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Maternal copper status and neuropsychological development in infants and preschool children

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Abstract

Introduction: Copper (Cu) is an essential element involved in biological processes; however, excessive Cu could be harmful because of its reactive nature. Very few studies have evaluated its potential neurotoxic effects. We aimed to evaluate the association between maternal Cu levels and children's neuropsychological development.

Methods: Study subjects were mother-child pairs from the Spanish INMA (i.e. Childhood and Environment) Project. Cu was measured by inductively coupled plasma mass spectrometry in serum samples taken at the first trimester of pregnancy (2003-2005). Neuropsychological development was assessed using the Bayley Scales of Infant Development (BSID) at 12 months (n=651) and the McCarthy Scales of Children's Abilities (MSCA) at 5 years of age (n=490). Covariates were obtained by questionnaires during pregnancy and childhood. Multivariate linear and non-linear models were built in order to study the association between maternal Cu and child neuropsychological development.

Results: The mean ± standard deviation of maternal Cu concentrations was 1606 ± 272 µg/L. In the multivariate analysis, a negative linear association was found between maternal Cu concentrations and both the BSID mental scale (beta=-0.051; 95% confidence intervals [CI]: -0.102, -0.001) and the MSCA verbal scale (beta=-0.044; 95%CI:-0.094, 0.006). Boys obtained poorer scores than girls, with increasing Cu at 12 months (interaction p-value=0.040 for the mental scale and 0.074 for the psychomotor scale). This effect modification disappeared at 5 years of age. The association between Cu and the MSCA scores (verbal, perceptive performance, global memory and motor, general cognitive, and executive function scales) was negative for those children with lowest maternal iron concentrations (<938µg/L). **Conclusion:** The Cu concentrations observed in our study were within the reference range established for healthy pregnant women in previous studies. The results of this study contribute to the body of scientific knowledge with important information on the possible neurotoxic capability of Cu during pregnancy.

Keywords: birth cohort, cognitive, neurodevelopment, metal, delayed effects, prenatal exposure

1. Introduction

Copper (Cu) is an essential trace element found in all organs and cells. On the one hand, Cu is a transition metal involved in numerous biological processes such as cellular respiration, antioxidant defence, connective tissue formation, neurotransmitter biosynthesis, peptide hormone maturation, pigmentation, keratinization and iron homeostasis (Uriu-Adams et al., 2010). On the other hand, excessive Cu could be harmful because of its highly reactive nature, leading to the possible production of hydroxyl radicals (Valko et al., 2005). However, most of the literature on the neurotoxicity of Cu is focused on nutritional deficiency and its effect on the brain.

The main source of Cu is the diet. Absorption is dependent on the amount ingested, its chemical form and the composition of other dietary components such as zinc. Liver and kidney contain high Cu levels, and fish, fruits, cereals, nuts and green vegetables are also important sources (Ellingsen et al., 2005). In human adults, the proportion of Cu absorption is inversely correlated with dietary copper intake: high dietary copper intake results in low relative Cu absorption (van den Berghe and Klomp, 2009). Under normal physiological conditions about 98% of Cu excretion is via bile and the remaining 2% is via urine (Wijmenga and Klomp, 2004).

Cu can be transferred from the mother to the foetus via the placenta and a substantial portion is accumulated and retained in the foetal liver (Gambling et al., 2003) to supply Cu during the first months of life, a period with a minimum intake of this nutrient. The Cu stores in the foetal liver therefore aid in preventing Cu deficiency during the early months of life (Harvey and McArdle, 2008). Some experimental studies conducted with rats have shown the importance of Cu and iron during pregnancy in order to ensure adequate brain development (Penland and Prohaska, 2004; Prohaska and Gybina, 2005). In humans, rare and severe alterations in Cu homeostasis have been associated with some neurological disorders, such as aceruloplasminemia, Alzheimer, Huntington or Menkel diseases (Desai and Kaler, 2008).

Very few epidemiological studies have evaluated the association between prenatal or early postnatal Cu levels and child neuropsychological development, and with heterogeneous results. Postnatal traffic-related Cu exposure was associated with poorer motor performance and altered basal ganglia assessed with magnetic resonance imaging in 8–12-year-old children from Barcelona (Spain) (Pujol et al., 2016), but maternal Cu levels measured during pregnancy in plasma in Łódź and Legnica (Poland) (Polanska et al., 2017) or urine in Sabadell (Spain) (Forns et al., 2014) were not associated with children's neuropsychological development assessed at 1–2 and 4 years old, respectively. Foetuses are especially vulnerable to the adverse effects of toxicants in comparison to adults, since their organs and systems are still developing and their detoxification mechanisms are not yet fully mature (Selevan et al., 2000). The nervous system has a long development time that extends from the embryonic period through adolescence and, thus, early exposure to toxicants could lead to developmental neurotoxicity (Rice and Barone S Jr, 2000).

The aim of this study is to evaluate the association between maternal Cu levels in serum samples during pregnancy and children's neuropsychological development assessed at 1 and 5–6 years of age in a Spanish birth cohort study. We additionally assessed the sociodemographic, environmental and dietary determinants of maternal Cu concentrations and evaluated the effect of interactions between Cu and other nutrients (iron, selenium and zinc) and children's sex on neuropsychological development.

2. Methods

2.1 Study population

Study subjects were participants in the INMA Project (Childhood and Environment Project: http://www.proyectoinma.org) – a multicentre birth cohort study that aims to investigate the effects of environmental exposures and diet during pregnancy on foetal and child health in different areas of Spain.

The study protocol has been reported elsewhere (Guxens et al., 2012). Briefly, pregnant women were recruited at the beginning of their pregnancy in the region of Valencia (n=855, 2003−2005). The inclusion criteria were: at least 16 years of age, 10–13 weeks of gestation, singleton pregnancy, no participation in an assisted fertility programme, intention of undergoing follow-up and delivery at the hospital of reference, and no impediment for communication.

When excluding the women who withdrew from the study (n=28), were lost to follow-up (n=5), or had induced or spontaneous abortions (n=31) or foetal deaths (n=4), a total sample of 787 (92%) women were followed up until delivery. Their children were enrolled at birth and monitored from then on (n=708, 83% at 12 months of age; n=536, 63% at 5 years of age). The final study population was made up of mothers with available Cu concentrations (n=656), and mother-child pairs with both maternal Cu concentrations in serum and child neuropsychological test scores at 12 months (n=651) and 5 years of age (n=490). Informed consent was obtained from all participants in each phase and the study was approved by the La Fe Hospital Ethics Committee.

2.2 Copper concentrations

Concentrations of Cu were determined in serum samples taken at the first trimester of pregnancy (mean ± standard deviation (SD) = 12.7 ± 1.5 weeks of gestation). After separation of serum by centrifugation, samples were stored at –80^{o}C and transported frozen to the Karolinska Institutet, Sweden, for analysis. Approximately 120 μg of serum was diluted 1:25 in an alkaline solution containing 2% 1-butanol (anhydrous, 99.8%, Sigma-Aldrich, Schnelldorf, Germany), 0.05% EDTA (99.995%, Sigma-Aldrich), 0.05% Triton X-100 (BioXtra, Sigma-Aldrich), 1% NH_4OH (25%, Romil, Cambridge, UK), and 20 ng/g of internal standards (Sc-45, Ge-72, Rh-103; CPI International, Amsterdam, Netherlands). Samples were then sonicated and centrifuged for 5 minutes each. The concentrations of serum Cu were determined by inductively coupled plasma mass spectrometry (ICPMS; Agilent 7700x, Agilent Technologies, Tokyo, Japan) with the collision/reaction cell system in helium mode. Analytical quality control was performed by inclusion of reference materials (Seronorm: Trace Elements serum lot MI0181, Trace Elements whole blood L-1 lot 1406263 and L-2 lot 1406264, and Medisafe serum L-2 lot 28342). The values obtained were within the analytical range for all reference materials. The limit of detection was 2.93 ng/g and no samples had concentrations below this value. Cu concentrations were corrected according to the variations in three daily measures of the Seronorm^{TM} (lot MI0181) reference material. The correction was performed by adding to each measure the difference between the daily mean of the reference measures and the overall mean of the reference measures (Amorós et al., 2018a).

2.3 Child neuropsychological development

The neuropsychological development of the children was assessed at around 12 months of age (mean ± SD = 12.3 ± 0.7, range = 11.4–19.5 months) and at 5–6 years of age (mean ± SD = 5.8 ± 0.16 years, range 5.5–6.9 years). For the measure at 12 months, the first edition of the Bayley Scales of Infant Development (BSID) was used. These scales assess age-appropriate mental and psychomotor development, including performance abilities, memory, early language skills, psychomotor skills and coordination. The BSID are composed of the mental scale (163 items) and the psychomotor scale (81 items). All testing was carried out at the children's reference hospital (La Fe Hospital, Valencia), in the presence of their mothers, by four trained psychologists.

For the measure at 5–6 years of age, a standardized version of the MSCA adapted to the Spanish population was used (McCarthy D, 2009). The verbal scale refers to cognitive tasks related to the processing of verbal information; the perceptual-performance scale refers to cognitive tasks related to perceptual information processing, including manual performance; the quantitative scale assesses numerical abilities; the global memory scale considers short-term retention of information (verbal, visual or numerical); the global motor scale refers to fine (e.g. drawing) and gross (e.g. balance or accuracy) abilities; the working memory scale refers to those cognitive tasks related to temporarily storing and managing the information required to carry out other cognitive tasks such as learning, reasoning and comprehension; and the executive function scale refers to those cognitive tasks that are critical to non-routine, goal-oriented situations that are performed by the pre-frontal cortex (Julvez et al., 2011, 2007). The sum of the first three scales provides a general cognitive scale. Testing was conducted by two psychologists using a strict protocol.

The raw scores of the two tests (BSID and MSCA) were standardized for the child's age in days at test administration and for psychologist. Standardized residuals were then typified by having a mean ± SD of 100 ± 15 points to homogenize the scales.

2.4 Other variables

The women completed two questionnaires during their pregnancy, one at the first trimester (mean ± SD) = 12.7 ± 1.5 weeks of gestation) and the other at the third trimester (mean ± SD = 32.4 ± 2.0 weeks of gestation). Questionnaires were administered by trained interviewers and focused on sociodemographic, dietary, environmental and lifestyle information during pregnancy. The maternal covariates and potential confounders collected were: country of birth (Spain, other), age (<25, 25–29, 30–34, ≥35 years), body mass index before pregnancy (Kg/m^2), level of education (primary, secondary, university), parity (0, 1, ≥2), area (urban, metropolitan, semi-urban, rural) and age (≤5, >5 years) of the residence, employment during pregnancy (non-worker, worker), smoking at the beginning of pregnancy (no, yes) and season of sampling (Spring, Summer, Autumn, Winter). We also obtained data on paternal age, employment and level of education.

Parental social class was defined during pregnancy as the highest occupational social class of both parents, 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).

Information on diet during pregnancy was collected by using a validated semiquantitative food frequency questionnaire (FFQ) (Vioque et al., 2013). We obtained data (expressed in grams per day) on the intake of seafood, meat, cereals and pasta, legumes, nuts, fruits, vegetables, eggs, dairy products, potatoes and bread. Energy-adjusted intakes were computed using the residual method (Willett et al., 1985). Information related to the child's gestational age, sex and anthropometric measures at birth was obtained from clinical records. Low birth weight was defined as less than 2,500 g, and preterm birth was considered to be less than 37 weeks of

gestation. Breastfeeding duration and attendance at nursery were obtained in a subsequent interview at the same time point as the neuropsychological development assessment when children were 12 months old. Breastfeeding (in weeks) was defined as receiving breast milk for at least 7 days, although it could be supplemented with any food or liquid, including nonhuman milk. The variable was categorized as non-breastfed vs. breastfed. Information about maternal and paternal working status, maternal and paternal smoking habits in the presence of the child, and a proxy of the maternal verbal intelligence quotient (IQ, based on the Similarities Subtest of the Weschler Adult Intelligence-Third Edition (WAIS-III)) was obtained at the same time point as the neuropsychological development assessment when children were 5–6 years old.
Iron, selenium and zinc concentrations were analysed in the same serum maternal samples and by using the same method as for Cu, except that selenium was measured with the ICPMS collision/reaction cell system in helium and hydrogen mode.

2.5 Statistical analysis

Univariate and multivariate linear regression models were built to examine the determinants of prenatal Cu exposure. In these models Cu was the dependent variable and the maternal characteristics were the independent ones (country of birth, age, BMI, educational level, parity, area of residence, employment during pregnancy, social class, smoking at the beginning of the pregnancy, season of sampling, gestational age, age of the residence, and intake of seafood, meat, cereals and pasta, legumes, nuts, fruits, vegetables, eggs, dairy products, potatoes and bread). The multivariate model was built following a backward elimination procedure, using all variables with a p-value < 0.1 for the univariate models as candidate covariates and retaining those with a p-value < 0.1 in the likelihood ratio test (LRT) for the multivariate model.
Multivariate linear regression models were built in order to assess the relationship between Cu concentrations (as an explanatory/independent variable) and the different scales of the BSID and the MSCA (outcome variables). For these models we corrected the Cu concentrations for the gestational age at sampling, as a preliminary exploration of the data showed a linear increase in the Cu concentration of 9.4 µg/L per gestational day (95% confidence interval [CI]: 4.9, 13.9, p-

value<0.001). In the first step, a core model was built for each scale with parental and child sociodemographic variables as possible covariates (specifically, parental age, educational level and working situation; maternal country of birth, parity, BMI before pregnancy, social class, and maternal intelligence; sex, breastfeeding, and attendance to nursery). These multivariate models were built following a backward elimination procedure, using all variables with a p-value < 0.1 for the univariate models as candidate covariates and retaining those with a p-value < 0.1 in the likelihood ratio test (LRT) for the multivariate models. In the second step, we introduced the Cu concentrations into these adjusted models and additional confounders were included if they changed the magnitude of the Cu main effect in a significant way, compared to the same potential confounder but randomized, that is, the same variable randomly reordered to simulate independence from Cu and the response variable, with a 5% significance level (Lee, 2014). The potential confounder variables were those found to be determinants of the maternal Cu status. For comparability purposes, final models for the subscales of BSID and MSCA were fitted for the same pool of variables: those that were retained in any of individual models following the described procedure. Generalized additive models (GAM) using natural cubic splines with one internal knot were employed to assess the linearity of the relationship between child neuropsychological development and Cu concentrations by graphical observation and the Akaike information criterion (AIC). More than one knot was tested but in the end only one was used to avoid overfitting the potential non-linear relationships.

Effect modification by sex of the child and other nutrients (selenium, iron, zinc) was also assessed. To do so, the interaction effect was tested using the LRT for the linear model, and AIC scores were compared for the GAM models with and without interaction. We dichotomized the nutrient variables according to the first tertile for iron (938 µg/L) and for zinc (553 µg/L) and the breakpoint observed in our previous study about maternal selenium and child neuropsychological development (selenium concentration of 85 µg/L) (Amorós et al., 2018b). Finally, some sensitivity analyses were performed by excluding preterm (n=33) and low birth weight (n=35) from the analysis and including the variable maternal serum selenium concentrations in the models due to its relationship with neurodevelopment observed in our population (Amorós et al., 2018a, 2018b). We considered associations or interactions as statistically significant when p-values were < 0.05. All the analyses were performed using the R, version 3.3.0, software. R packages mgcv and ggplot2 were used to implement the GAM models and to plot the graphs, respectively.

3. Results

The characteristics of the mothers and children are shown in Table 1. Eighty-eight per cent of the women were born in Spain, 25% had finished university studies and 48% belonged to the lowest social class. Fifty-two per cent of the children were boys and 15% were not breastfed. The mean ± SD of maternal Cu concentration was 1606 ± 272 μg/L. The maternal factors associated with higher Cu concentrations in the multivariate model were age (higher in the 25−29-year-old group), higher BMI before pregnancy, being multiparous and higher gestational age at blood sampling (Table 1). The higher age of the residence and meat intake were positively associated with Cu concentrations in the univariate models but they did not remain significant in the multivariate models (Table 1). Linear models including Cu showed a better fit with the data (lower AIC score) for the association with children's mental (BSID) and verbal (MSCA) scales, in comparison to the same multivariate models not including Cu or the GAM models, where the relation between the outcome and Cu was considered to be non-linear. For the rest of the scales, the linear model without including Cu as an explanatory variable had a lower AIC score than both the linear model including the Cu and the non-linear one. The estimated splines of the GAM in Figure 1 and 2 showed the linear relationship between Cu and the mental scale at 12 months (BSID) and the verbal scale at 5 years (MSCA). The estimated splines of the GAM showed associations with a range from an inverted U to horizontal shapes for the other scales (Figure 1 and 2).

Table 2 shows the coefficients for the multivariate linear regression models between maternal Cu concentrations and the BSID and MSCA scores. These models were adjusted for parental and child sociodemographic, environmental and life style characteristics (Table 2). For BSID at 12 months, the association between maternal Cu concentrations and mental scale was negative

(Beta: -0.051; 95%CI: -0.102, -0.001) and it reached the significance level (p=0.045), whereas for the psychomotor scale this association was close to null (Beta: 0.003; 95%CI: -0.044, 0.051). Regarding the MSCA at 5 years, maternal Cu concentrations were marginally and negatively associated with the verbal scale (Beta: -0.044; 95%CI: -0.094, 0.006, p-value=0.086). For the other scales, the associations were negative (except for working memory) but not statistically significant in any case.

When we tested sex as a potential effect modifier, a statistically significant or close to significant linear interaction was observed for the mental (p=0.040) and psychomotor (p=0.074) scales, respectively, of the BSID at 12 months (Table 2). The linear regression coefficients were negative for males (Beta= -0.114; 95%CI: -0.185, -0.043 for the mental, and Beta= -0.058; 95%CI: -0.121, 0.005 for the psychomotor scale) and positive but non-significant for females (Beta: 0.015; 95%CI: -0.058, 0.087 for the mental scale and Beta: 0.050; 95%CI: -0.022, 0.121 for the psychomotor scale). The GAM plotted in Figure 3 showed an inverse linear relationship between Cu and the scores for boys, this association being flat or slightly ascending for girls. This interaction disappeared at 5 years of age (Table 2 and Figure 4).

We also evaluated the maternal concentrations of other nutrients (iron, selenium and zinc) as potential effect modifiers. We observed statistically significant interactions for iron (Table 3). The association between Cu and the scores obtained by the children for the verbal, perceptual performance, global memory, global motor, general cognitive and executive function scales at 5 years of age was negative for those children whose mothers had iron levels below the first tertile (938 μg/L). Similar pattern was observed for zinc but the only statistically significant interaction was found for the perceptive manipulative scale at 5 years of age. Selenium did not modify the associations between Cu and outcomes (data not shown).

Sensitivity analyses excluding preterm and low birth weight infants from the models provided a similar pattern to that for the whole sample (Supplemental material Table 1). When the variable maternal selenium concentrations was included in the multivariate models the coefficients for the association between Cu and the mental (BSID) and verbal (MSCA) scores remained negative but the p-values ($p<0.1$) became more distant from statistical significance (Supplemental material Table 2).

4. Discussion

In this Spanish birth cohort study, we observed a negative association between maternal Cu concentrations and some domains of child neuropsychological development assessed at 12 months and 5 years of age. Boys seemed to be more susceptible to Cu neurotoxicity, since they obtained poorer scores than girls on the mental scale at 12 months of age with increasing maternal Cu. The association between Cu and the scores obtained by the children at 5 years of age was negative for those whose mothers had lower iron levels. The mean ± SD of Cu concentration in our population was 1606 ± 272 μg/L. These concentrations were similar to those observed for healthy pregnant women in Jordania (mean ± SD: 1750 ± 420 μg/L) (Awadallah et al., 2004) and in another study in Spain (1470.53 ± 340.61 μg/L) (Izquierdo Alvarez et al., 2007), higher than healthy pregnant women in China (median: 1026.3 μg/L) (Zhang et al., 2013) and lower than pregnant women in Poland (mean: 1980 μg/L) (Polanska et al., 2017). All of these studies measured Cu in plasma or serum at the first trimester of pregnancy.

Although there is no consensus on Cu reference ranges for pregnancy, some studies have established them in different populations. Thus, the reference ranges established for healthy women in the first trimester of pregnancy were 936.1–3033.2 μg/L in Australia (Wilson et al., 2018), 340.54–2250.70 μg/L in Turkey (Kilinc et al., 2010) and 890.7–3660.0 μg/L in China (Liu et al., 2017). Abbassi-Ghanavati et al. (2009) performed a review of different studies published between 1975 and 2008 on Cu during pregnancy and established the reference range for the first trimester as 1120–1990 μg/L (Abbassi-Ghanavati et al., 2009). The Cu concentrations observed in our study were within the ranges established in all of these previous studies.

The literature exploring the association between prenatal or early postnatal Cu concentrations and child neuropsychological development is very scarce and the results obtained are controversial. Forns et al. (2014) explored the association between urinary Cu measured at the first and the third trimester of pregnancy and child neuropsychological development assessed at 4 years of age in the INMA study in Sabadell, Spain (n=485), but did not find any significant association (Forns et al., 2014). A possible explanation for this lack of significant results could be that the biomarker used for the Cu exposure assessment is not a good proxy of the Cu transferred from the mother to the foetus, since only 2% of the Cu is excreted via urine (Ellingsen, DG., Moller LB., Aaseth J., 2005).

In another study conducted in a Polish mother-child cohort (n=539), Cu was measured in plasma at each trimester of pregnancy, at delivery and in cord blood (Polanska et al., 2017). The authors did not find any statistically significant association between the different measures of Cu and any of the different domains (cognitive, language and psychomotor) of the Bayley test assessed at 1–2 years of age. Cu levels observed in this population (mean: 1980±570 µg/L in plasma) was a bit higher that in our study.

Pujol et al. (2016) measured indoor and outdoor airborne Cu at schools in Barcelona (Spain) and used magnetic resonance imaging to assess children's behaviour with the Attentional Network Test at 9 years of age and anatomical damage in the brain (n=263). They observed that higher Cu exposure was associated with poorer motor performance and altered structure of the basal ganglia (Pujol et al., 2016). A further study on the same population described a genetic component influencing the association between airborne Cu and children's inattentiveness (Alemany et al., 2017). Both outdoor and indoor Cu exposure increased inattentiveness in rs1061472-CC and rs1801243-CC carriers for the ATPase copper transporting beta (*ATP7B*) gene. This gene encodes an ATPase that regulates the amount of Cu leaving the cell.

A case-control study conducted in Bratislava (Slovakia) reported higher plasma Cu levels and Cu/Zn ratio in 6–7 year old children with Attention-deficit hyperactivity disorder (ADHD) than in controls (Viktorinova et al., 2016). However, the limited sample size of this study (n=58 children with ADHD and n=50 healthy) and the lack of multivariate logistic models warrant the need to confirm these results by further studies.

We observed that children's sex modified the association between maternal Cu and the scores obtained by the children for the mental scale in the Bayley test. Boys obtained poorer scores than girls with increased maternal Cu. This modifying effect was similar for the psychomotor scale but the p-value for the interaction was only close to the significance level ($p = 0.07$), although this sex-related modifying effect diminished at 5 years of age. Similarly, an inverse association was observed between children's blood Cu concentrations and a poorer working memory in boys, but not in girls, at 12 years of age in China (Zhou et al., 2015). In addition, an experimental study conducted with mice seems to support the hypothesis that males are more sensitive to the toxic effects of Cu, male mice being the ones that experimented more severe toxic symptoms in behavioural observation, pathological examination and blood biochemical assay due to exposure to nano-copper particles (Chen et al., 2006). However, another study conducted with adolescents in Belgium observed that urinary Cu was related to poorer attention and short-term memory in girls, but not in boys (Kicinski et al., 2015).

A possible mechanism of these sex-related differences in Cu neurotoxicity could be the interaction with hormones. Some epidemiological studies have evidenced the endocrine disrupting capability of Cu. Thus, Jain (2014) observed a gender differential effect of Cu on thyroid hormones in a US population: Cu was associated with an increase in free thyroxine (FT4) in males and an increase in total triiodothyronine (TT3) in females (Jain, 2014). Chang et al. (2011) observed a negative correlation between Cu levels and total testosterone in 40−60-year-old men (Chang et al., 2011). More research is highly warranted on this issue, as gender seems to play a role in the influence of Cu neurotoxicity.

We also observed that the association between maternal Cu and child neuropsychological development assessed at 5 years of age was modified by maternal iron concentrations. Specifically, this association was negative for children whose mothers had iron levels below the first tertile (938µg/L), even though this level is not considered as iron-deficient (the thresholds used to classify individuals as iron deficient typically range from 500-600 µg/L in plasma) (World Health Organization (WHO)/Centers for Disease Control (CDC), 2004). One of the transport mechanisms of iron, and other metals such as Cu, into the brain and in the proximal portion of the small intestine seems to be mediated by the same divalent metal transporter, the divalent metal-ion transporter 1 (DMT1) (Skjørringe et al., 2012). The interaction between the Cu and iron homeostasis has been observed in rat duodenum where *DMT1* expression was strongly induced in response to dietary iron deficiency and significantly higher liver Cu levels were additionally observed (Collins et al., 2005). Similarly, Garcia et al. (2007) observed that the brains of young rats subjected to iron deficiency had elevated copper levels (Garcia et al., 2007). We also observed effect modification with the maternal zinc levels, but the interaction between zinc and Cu was only statistically significant for one of the scales at 5 years old. In this case, all the women with zinc levels below the first tertile (553 µg/L) would be considered as zinc-deficient, since the suggested lower cut-off for zinc concentrations is 700 µg/L (Hess et al., 2007). The DMT1 is also able to transport zinc, but with less affinity than for iron or Cu (Espinoza et al., 2012), and some inhibitory interaction between Cu and zinc has been also observed (Nadella et al., 2006; Ojo et al., 2009). Although these results could be of interest to understand the possible mechanisms of Cu neurotoxicity, they should be taken with caution and confirmed by further studies.

In our population, higher maternal Cu concentrations were associated with age, BMI before pregnancy, parity, gestational age at blood sampling, and social class. Increasing levels of Cu during pregnancy have been reported previously by other studies (Aaseth et al., 2001; Izquierdo Alvarez et al., 2007; Polanska et al., 2017). The reason for this increase in Cu concentrations could be related to the elevation of serum ceruloplasmin throughout pregnancy (Skarżyńska et al., 2018), which is a copper-containing protein with both antioxidant and prooxidant properties (Uriu-Adams and Keen, 2005), or to the iron depletion through pregnancy (Gulec and Collins, 2014).

The association between Cu status and BMI observed in our study could be explained by the relationship between Cu and lipids found in previous studies. Thus, a positive association between Cu and triglycerides has been observed in umbilical cord serum (Bastida et al., 2000; Wells et al., 2014) and between Cu and cholesterol in adults (Ghayour-Mobarhan et al., 2005).

Nulliparous women those with an age between 25 and 29 years and those belonging to the lowest social class had the highest Cu concentrations. The relationship between maternal age and some indicator of economic status (such as income or automobile possession) and cord blood Cu concentrations was examined by Parajuli et al. (2012); however they did not obtain any statistically significant result (Parajuli et al., 2012). The literature on this topic is very scarce.

A limitation of our study could be the drop in participation rate from birth to the age of 5 years, when we assessed neurodevelopment (61% of the children recruited at birth were monitored at 5 years of age). We evaluated the parental differences between the included and the excluded population and observed significant differences for parental age, BMI before pregnancy, maternal education and social class. Overall, parents whose children were evaluated at 5 years of age were older, better educated and belonged to a higher social class. The loss to follow-up in cohort studies could represent another bias in estimating some exposure-outcome associations; additionally, this loss is usually more frequent among the less advantaged population (Howe et al., 2013).

As a positive feature of our study, its longitudinal nature has made it possible to obtain sufficient information on maternal and child characteristics that may be related to Cu exposure and neuropsychological development, including the interactions with other nutrients. In addition, because the study population was followed up over time, it was possible to detect changes in certain variables such as smoking, which may affect children's cognitive development. We have also obtained a longitudinal assessment of child neuropsychological development, which has allowed us to evaluate whether Cu neurotoxicity persists over time.

In conclusion, this study provides some evidence of the adverse effects of prenatal exposure to Cu on child neuropsychological development. We observed a negative association between maternal Cu status at the first trimester of pregnancy and mental development assessed at 12 months of age. This effect persisted until 5 years of age, when we observed the same association with the verbal scale. In addition, boys seemed to be more sensitive to Cu exposure than girls, obtaining poorer scores on the mental scale at 12 months of age. Some nutrient, such as iron, seems to influence the association between Cu and child neuropsychological development. Cu is a trace element that is necessary for foetus and child development; however, its oxidant capabilities could trigger deleterious effects. In fact, we have observed these associations at levels within the reference range established by previous studies. The results of this study add important information to the body of scientific knowledge on the possible neurotoxic capability of Cu during pregnancy. However, further studies of a similar nature are warranted to confirm these results and the possible sex-specific differences in exposure to this metal.

Figure captions:

Figure 1: Generalized additive models of the association between maternal serum Cu concentrations and the children's scores for the Bayley Scales of Infant Development at 12 months of age

Figure footnote: Bayley scales adjusted for sex, BMI before pregnancy, parity, type of zone of residence, parental age, BMI before pregnancy, social class, season of sampling and attendance at nursery.

Figure 2: Generalized additive models of the association between maternal serum Cu concentrations and the children's scores on the McCarthy Scales of Children's Abilities at 5–6 years of age

Figure footnote: McCarthy scales adjusted for BMI before pregnancy, sex, maternal country of birth, maternal age, parental educational level, parity, type of zone, maternal working situation during pregnancy, social class, breastfeeding, smoking during pregnancy, maternal intelligence, main care provider, maternal smoking at evaluation and paternal working situation at evaluation.

Figure 3: Generalized additive models of the association between maternal serum Cu concentrations and the children's scores for the Bayley Scales of Infant Development at 12 months of age according to sex

Figure footnote: models adjusted for the same variables as Figure 1

Figure 4: Generalized additive models of the association between maternal serum Cu concentrations and the children's scores on the McCarthy Scales of Children's Abilities at 5–6 years of age according to sex

Figure footnote: models adjusted for the same variables as Figure 2

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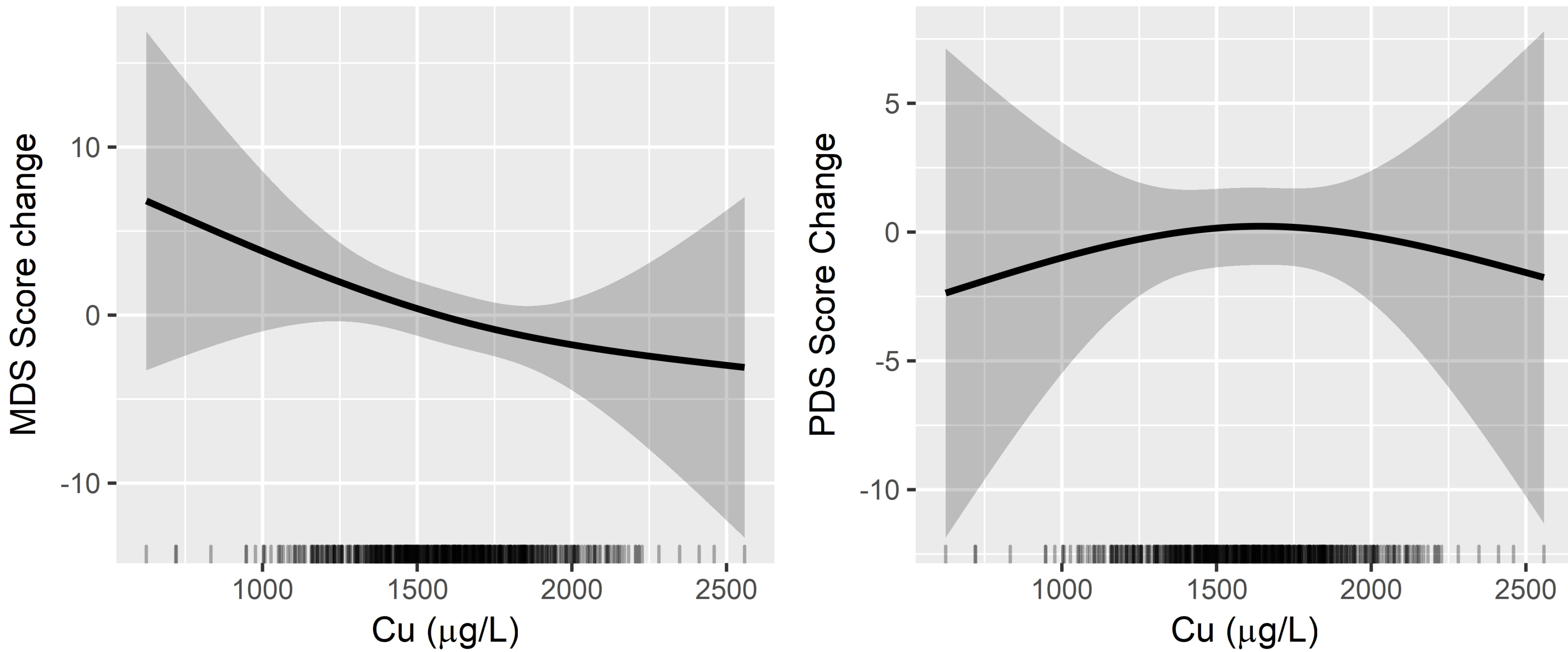
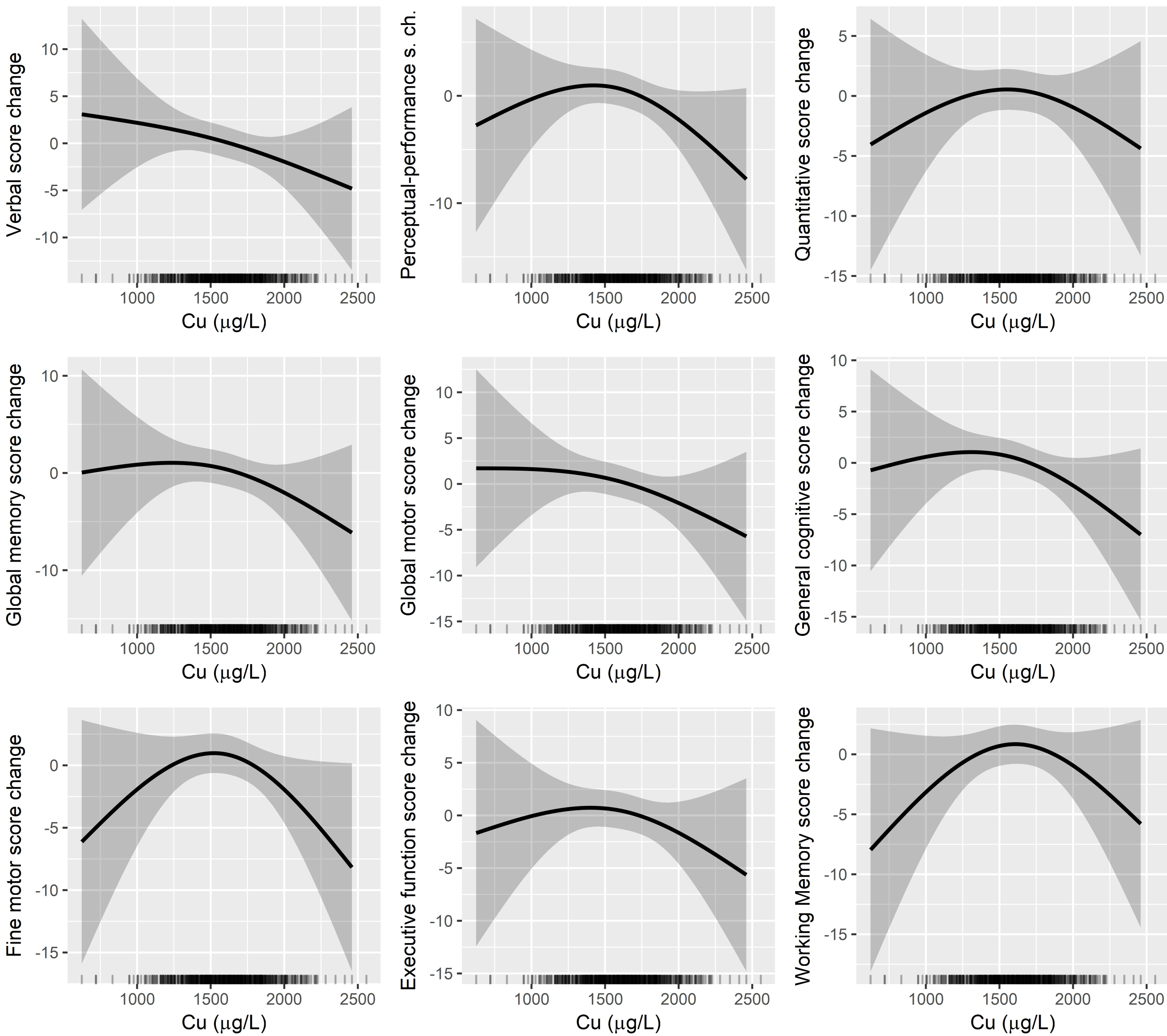
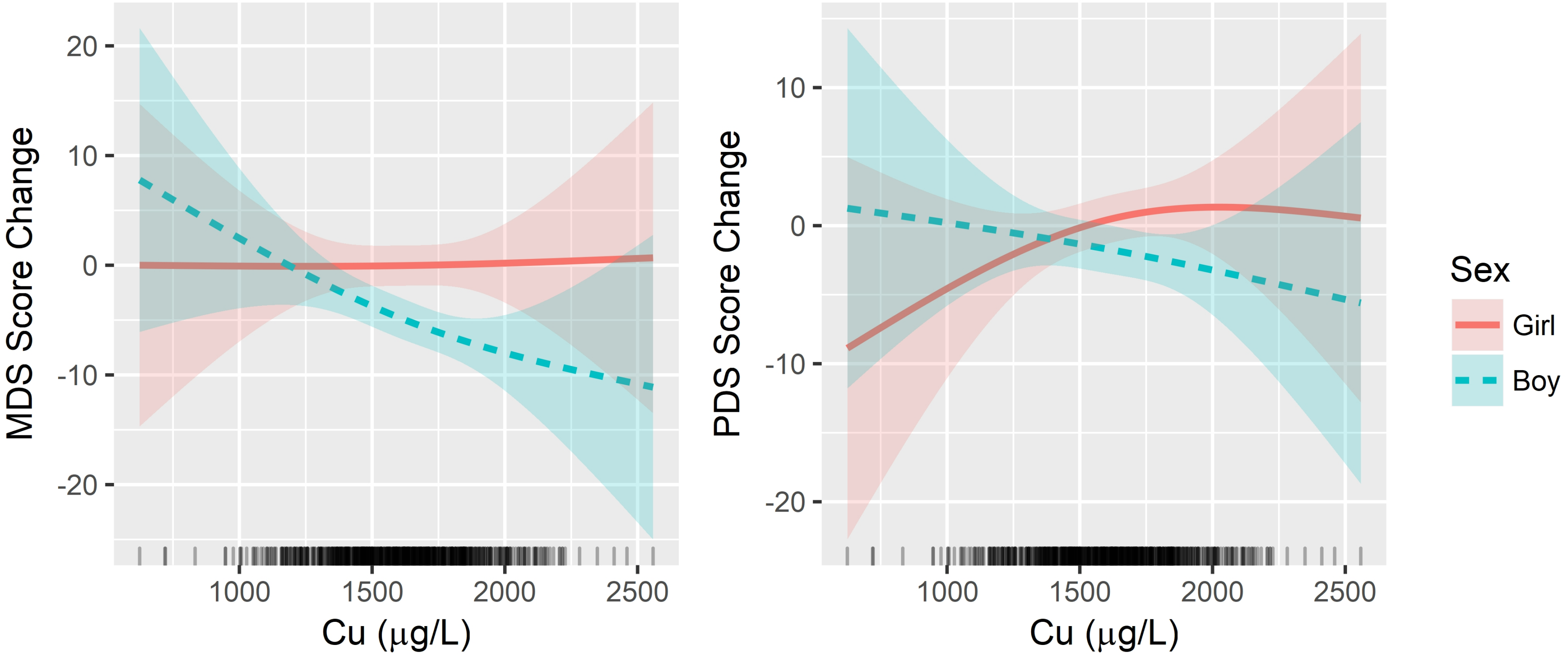


MDS Score change
10
0
-10
1000
1500
2000
2500
Cu (μg/L)
PDS Score Change
5
0
-5
-10
1000
1500
2000
2500
Cu (μg/L)


Verbal score change
10
5
0
-5
-10
1000
1500
2000
2500
Cu (μg/L)
Perceptual-performance s. ch.
0
-10
1000
1500
2000
2500
Cu (μg/L)
Quantitative score change
5
0
-5
-10
-15
1000
1500
2000
2500
Cu (μg/L)
Global memory score change
10
0
-10
1000
1500
2000
2500
Cu (μg/L)
Global motor score change
10
5
0
-5
-10
-15
1000
1500
2000
2500
Cu (μg/L)
General cognitive score change
10
5
0
-5
-10
-15
1000
1500
2000
2500
Cu (μg/L)
Fine motor score change
0
-5
-10
-15
1000
1500
2000
2500
Cu (μg/L)
Executive function score change
10
5
0
-5
-10
-15
1000
1500
2000
2500
Cu (μg/L)
Working Memory score change
0
-5
-10
-15
1000
1500
2000
2500
Cu (μg/L)


MDS Score Change
20
10
0
-10
-20
1000
1500
2000
2500
Cu (μg/L)
PDS Score Change
10
0
-10
-20
1000
1500
2000
2500
Cu (μg/L)
Sex
Girl
Boy

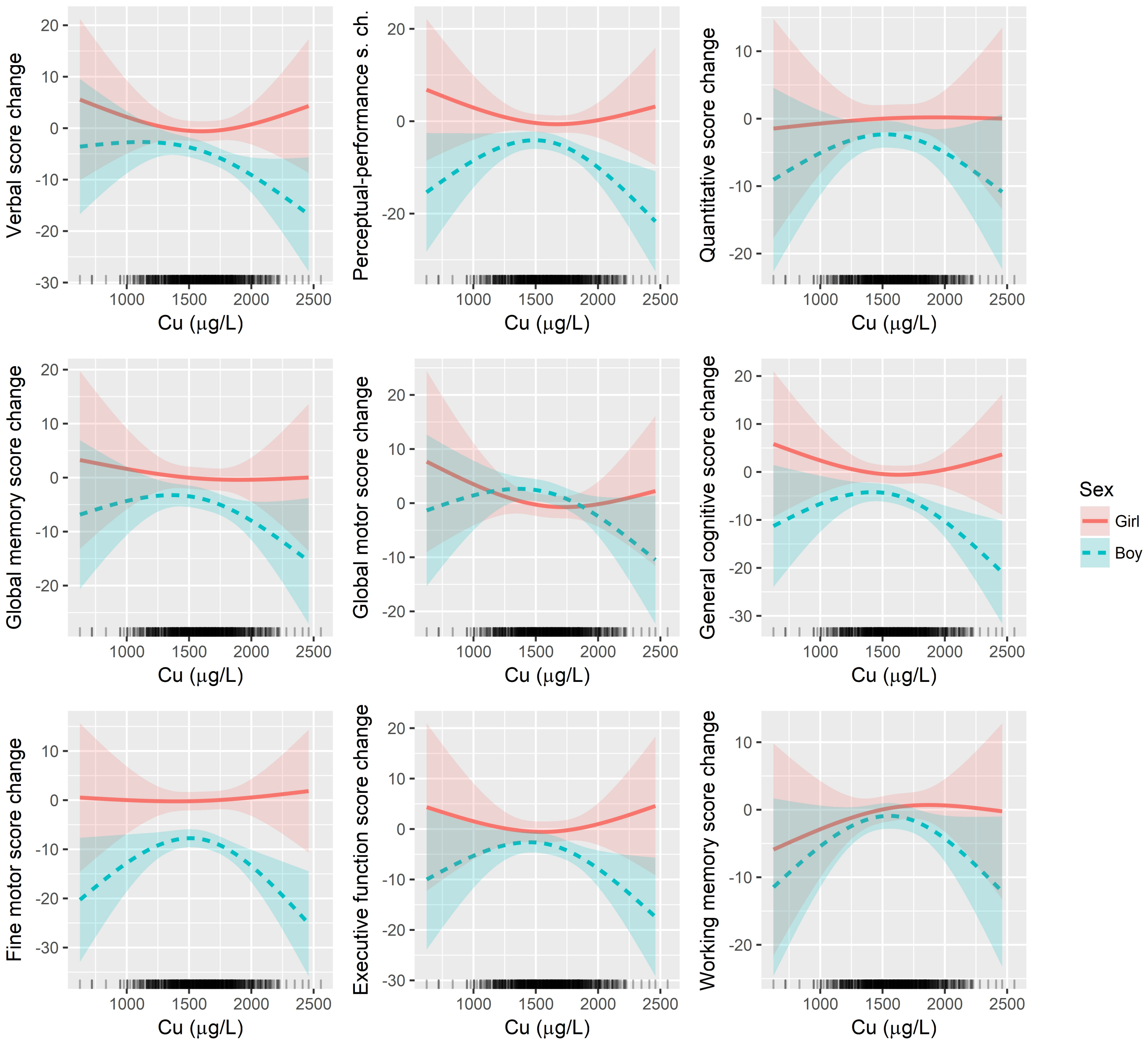

Verbal score change
Perceptual-performance s. ch.
Quantitative score change
Global memory score change
Global motor score change
General cognitive score change
Fine motor score change
Executive function score change
Working memory score change
Cu (μg/L)
Sex
Girl
Boy

Table 1: Maternal sociodemographic, environmental and dietary characteristics associated with maternal Cu concentrations. The INMA Project (Valencia, Spain, 2003−2005).

		N (%)	Univariate analysis				Multivariate analysis			
			Beta	95%CI		p-value	Beta	95%CI		p-value
Country of birth	**Spain**	579 (88.3)								
	Other	77 (11.7)	37.62	-27.06	102.3	0.255				
Age (years)	**<25**	68 (10.4)								
	25-29	223 (34.0)	61.97	-11.61	135.55	0.099	82.46	12.17	152.75	0.022
	30-34	267 (40.7)	14.41	-57.74	86.56	0.696	22.32	-48.4	93.04	0.536
	≥35	98 (14.9)	87.92	4.09	171.75	0.04	66.56	-17.82	150.94	0.122
BMI (Kg/m^2)		23.8 (4.6)[1]	16.69	12.34	21.03	<0.001	15.2	10.87	19.53	<0.001
Educational level	**Primary**	207 (31.6)								
	Secondary	285 (43.4)	-48.32	-96.81	0.17	0.051				
	University	164 (25.0)	-76.69	-132.2	-21.18	0.006				
Parity	**0**	361 (55.0)								
	1	243 (37.0)	73.33	29.56	117.1	0.001	60.46	16.22	104.7	0.007
	≥2	52 (7.9)	122.94	44.7	201.18	0.002	83.89	4.86	162.92	0.037
Area of residence	**Urban**	62 (9.5)								
	Metropolitan	315 (48.1)	-18.83	-92.96	55.3	0.619				
	Semi-Urban	240 (36.6)	-11.03	-87.04	64.98	0.776				
	Rural	38 (5.8)	20.75	-89.17	130.67	0.711				
Employment during pregnancy	**Non-worker**	108 (16.5)								
	Worker	548 (83.5)	-18	-74.17	38.17	0.53				
Social Class	**I+II**	156 (23.8)								
	III	181 (27.6)	-6.74	-64.78	51.31	0.82	-23.7	-79.36	31.96	0.404
	IV+V	319 (48.6)	51.8	-0.11	103.7	0.051	29.67	-21.72	81.06	0.258
Smoking at the beginning of pregnancy	**No**	395 (60.2)								
	Yes	261 (39.8)	-33.39	-75.88	9.1	0.124				
Season of sampling	**Spring**	220 (33.6)								
	Summer	168 (25.6)	-18.5	-73.24	36.24	0.508				
	Autumn	113 (17.3)	21.42	-40.42	83.26	0.497				
	Winter	154 (23.5)	-1.99	-58.13	54.15	0.944				
Gestational age (weeks)		12.7 (1.5)[1]	9.41	4.94	13.88	<0.001	7.64	3.35	11.93	<0.001
Age of the residence (years)	**≤5**	194 (29.7)								
	>5	459 (70.3)	56.32	11.18	101.46	0.015				
Seafood		74.5 (35.1)[1]	0.15	-0.44	0.75	0.613				
Meat		121.7 (40.8)[1]	0.59	0.08	1.1	0.0235				
Cereals and pasta		11.4 (45.9)[1]	0.22	-0.24	0.67	0.356				
Legumes		28.3 (21.9)[1]	-0.23	-1.19	0.73	0.635				

Nuts	4.2 (7.0) [1]	-1.31	-4.32	1.7	0.394
Fruits	273.1 (173.6) [1]	0	-0.12	0.12	0.979
Vegetables	202.5 (108.3) [1]	-0.11	-0.31	0.08	0.252
Eggs	19.4 (9.6) [1]	-0.36	-2.54	1.81	0.745
Dairy products	431.6 (218.1) [1]	-0.01	-0.11	0.08	0.804
Potatoes	54.4 (35.2) [1]	0.43	-0.16	1.03	0.153
Bread	83.2 (49.8) [1]	0.16	-0.27	0.58	0.472

For interpretability of the parameters of the model: the sample mean ± SD of maternal Cu is 1606 ± 272 µg/L

[1]mean (standard deviation)

Dietary variables expressed in grams per day

Table 2: Linear regression analysis between maternal Cu concentrations (increase of 10 µg/L) and the scores for the Bayley scales at 12 months and the McCarthy Scales of Children's Abilities at 5 years of age for all children and stratified by children's sex.

		All children				Male			Female			
Bayley test		**Beta**	**95%CI**		**p-value**	**Beta**	**95%CI**		**Beta**	**95%CI**		**p-value Cu*sex**
	Mental	-0.051	-0.102	-0.001	0.045	-0.114	-0.185	-0.043	0.015	-0.058	0.087	0.040
	Psychomotor	0.003	-0.044	0.051	0.890	-0.058	-0.121	0.005	0.050	-0.022	0.121	0.074
McCarthy scales												
	Verbal	-0.044	-0.094	0.006	0.086	-0.079	-0.153	-0.006	0.014	-0.058	0.087	0.118
	Perceptual performance	-0.032	-0.082	0.017	0.198	-0.040	-0.111	0.032	0.023	-0.092	0.047	0.469
	Quantitative	-0.006	-0.058	0.046	0.827	-0.010	-0.086	0.067	0.005	-0.070	0.080	0.631
	Global Memory	-0.037	-0.089	0.016	0.171	-0.049	-0.127	0.029	-0.014	-0.088	0.059	0.457
	Global Motor	-0.043	-0.096	0.011	0.119	-0.055	-0.134	0.024	-0.036	-0.111	0.039	0.513
	General cognitive	-0.038	-0.087	0.011	0.127	-0.059	-0.132	0.013	-0.001	-0.070	0.067	0.226
	Fine motor	-0.018	-0.067	0.031	0.468	-0.029	-0.093	0.036	-0.001	-0.077	0.076	0.296
	Executive function	-0.025	-0.079	0.028	0.354	-0.053	-0.134	0.028	0.013	-0.060	0.086	0.266
	Working memory	0.005	-0.045	0.056	0.835	-0.017	-0.091	0.057	0.023	-0.048	0.094	0.406

[1]p-value for the interaction between Cu and sex.

Bayley scales adjusted for sex, BMI before pregnancy, parity, type of zone of residence, parental age, BMI before pregnancy, social class, season of sampling and attendance at nursery.

McCarthy scales adjusted for BMI before pregnancy, sex, maternal country of birth, maternal age, parental educational level, parity, type of zone, maternal working situation during pregnancy, social class, breastfeeding, smoking during pregnancy, maternal intelligence, main care provider, maternal smoking at evaluation and paternal working situation at evaluation.

Table 3: Linear regression analysis between maternal Cu concentrations (increase of 10 µg/L) and the scores for both the Bayley scales at 12 months and the McCarthy Scales of Children's Abilities at 5 years of age, stratified by maternal iron and zinc concentrations (<1st tertile vs. >1st tertile).

	Iron							Zinc						
	<1st tertile			>1st tertile			pvalue	<1st tertile			>1st tertile			pvalue
	Beta	95%CI		Beta	95%CI		Cu*Fe	Beta	95%CI		Beta	95%CI		Cu*Zn
Bayley test														
Mental	-0.010	-0.096	0.076	-0.063	-0.123	-0.002	0.696	-0.051	-0.144	0.041	-0.046	-0.104	0.012	0.318
Psychomotor	-0.032	-0.126	0.062	0.015	-0.040	0.069	0.396	0.008	-0.080	0.096	-0.004	-0.060	0.051	0.898
McCarthy scales														
Verbal	-0.108	-0.190	-0.025	-0.018	-0.078	0.041	0.024	-0.039	-0.122	0.044	-0.057	-0.116	0.002	0.770
Perceptual performance	-0.126	-0.207	-0.045	-0.007	-0.064	0.050	0.013	-0.132	-0.216	-0.047	0.005	-0.050	0.061	0.028
Quantitative	-0.039	-0.123	0.046	0.020	-0.041	0.080	0.241	-0.051	-0.137	0.034	0.031	-0.028	0.091	0.143
Global Memory	-0.105	-0.192	-0.019	0.007	-0.054	0.068	0.026	-0.049	-0.141	0.043	-0.016	-0.075	0.043	0.677
Global Motor	-0.119	-0.206	-0.032	0.009	-0.052	0.071	0.025	-0.045	-0.134	0.044	-0.030	-0.091	0.032	0.698
General cognitive	-0.119	-0.201	-0.036	0.000	-0.058	0.059	0.008	-0.071	-0.156	0.014	-0.017	-0.075	0.040	0.290
Fine motor	-0.062	-0.150	0.026	0.019	-0.037	0.076	0.146	-0.069	-0.153	0.016	0.022	-0.036	0.081	0.120
Executive function	-0.091	-0.185	0.004	0.007	-0.056	0.071	0.025	-0.066	-0.156	0.024	-0.002	-0.068	0.063	0.205
Working memory	-0.021	-0.107	0.065	0.028	-0.033	0.089	0.170	-0.028	-0.111	0.056	0.035	-0.027	0.097	0.094

[1]p-value for the interaction between Cu and Fe.
Bayley scales adjusted for sex, BMI before pregnancy, parity, type of zone of residence, parental age, BMI before pregnancy, social class, season of sampling and attendance at nursery.
McCarthy scales adjusted for BMI before pregnancy, sex, maternal country of birth, maternal age, parental educational level, parity, type of zone, maternal working situation during pregnancy, social class, breastfeeding, smoking during pregnancy, maternal intelligence, main care provider, maternal smoking at evaluation and paternal working situation at evaluation.

Supplemental Table 1: Sensitivity analysis of the association between maternal Cu concentrations (increase of 10 µg/L) and the scores for the Bayley scales at 12 months and the McCarthy Scales of Children's Abilities at 5 years of age, excluding preterm and low birth weight infants

		Term infants				Appropiate weight			
Bayley test		**beta**	**95%CI**		**p-value**	**beta**	**95%CI**		**p-value**
	Mental	-0.043	-0.093	0.007	0.093	-0.053	-0.103	-0.002	0.040
	Psychomotor	0.010	-0.038	0.059	0.679	0.006	-0.042	0.053	0.819
McCarthy scales									
	Verbal	-0.049	-0.101	0.003	0.065	-0.053	-0.103	-0.002	0.042
	Perceptual performance	-0.044	-0.094	0.007	0.089	-0.033	-0.084	0.017	0.194
	Quantitative	-0.019	-0.072	0.034	0.474	-0.006	-0.059	0.046	0.810
	Global Memory	-0.046	-0.100	0.009	0.098	-0.043	-0.097	0.011	0.117
	Global Motor	-0.055	-0.109	-0.001	0.046	-0.039	-0.093	0.015	0.152
	General cognitive	-0.050	-0.100	0.001	0.052	-0.043	-0.093	0.006	0.082
	Fine motor	-0.031	-0.080	0.019	0.225	-0.023	-0.072	0.027	0.372
	Executive function	-0.035	-0.089	0.020	0.210	-0.025	-0.078	0.028	0.359
	Working memory	-0.005	-0.056	0.046	0.850	0.005	-0.046	0.055	0.857

Bayley scales adjusted for sex, BMI before pregnancy, parity, type of zone of residence, parental age, BMI before pregnancy, social class, season of sampling and attendance at nursery.
McCarthy scales adjusted for BMI before pregnancy, sex, maternal country of birth, maternal age, parental educational level, parity, type of zone, maternal working situation during pregnancy, social class, breastfeeding, smoking during pregnancy, maternal intelligence, main care provider, maternal smoking at evaluation and paternal working situation at evaluation

Supplemental Table 2: Sensitivity analysis of the association between maternal Cu concentrations (increase of 10 µg/L) and the scores for the Bayley scales at 12 months and the McCarthy Scales of Children's Abilities at 5 years of age, including maternal serum selenium concentrations in the models

		beta	95%CI		p-value
Bayley test					
	Mental	-0.047	-0.098	0.005	0.074
	Psychomotor	0.008	-0.040	0.056	0.738
McCarthy scales					
	Verbal	-0.042	-0.093	0.009	0.107
	Perceptual performance	0.008	-0.040	0.056	0.738
	Quantitative	-0.042	-0.093	0.009	0.107
	Global Memory	-0.028	-0.079	0.022	0.265
	Global Motor	-0.008	-0.061	0.045	0.767
	General cognitive	-0.033	-0.086	0.021	0.228
	Fine motor	-0.038	-0.092	0.017	0.175
	Executive function	-0.036	-0.085	0.014	0.158
	Working memory	-0.015	-0.064	0.035	0.559

Bayley scales adjusted for sex, BMI before pregnancy, parity, type of zone of residence, parental age, BMI before pregnancy, social class, season of sampling and attendance at nursery.
McCarthy scales adjusted for BMI before pregnancy, sex, maternal country of birth, maternal age, parental educational level, parity, type of zone, maternal working situation during pregnancy, social class, breastfeeding, smoking during pregnancy, maternal intelligence, main care provider, maternal smoking at evaluation and paternal working situation at evaluation