurately model the multivariate, temporal, and inter-individual variability of the data, confounders, and covariates, which is essential in longitudinal settings, and, at the same time, to obtain plausible effects of Hg exposure on behaviour.

We observed an association between hair tHg concentrations and children's behaviour at 9 years old, with higher hair tHg concentrations linked to worse scores on the CBCL internalizing and total problems scales. However, this association was not observed at 11 or 15 years old. Additionally, sex differences were notable: boys exhibited an association with increased internalizing and total problems at 9 years old, followed by a

decrease in internalizing problems at 11 and externalizing problems at 15. In contrast, no significant associations were found in girls at any age.

These findings suggest that the relationship between hair tHg concentrations and behaviour may vary by developmental stage and sex. Moreover, the study underscores the importance of considering sex differences when assessing the effects of Hg exposure on behaviour. However, the disappearance of associations at older ages, coupled with the lack of consistent patterns across all age groups, warrants further research to clarify the complex interactions between Hg exposure and behaviour during childhood and adolescence.

In light of these findings, interventions and policy recommendations aimed at reducing Hg exposure, particularly during critical developmental periods, may be necessary to protect children's behavioural health.

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Authors contribution

GR-M contributed to methodology and formal statistical analysis; AO-C contibuted to methodology and writing; AJS-P contributed to conceptualization, methodology, visualization, support in statistical analysis, reviewing of manuscript and obtaining funding;

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Appendix

GPs are non-parametric probabilistic models for multidimensional functions (Rasmussen and Williams, 2006; Neal, 1997). GPs are reliable and powerful models as they consider high-order interactions between multiple covariables and infer and exploit the covariance structure of data to estimate the underlying functions. In this work $f(t,x)$ is a GP function of time t and confounder and adjustment covariates x,

$$f(t,x) \sim \mathrm{GP}(0, k(t,x,t',x'),$$

with k being a squared exponential covariance function (Rasmussen and Williams, 2006):

$$k(t,t',x,x') = \sigma^2 \exp\left(-\frac{1}{2}\frac{(t-t')^2+(x'-x')^2}{\ell^2}\right),$$

where ℓ is the lengthscale parameter that controls the smoothness of the underlying GP function, or grade of decay in the correlation between pairs of observations as a function of the square distance $((t-t')^2 + (x'-x')^2)$ between them in terms of the time t and covariates x. The parameter $\sigma > 0$ is the marginal variance of the GP function. The defining property of a GP is that the collection of function values $f(t_i,x_i)_1^n$ follows a multivariate normal with an arbitrary mean function (Let us considerer a null mean function for simplicity) and a variance-covariance matrix K,

$$f(t_1,x_1),\ldots,f(t_n,x_n) \sim \mathrm{Normal}(0,K),$$

where each element (i, j) of matrix K is given by the covariance function,

$$K_i j = k\left(t_i, x_j, t_j, x_j\right).$$

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Table 1. Sociodemographic characteristics and lifestyle among mothers and their 9-, 11- and 15-years children of the INMA cohort study (*n* = 870).

Variables	**9 years (*n* = 368)**	**11 years (*n* = 297)**	**15 years (*n* = 205)**
Mother			
Age (years)	30 (5)[1]	31 (6)	30 (5)
Work, *n* (%)			
Employed	256 (69.6)	203 (68.4)	162 (79.0)
No-employed	112 (30.4)	94 (31.6)	43 (21.0)
Social class[2], *n* (%)			
I+II (highest)	102 (27.7)	94 (31.6)	66 (32.2)

III	103 (28.0)	91 (30.6)	64 (31.2)
IV+V (lowest)	163 (44.3)	112 (37.7)	75 (36.6)
Parity, *n* (%)			
1	214 (58.2)	176 (59.3)	118 (57.6)
2	136 (37.0)	103 (34.7)	74 (36.1)
>2	18 (4.9)	18 (6.1)	13 (6.3)
Smoke during pregnancy, *n* (%)			
Yes	76 (20.7)	65 (21.9)	39 (19.0)
No	292 (79.3)	232 (78.1)	166 (81.0)
WAIIS (IC measure)	9.8 (3.8)	10.5 (4.4)	10.5 (4.4)
Children			
Age (years)	9.1 (0.3)	10.9 (0.2)	15.6 (0.6)
Gestational age (weeks)	39.8 (1.7)	39.7 (1.8)	39.8 (1.8)
Sex, *n* (%)			
Male	182 (49.5)	131 (44.1)	97 (47.3)
Female	186 (50.5)	166 (55.9)	108 (52.7)
z-BMI, (Kg/m^2)	0.8 (2.0)	0.8 (2.0)	0.4 (1.4)
Hair tHg levels (μg/g)	1.0 (1.2)	0.9 (0.9)	0.6 (0.7)
Fish consumption (g/day)	4.8 (3.1)	5.5 (4.0)	5.1 (4.1)
Breastfeeding (weeks)	13.9 (19.6)	14.9 (18.6)	17.1 (17.1)
Passive exposure to tobacco smoke, *n* (%)			
Yes	183 (49.7)	131 (44.1)	83 (40.5)
No	185 (50.3)	166 (55.9)	122 (59.5)

BMI, body mass index. [1]Median (IQR) (all such values). [2]Social Class = I-II (managers, professionals), III (technicians and associate professionals, clerical support workers, skilled agricultural, forestry and fishery workers), IV-V (craft and related trades workers, plant and machine operators and assemblers).

Table 2. Mean and variability of CBCL scale scores by follow-up visits for the study population.

Scale	**9 years (*n* = 368)**	**11 years (*n* = 297)**	**15 years (*n* = 205)**
Internalised[1]	7.53 (5.63)	7.41 (5.88)	8.76 (6.87)
Externalized[1]	7.55 (5.97)	7.01 (6.13)	7.84 (7.90)
Total problems[1]	28.98 (18.74)	27.38 (19.22)	28.78 (21.06)

[1]Mean (SD)

Table 3. Mean and 95% credible intervals of the effect coefficients of Hg exposure on CBCL scales (internalizing, externalizing and total problems) by age visit.

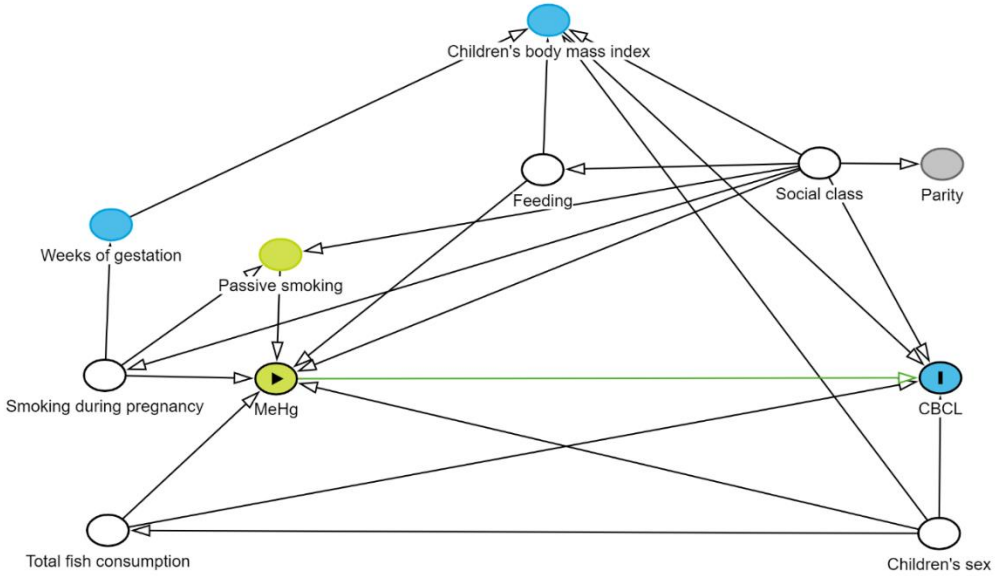
Scale	9 years		11 years		15 years	
		Pr<0		Pr<0		Pr<0
Internalised	8.04 (2.52; 13.87)	0.001	-1.86 (-8.21; 4.92)	0.77	1.91 (-7.20; 10.09)	0.46
Externalized	4.32 (-0.41; 9.82)	0.07	2.90 (-6.12; 12.33)	0.35	-7.89 (-19.97; 3.54)	0.83
Total problems	7.41 (1.99; 12.92)	0.002	0.09 (-7.10; 7.45)	0.49	-0.15 (-9.22; 8.84)	0.51

Table 4. Mean and 95% credible intervals of the effect coefficients of Hg exposure on CBCL scales (internalizing, externalizing and total problems) by age visit and sex.

Scale		9 years		11 years		15 years	
			Pr<0		Pr<0		Pr<0
Internalised	G	5.04 (-0.98; 13.23)	0.08	4.67 (-4.86; 15.63)	0.29	2.1 (-9.79; 16.32)	0.46
	B	10.92 (1.65; 17.82)	0.02	-11.77 (-21.50; -1.53)	0.98	1.78 (-9.01; 14.23)	0.48
Externalized	G	2.01 (-6.92; 9.34)	0.44	7.12 (-5.23; 20.03)	0.25	3.87 (-11.64; 21.14)	0.41
	B	7.15 (-2.05; 18.76)	0.09	-7.90 (-22.03; 9.11)	0.69	-17.10 (-27.34; -6.43)	0.00
Total problems	G	4.31 (0.01; 9.04)	0.05	5.87 (-3.56; 16.51)	0.17	5.00 (-7.01; 17.74)	0.31
	B	9.87 (1.94; 19.05)	0.01	-8.73 (-18.86; 2.34)	0.89	-4.92 (-9.09; 5.04)	0.65

B: Boys; G: Girls; The effect coefficients represent the percent increase in CBCL score scales for each 1 µg/g of Hg; Pr<0: probability of effect posterior distribution estimate to be less than zero.

Figure 1. Directed acyclic graph (DAG) between Hg exposure and behaviour problems scales (CBCL).



Nodes (Circles):

MeHg (Methylmercury): Exposure variable of primary interest.

CBCL (Child Behavior Checklist): Outcome variable measuring children's behavioural outcomes.

Children's sex, total fish consumption, smoking during pregnancy, passive smoking, weeks of gestation, feeding, social class, parity, children's body mass index (BMI): Additional variables included in the analysis.

Arrows:

Directional Arrows: Indicate the hypothesised causal relationships between variables. For instance, an arrow from "MeHg" to "CBCL" suggests that exposure to methylmercury is believed to affect the CBCL outcomes.

Double Arrows (Bidirectional Arrows): Represent potential feedback loops or mutual influence between variables.

Colours:

Light green (MeHg): Denotes the primary exposure variable in the study.

Blue (CBCL, Children's body mass index, Weeks of gestation): Indicates key outcome variables and significant covariates.

Green (Arrows): Highlights the primary causal pathway from exposure (MeHg) to outcome (CBCL).

White (Feeding, Social class, Smoking during pregnancy, Children's sex, Total fish consumption): Represents the minimum set of adjustment variables necessary to control for confounding in the analysis.

Other colours (Light blue, Light green, Grey): Represent additional variables considered in the DAG, such as passive smoking, parity, etc.

This DAG visually summarises our analytical framework and guides the selection of covariates to adjust for in our statistical models to ensure unbiased estimation of the causal effect of MeHg on children's behavioural outcomes.

Figure 2. Histograms of the discrete scores of CBCL, internalizing, externalizing, and total problems scales, by age's visit.

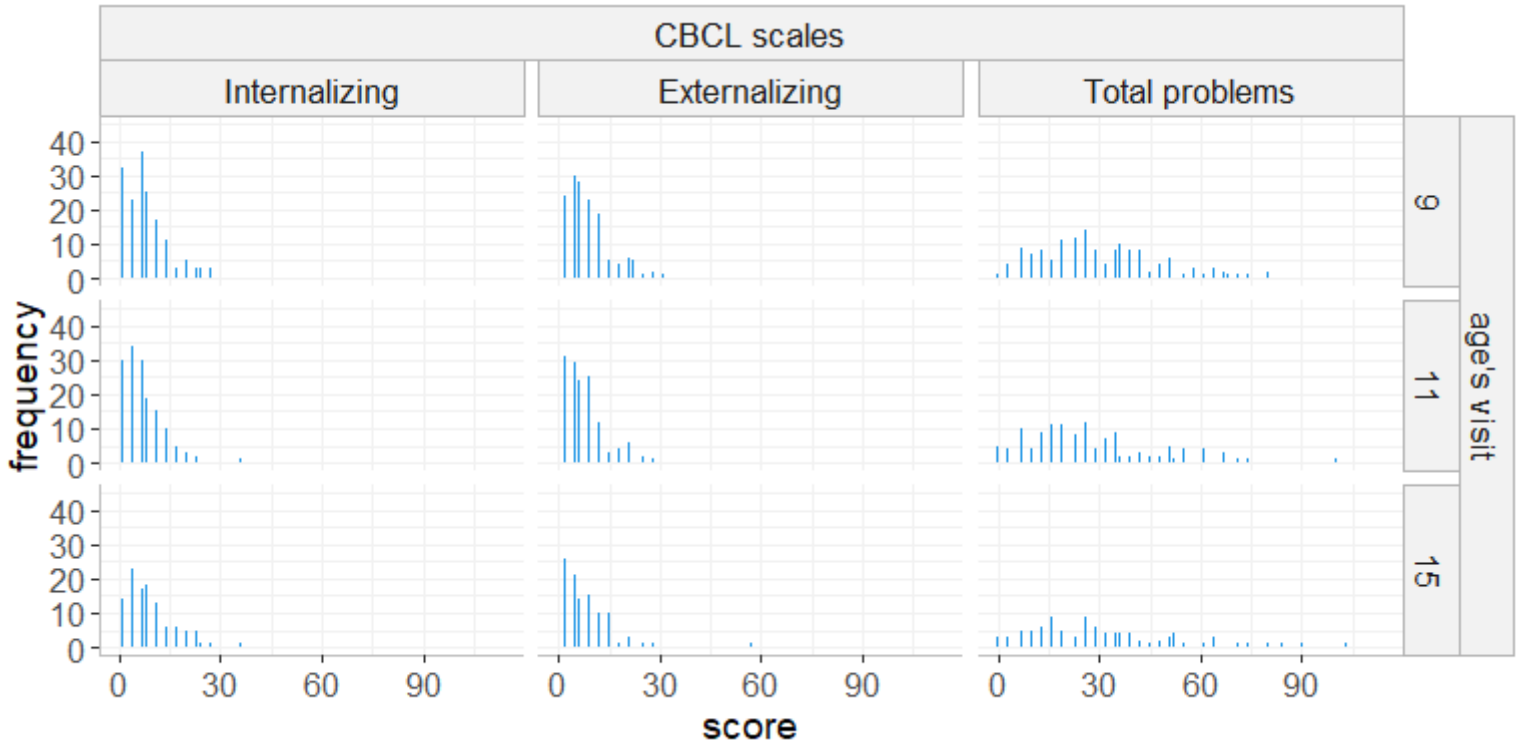


Figure 3. Effect of Hg exposure on CBCL, internalizing, externalizing, and total problems scales, by age's visit: Percent increase in CBCL score scales per 1 mg/g Hg exposure.

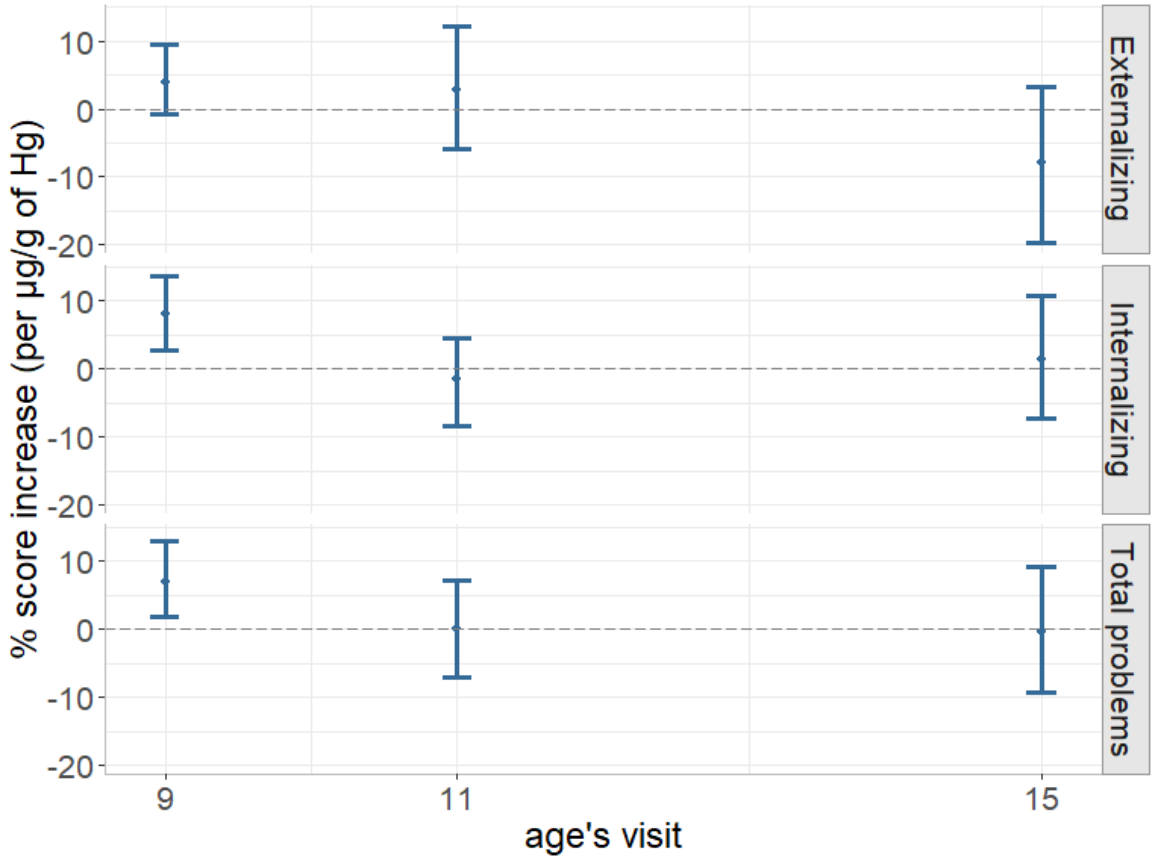


Figure 4: Effect of Hg exposure on CBCL, internalizing, externalizing, and total problems scales, by age's visit and differentiated by sex: Percent increase in CBCL score scales per 1 mg/g tHg exposure.

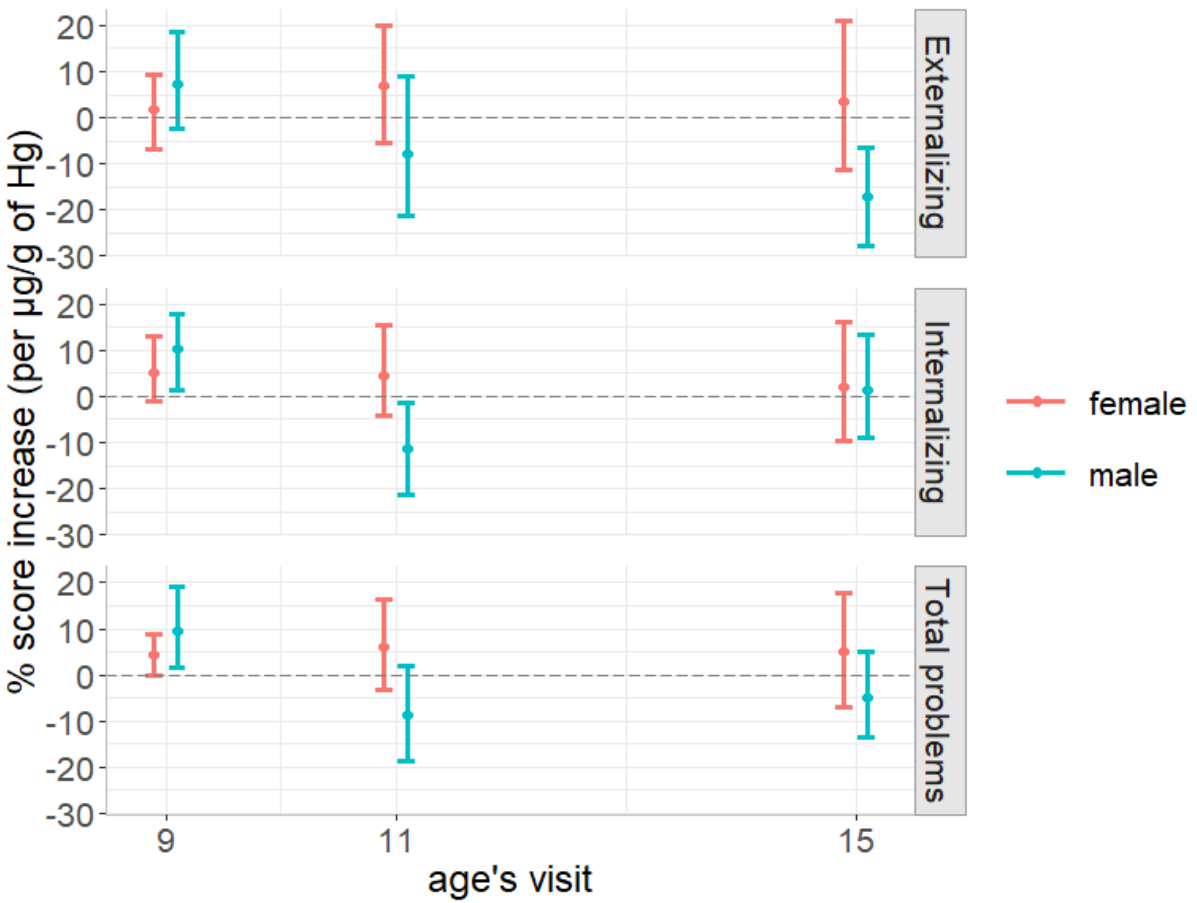


Figure S1. Flowchart of participants included in the present analysis from the "*INfancia y Medio Ambiente*" (INMA) Cohort Study.

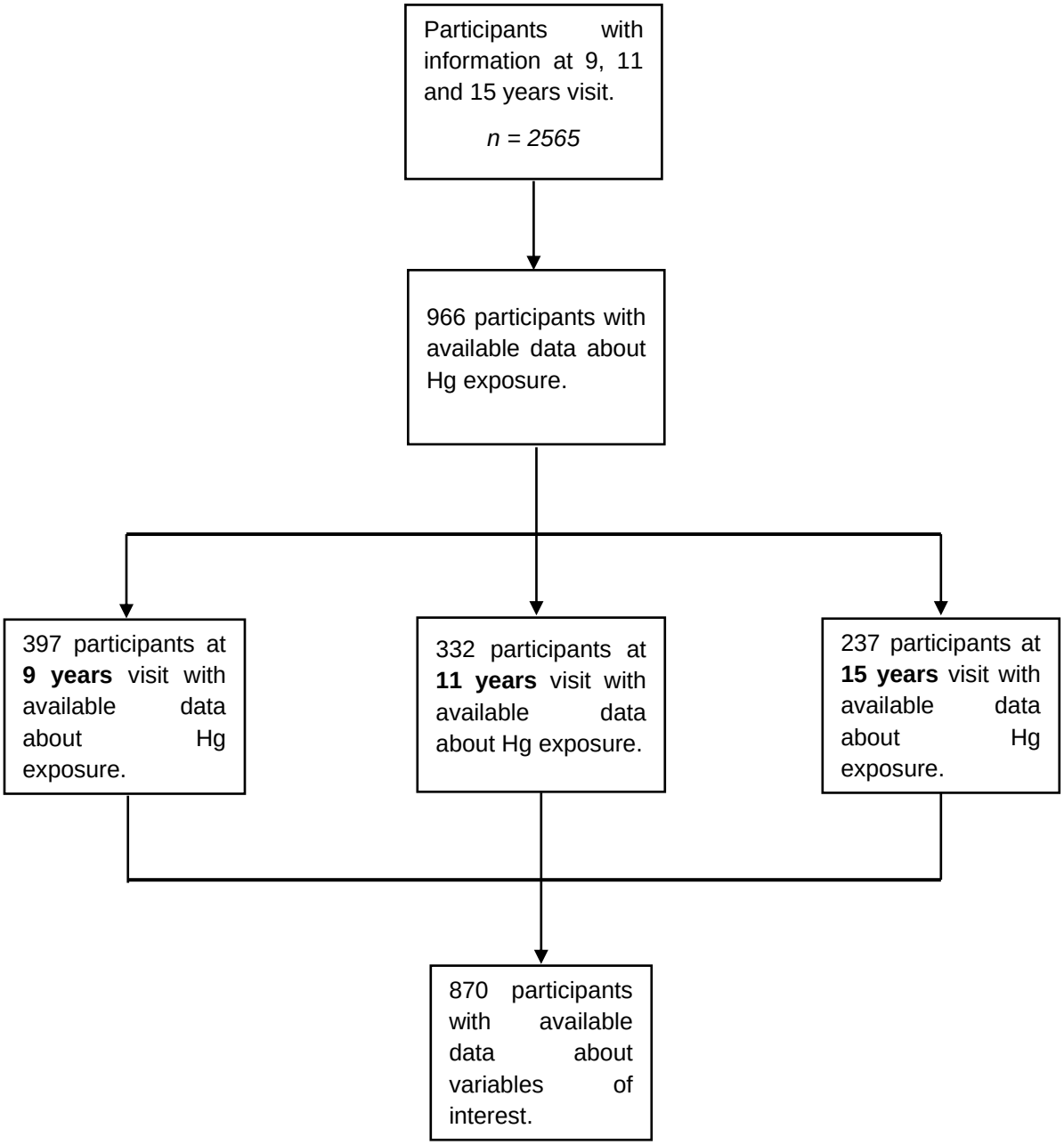


Table S1. Sociodemographic characteristics among mothers and their 9-, 11- and 15- years old children included and excluded from the study.

	9 years		11 years		15 years	
Variables	**Included (*n* = 368)**	**Excluded***	**Included (*n* = 297)**	**Excluded***	**Included (*n* = 205)**	**Excluded***
Mother						
Age (years)	30 (5)[1]	29.5 (7)	31 (6)	29 (7)	30 (5)	30 (6)
Work, *n* (%)						
Employed	256 (69.6)	36 (59.0)	203 (68.4)	53 (67.9)	162 (79.0)	65 (80.2)
Unemployed	112 (30.4)	25 (41.0)	94 (31.6)	25 (32.1)	43 (21.0)	16 (19.8)
Social class[2], *n* (%)						
I+II (highest)	102 (27.7)	61 (17.2)	94 (31.6)	69 (16.2)	66 (32.2)	97 (18.8)
III	103 (28.0)	94 (26.6)	91 (30.6)	106 (24.9)	64 (31.2)	133 (25.7)
IV+V (lowest)	163 (44.3)	199 (56.2)	112 (37.7)	205 (58.8)	75 (36.6)	287 (55.5)
Children						
Age (years)	9.1 (0.3)	9.2 (0.4)	10.9 (0.2)	11.0 (0.4)	15.6 (0.6)	15.6 (0.6)
Sex, *n* (%)						
Male	182 (49.5)	200 (56.5)	131 (44.1)	251 (59.1)	97 (47.3)	285 (55.1)
Female	186 (50.5)	154 (43.5)	166 (55.9)	174 (40.9)	108 (52.7)	232 (44.9)
z-BMI, (kg/m^2)	0.8 (2.0)	0.9 (2.3)	0.8 (2.0)	0.9 (2.1)	0.4 (1.4)	0.3 (1.7)

BMI, body mass index. [1]Median (IQR) (all such values). [2]Social Class = I-II (managers, professionals), III (technicians and associate professionals, clerical support workers, skilled agricultural, forestry and fishery workers), IV-V (craft and related trades workers, plant and machine operators and assemblers). *The sample size of the excluded population depends on the variable described.

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