Inorganic arsenic exposure and neuropsychological development of children of 4-5 years of age living in Spain

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Abstract

Early-life exposure to inorganic arsenic (iAs) may adversely impact health later in life. To date, evidence of iAs adverse effects on children's neurodevelopment comes mainly from populations highly exposed to contaminated water with conflicting results. Little is known about those effects among populations with low iAs exposure from food intake. We investigated the cross-sectional association between exposure to iAs and neurodevelopment scores among children living in Spain whose main route of exposure was diet. Arsenic species concentrations in urine from 400 children was determined, and the sum of urinary iAs, dimethylarsinic acid, and monomethylarsonic acid was used to estimate iAs exposure. The

McCarthy Scales of Children's Abilities was used to assess children's neuropsychological development at about 4-5 years of age. The median (interquartile range) of children's sum of urinary iAs, MMA, and DMA was 4.85 (2.74 - 7.54) μg/L, and in adjusted linear regression analyses the natural logarithm transformed concentrations showed an inverse association with children's motor functions (**β**, [95% confidence interval]; global scores (-2.29, [-3.95, -0.63]), gross scores (-1.92, [-3.52, -0.31]) and fine scores (-1.54, [-3.06, -0.03]). In stratified analyses by sex, negative associations were observed with the scores in the quantitative index (-2.59, [-5.36, 0.17]) and working memory function (-2.56, [-5.36, 0.24]) only in boys. Our study suggests that relatively low iAs exposure may impair children's neuropsychological development and that sex-related differences may be present in susceptibility to iAs related effects; however, our findings should be interpreted with caution given the possibility of residual confounding.

Keywords: urinary arsenic species, inorganic arsenic, children, neuropsychological development, dietary arsenic, developmental toxicology, neurodevelopment, environment, and McCarthy Scales of Children's Abilities.

Introduction

Arsenic is a ubiquitous element in the environment that occurs in different oxidation states (−3, 0, +3, +5) in both organic and inorganic forms that constitute total arsenic (referred to as "arsenic" in this study) (WHO, 2001). Intake of inorganic arsenic (iAs), including arsenite (AsIII) and arsenate (AsV), is an established cause of cancer of the lung, skin, and bladder and a possible cause of others, with accumulating evidence of effects on non-cancer health outcomes such as neurological, cardiovascular, respiratory, and metabolic diseases (IARC, 2012; Nachman et al., 2017; Sanchez et al., 2016; Tsuji et al., 2015). The metabolism of iAs involves a series of reduction and oxidative methylation processes catalyzed by the enzyme arsenic-methyltransferase with S-adenosylmethionine as the methyl group donor that results in the formation of the pentavalent monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) that are primarily excreted in the urine (Antonelli et al., 2014; Jansen et al., 2016; Tseng, 2009). The trivalent forms of iAs, MMA, and DMA are considered to be more toxic forms with MMAIII having the highest toxicity followed by iAsIII (Tseng, 2009). Direct ingestion of DMA and MMA in the pentavalent form may be excreted in the urine unchanged potentially posing less toxic effects (Buchet et al., 1981; Cohen et al., 2006; Meharg et al., 2014; Molin et al., 2015; Tseng, 2009). The sum of urinary iAs and methylated arsenic species concentrations (i.e. MMA and DMA) is considered a reliable biomarker of short-term exposure to iAs from all sources, and it also appears to be a reliable source of long-term exposure among individuals with consistent patterns of exposure such as child populations whose diet is generally of lower food diversity (EFSA, 2009; Kile et al., 2009; Marchiset-Ferlay et al., 2012; Navas-Acien et al., 2009; Signes-Pastor et al., 2017b). Oxidative stress is considered to be a potential mechanism of iAs toxicity, and increasing evidence suggests that this mechanism may be responsible for iAs related neurotoxicity and impaired neurodevelopment (Grandjean and Landrigan, 2014; Tolins et al., 2014).

A growing number of epidemiologic studies suggest that children's iAs exposure adversely impacts health later in life, including neurodevelopment (EFSA, 2009; Freire et al., 2018; Grandjean and Landrigan, 2014; Nachman et al., 2017; Tolins et al., 2014; Tsuji et al., 2015; Wasserman et al., 2014); however, the consistency and generalizability of these findings has not been established yet, especially among populations whose main exposure source is diet. This includes the Spanish population for whom ingested iAs and organic arsenic is likely to be associated with rice and marine product consumption, respectively (Cubadda et al., 2016; EFSA, 2009; Kurzius-Spencer et al., 2014, 2013; Navas-Acien et al., 2009; Signes-Pastor et al., 2017b). Among populations whose main exposure route to iAs is from food intake, consumption of fish/seafood products needs to be carefully taken into account. These contain high concentrations of arsenobetaine (AsB), a putative non-toxic organic form excreted in urine unchanged, which may cause exposure misclassification of iAs if total urinary arsenic is used as a biomarker of exposure (Forns et al., 2014; Molin et al., 2015; Navas-Acien et al., 2011). Biotransformation of other fish/seafood organosenicals excreted in urine as DMA or direct ingestion of DMA or MMA similarly can be problematic in the assessment of iAs intake (Jones et al., 2016; Meharg et al., 2014; Molin et al., 2015). Currently there is a lack of information regarding the association between early-life neuropsychological development and iAs exposure based on urinary arsenic speciation among populations with access to arsenic drinking water lower than the WHO guideline value of 10 μg/L (Forns et al., 2014; WHO, 2011). In water, arsenic is mostly present as iAs, and relatively low levels of arsenic drinking water have been negatively associated with school-age children's full intelligence quotient (IQ) in the U.S. (Wasserman et al., 2014; WHO, 2011). In water arsenic-contaminated areas of Bangladesh, India and Mexico inverse associations were reported between iAs exposure, assessed using arsenic concentrations in water, urine and blood, and children's cognitive function (Hamadani et al., 2011; Mst. Nasrin Nahar et al., 2014; Parvez et al., 2011; Rosado et al., 2007; Wasserman et al., 2011; WHO, 2011). However, other studies in Bangladesh focused on areas with arsenic-contaminated water have not found evidence of child neuropsychological development in relation to urinary arsenic (Hamadani et al., 2010; Tofail et al., 2009). Further, although a few studies have suggested sex-related differences in iAs-associated neurodevelopmental outcomes, this has not always been observed, and thus further investigations are needed (Hamadani et al., 2011; Llop et al., 2013; Rosado et al., 2007; Sanchez et al., 2016).

In populations with access to low arsenic drinking water, i.e. < 10 μg/L, food is considered to be the major source of iAs exposure (Cubadda et al., 2016; EFSA, 2009; Kurzius-Spencer et al., 2014, 2013), and yet little is known regarding the potential association between dietary iAs exposure and childhood neuropsychological development. In this study, we investigated whether early-life exposure to dietary iAs levels adversely affects children's neuropsychological development. We focused on a population of children of approximately 4-5 years of age living in Spain for whom diet is expected to be the major iAs exposure source (Signes-Pastor et al., 2017b, 2017a). We further explored the possibility of sex-related differences in susceptibility to iAs related neuropsychological outcomes.

Material and methods

Study population.

The study population was derived from the mother-child pair participants in the INMA – INfancia y Medio Ambiente - Environment and Childhood project, a prospective population-based birth cohort study conducted in multiple regions around Spain (www.proyectoinma.org). The general design of INMA has been previously described in detail (Guxens et al., 2012). Briefly, women participants of the INMA project were recruited at the beginning of their pregnancy (2003 - 2006) at their reference primary health care centers or public hospitals and were followed-up until delivery (n = 2,625). All women met the inclusion criteria of ≥ 16 years old, singleton pregnancy, non- assisted conception and delivery scheduled at the reference hospital. Their children were enrolled at birth and were followed-up during infancy and childhood. Informed consent was obtained from all participants in each phase, and the hospitals ethics committees in the participating regions approved the study. For the present study, 100 children, evenly distributed between boys and girls, were randomly selected to provide a urine sample from each sub-cohort located in the Spanish regions of Asturias, Gipuzkoa, Sabadell, and Valencia (overall n = 400) (Signes-Pastor et al., 2017b, 2017a). To date a total of 400 INMA 4-year-old children have had urinary arsenic species concentrations analyzed.

Neuropsychological assessment.

Overall, children's neuropsychological development was assessed at the median age of 4.5 years (standard deviation of 0.6 years) with a standardized version of the McCarthy Scales of Children's Abilities (MSCA) adapted to the Spanish population (McCarthy, 2009). The MSCA was selected because of its reliability and validity, and wide use in research related to environmental health and neurodevelopment including prior studies by INMA (Andiarena et al., 2017; Forns et al., 2012; Nagle, 1979). For children from the sub-cohorts of Asturias, Gipuzkoa and Sabadell (n = 300) the MSCA test was performed at the same time urine samples were collected, along with children's weight and height measured and a food frequency questionnaire (FFQ) at a median age of 4.4 years (standard deviation of 0.2 years); for the Valencian children (n = 100) the neuropsychological assessment was carried out at the median age of 5.8 years (standard deviation of 0.1 years). The urine samples, children's weight, and height, and the FFQ for the Valencian children were collected at a median age of 4.4 years (standard deviation of 0.1 years). Trained psychologists administered the MSCA test. The MSCA test included a battery of 18 subtests (i.e. construction with cubes, puzzle, pictorial memory, vocabulary, calculation, beating sequence, verbal memory, right-left orientation, leg coordination, arm coordination, imitative action, copying of drawings, drawing of a child, numerical memory, verbal fluency, counting and distribution, opposites, and concept formation). The MSCA subtests were grouped into the original function scales of general cognitive, verbal, perceptive-performance, quantitative index, memory, and motor function. With further classification of the MSCA subtests, we obtained the new function scales of executive, working memory, visual and verbal span, verbal memory, gross motor, fine motor,

and cognitive function of the posterior cortex as described in detail previously (Julvez et al., 2011). We previously calculated and reported high intraclass coefficients for the original function scales (> 0.78), and reasonably high Cronbach's alpha coefficients (≥ 0.70) with the new function scales. Further details appear in the prior INMA publication (Valera-Gran et al., 2017).

Sample preparation and chemical analyses.

Arsenic speciation analyses were carried out in spot urine samples (Signes-Pastor et al., 2017a). Urine samples were collected in 100 mL polypropylene containers and immediately stored at or below -20°C, then a 5 mL aliquot from each child in the study were shipped on dry ice to the Institute for Global Food Security at Queen's University Belfast (QUB), Northern Ireland, for arsenic speciation analyses including AsB, DMA, MMA, and iAs. Before speciation, urine samples were centrifuged, and analytical grade hydrogen peroxide was added to convert any arsenite to arsenate to facilitate subsequent chromatographic detection by ion chromatography (IC) with inductively coupled plasma mass spectrometry (ICP-MS). In each analytic batch, blank and replicate samples of the urine lyophilized material ClinChek® - Control level I (Recipe Chemicals + Instruments GmbH in Munich, Germany) were included for quality control. Urine samples were normalized for urine dilution using specific gravity measured with a clinical refractometer. The average recovery percentages and standard deviations of the arsenic species based on several replicate samples of the urine lyophilized material ClinChek®- Control level I (n = 33) were 115 ± 2% for i-As, 97 ± 2% for MMA, 94 ± 2% for DMA, and 90 ± 2% for AsB. The mean and range concentrations of the arsenic species reference values in the urine lyophilized material ClinChek® - Control level I are as follows: 4.55 (2.73 - 6.37) µg/L for i-As, 2.50 (1.50 - 3.50) µg/L for MMA, 9.8 (5.88 - 13.7) µg/L for DMA, and 16.8 (12.6 - 21.0) µg/L for AsB. The limit of detection (LOD) for arsenic speciation, calculated from DMA calibration, was 0.011 µg/L (Signes-Pastor et al., 2017a).

Questionnaire.

In the 1st trimester of pregnancy a maternal questionnaire was administered to gather information regarding parental sociodemographic and socioeconomic characteristics such as the number of previous live births (i.e. 0, 1, 2, or 3), maternal age at conception (years), maternal highest attained level of education (i.e. primary, secondary, or university), and social class according to the International Standard Classification of Occupants (ISCO88) (i.e. upper - I+II, middle - III, or lower - IV+V) (International Labor Office (ILO), 2012). Trained staff measured children's weight (kg) and height (m) at the same time the urine samples were collected following standard protocols to calculate the body mass index (BMI) in kg/m^2. At the same time, parents reported children's diet including consumption of rice and fish/seafood with a validated FFQ (Signes-Pastor et al., 2017b; Vioque et al., 2016). All the aforementioned covariates were among those considered while identifying potential confounders (see Supplemental Material, **Figure S1**, for further details)

Statistical analyses.

For all statistical analyses, observations with missing data for at least one covariate were excluded in addition to children who did not complete the neuropsychological development test. Summary statistics were calculated for each variable: median (range and interquartile range) for continuous variables and n (%) of each level for categorical variables. We calculated the sum of iAs (i.e. arsenite and arsenate), DMA and MMA (referred to as "sum of urinary arsenic" in this study) as a biomarker of iAs exposure. The distribution of children's urinary arsenic species concentrations and sum of urinary arsenic were right skewed, so they were natural logarithm transformed (ln-transformed) before statistical analysis. All scores from the neuropsychological MSCA function scales were standardized to a mean of 100 points with a standard deviation of 15.

The association between children's sum of urinary arsenic concentrations ln-transformed (continuous) and neuropsychological function scores was firstly assessed using univariate linear regression models (Model 0 in Supplemental Material, **Table S1**). Then, multiple linear regression models adjusted for potential confounders were computed (Model 1 in **Table 3** and in Supplemental Material, **Table S1**). The potential confounders were identified using the directed acyclic graph (Textor et al., 2017), and the selected minimally sufficient adjustment set contained: maternal highest attained level of education (i.e. primary, secondary, or university), child's sex (i.e. girls or boys), BMI (continuous), age at MSCA testing (continuous) and calorie adjusted consumption of rice and fish/seafood (continuous) (Supplemental Material, **Figure S1**). The adjusted models were also used to explore the association between children's sum of urinary arsenic concentrations and the neuropsychological scores according to sex in stratified analysis and by including the main effects along with the interaction term (i.e. ln-transformed sum of urinary arsenic concentrations * sex). We carried out multiple sensitivity analyses in the models: i) children's sum of urinary arsenic concentrations were calibrated for fish/seafood consumption using a mathematical method previously described that uses AsB as a biomarker of fish/seafood intake (Model 2 in Supplemental Material, **Table S1**) (Jones et al., 2016), ii) influential points identified with the Bonferroni outlier test of the "car" package were excluded (Fox and Weisberg, 2011), iii) children's hair mercury concentrations analyzed at 4 years were added in the core models as potential confounder (Model 3 in Supplemental Material, **Table S1**), iv) analysis restricted to children with low urinary AsB (i.e. < 1 µg/L) as an indicator of exclusion of fish/seafood consumption (Model 4 in Supplemental Material, **Table S1**) (Jones et al., 2016), vi) and finally, we explored the association between children's ln-transformed sum of urinary arsenic concentrations and the neuropsychological scores adjusting for sub-cohort location (i.e. Asturias, Gipuzkoa, or Sabadell) in addition to the potential confounders described in the core models (Supplemental Material, **Table S2**). Children from Valencia were excluded in the sub-cohort adjusted models to circumvent collinearity between sub-cohort location and age at MSCA test. All analyses were carried out with the R software for statistical computing version 3.5.1 (R Core Team, 2014). A threshold of p-value < 0.05 was used to define associations as statistically significant.

Results

Of the 400 children evaluated, 361 (90%) were ultimately included in the analyses because they did not contain missing values in neither neuropsychological development test nor other covariates. Our study sample contained 185 (51%) girls and 176 (49%) boys. Children's median (interquartile range) sum of urinary arsenic concentrations was 4.85 (2.74 - 7.54) μg/L overall, and 4.76 (2.36 - 7.48) μg/L and 4.96 (3.09 - 7.60) μg/L for the girls and boys, respectively. Almost all children reported school attendance at 4 years across all sub-cohort locations. Refer to **Table 1** for further details.

We also assessed characteristics of the study population stratified by the median concentration of 4.85 μg/L of the sum of urinary arsenic. Children with ≥ 4.85 μg/L also had higher concentrations of urinary AsB with a median of 15.95 μg/L versus 5.41 μg/L ($p < 0.001$). We did not observe statistically significant differences with other characteristics of the study population (**Table 2**).

We observed a negative linear association between ln-transformed sum of urinary arsenic concentrations and the scores from the original global motor function (**β** = -2.29, 95% confidence interval (CI) = [-3.95, -0.63], $p = 0.007$), the derived gross motor function (**β** = -1.92, 95% CI = [-3.52, -0.31], p = 0.020) and fine motor function (**β** = -1.54, 95% CI = [-3.06, -0.03], $p = 0.046$) after adjustment for maternal highest attained level of education, child's sex, BMI, age at MSCA testing, and calorie adjusted consumption of rice and fish/seafood (**Table 3**). We did not observe any clear association with the remaining MSCA function scores and children's ln-transformed sum of urinary arsenic concentrations (**Table 3**).

In the stratified analyses by sex, we found negative trends between boy's ln-transformed sum of urinary arsenic concentrations and the scores of quantitative index and with the derived working memory function (**β** = -2.59, 95% CI = [-5.36, 0.17], $p = 0.066$, and **β** = -2.56, 95% CI = [-5.36, 0.24], $p = 0.073$, respectively), which were supported by low p-values in the interaction term ln-transformed sum of urinary arsenic concentrations and sex in the core models ($p = 0.065$ and $p = 0.052$, respectively). Further, we observed a stronger negative trend with an average of 5-fold higher regression coefficient between ln-transformed sum of urinary arsenic concentrations and the remaining neuropsychological function scores in boys compared to girls, but they did not achieve statistical significance (**Table 3**).

We did not observe any major changes in the regression coefficients between models (i.e. unadjusted (Model 0), adjusted for confounders (Model 1), with calibrated children's sum of urinary arsenic for consumption of fish/seafood (Model 2), and adjusted for children's hair mercury concentrations (Model 3 in Supplemental Material, **Table S1**). The restrictive analysis including only children who did not consume fish/seafood also followed similar trends; however, wider confidence intervals were observed owing to the small dataset ($n = 49$) (Model 4 in Supplemental Material, **Table S1**). The results from the adjusted sub-cohort location models, excluding children from Valencia, followed the trend of our primary findings; however, the regression coefficients were attenuated (Supplemental Material, **Table S2**). The mathematically calibrated urinary arsenic species concentrations (i.e. iAs, DMA and MMA) and

their sum removed any association with urinary AsB concentrations and had Pearson's correlation coefficients (r) < 0.017, $p = 0.745$). Calibrated children's ln-transformed sum of urinary arsenic concentrations did not appreciably alter the association with the scores in the original scale of global motor function (**β** = -2.11, 95% CI = [-3.86, -0.36], $p = 0.018$) and strengthened the negative association between ln-transformed sum of urinary arsenic concentrations and children's scores on fine motor function (**β** = -1.82, 95% CI = [-3.41, -0.22], $p = 0.026$) (Model 2 in Supplemental Material, **Table S1**). In contrast, the regression coefficient between calibrated children's ln-transformed sum of urinary arsenic concentrations and the scores in gross motor was modestly attenuated and lost statistical significance (**β** = -1.38, 95% CI = [-3.08, 0.32], $p = 0.112$) (Model 2 in Supplemental Material, **Table S1**). Similar results were obtained when adjusting for children's hair mercury concentrations (Model 3 in Supplemental Material, **Table S1**). We did not observe any major change in the sensitivity statistical analyses when excluding the identified outliers ($n = 10$) (data not shown).

Discussion

In this study, sum of urinary arsenic concentrations including iAs, MMA, and DMA were used as a biomarker of iAs exposure. We observed that the sum of urinary arsenic concentrations was negatively associated with the scores in the neuropsychological assessment of global, gross and fine motor function among children of approximately 4-5 years of age living in Spain after adjusting for potential confounding factors. Our findings also suggest that boys may be more susceptible to iAs neurotoxicity. In particular, we found a stronger negative trend between ln-transformed sum of urinary arsenic concentrations and children's scores in the neuropsychological quantitative and working memory function scales for boys compared to girls.

In Spain, drinking water usually complies with the EU drinking water iAs regulation, set at 10 µg/L (The Council of the European Union, 1998) with a reported median level < 1 µg/L (Espejo-Herrera et al., 2013; Palau Miguel and Guevara Alemany, 2011). Thus, diet is expected to be the main source of iAs exposure for our study population (Davis et al., 2017; Signes-Pastor et al., 2017b). Spain is the second largest producer of rice in the EU and rice consumption is strongly rooted in the Spanish gastronomic culture (Comission, 2015; Signes-Pastor et al., 2017b). Rice contains about 10-fold higher iAs compared to other cereals and the concentrations vary geographically (Meharg et al., 2009; Meharg and Zhao, 2012). We have previously reported that rice consumption in our study population was correlated with an increase of urinary iAs, and more weakly with the sum of urinary arsenic concentrations (Signes-Pastor et al., 2017b). Using the median cut point as in **Table 2**, the difference was not statistically significant, which may be in part because the concentrations of arsenic in rice vary widely and in our previous work in Spain ranges from 37 to 407 µg/kg (Signes-Pastor et al., 2016). Also, lack of associations or strong correlations may be related to misclassification of reporting of rice intake using a FFQ that asks about intake over the past year, and not the time period reflective of urinary excretion of arsenic (e.g., the past few days). Fish/seafood

consumption is also an important part of the Spanish diet, and it contributes to the ingestion of AsB, and tends to dominate exposure to organic arsenic from food intake in the Spanish and other populations with similar gastronomic cultures (Navarro Serrano et al., 2016; Taylor et al., 2016). In this study, the AsB concentrations contributed to over half of the sum of all urinary arsenic species analyzed (i.e. median (interquartile range) of [AsB /(iAs + MMA + DMA + AsB) *100] equals 67.0% (41.4% - 86.8%)) and was correlated with children's fish/seafood consumption (Signes-Pastor et al., 2017b), and thus, was critical to remove from our analysis of iAs exposure.

Numerous studies have reported detrimental effects on neuropsychological development of children living in areas with arsenic-contaminated drinking water with urinary arsenic concentrations 1-2 orders of magnitude higher compared to the levels found in this study (Mst Nasrin Nahar et al., 2014; Mst. Nasrin Nahar et al., 2014; Parvez et al., 2011; von Ehrenstein et al., 2007; Wasserman et al., 2011; WHO, 2011). Although iAs exposure in our study population was low, we observed negative associations between iAs exposure and children's scores in the neuropsychological motor function scales that involve skills such as playing with a ball and drawing. For each interquartile range increase in exposure, we found a decrease of over 2 points in the scores for global motor and gross motor scores, and 1.5 points in the scores for the fine motor function.

Only a few studies have been conducted in populations with low drinking water arsenic concentrations (Forns et al., 2014; Freire et al., 2018; Wasserman et al., 2014). In a cross-sectional study from Maine, among ~10-year-old children, home tap water with arsenic ≥ 5 µg/L was associated with reductions in full-scale IQ, and with all index scores, i.e. working memory, perceptual reasoning, and verbal comprehension (Wasserman et al., 2014). A recent study from INMA has reported that arsenic levels in placenta were associated with decrements in global and verbal executive function and quantitative abilities and could also be a risk factor for motor impairment in children of 4-5 years of age (Freire et al., 2018). Another prior study from INMA carried out in the sub-cohort of Sabadell did not find associations between maternal urinary arsenic concentrations during pregnancy and children's neuropsychological development at the age of 4 years (Forns et al., 2014). However, total urinary arsenic concentrations including AsB was used leaving open the likelihood of exposure misclassification (Feldmann and Krupp, 2011; Jones et al., 2016; Molin et al., 2015, 2014, 2012; Signes-Pastor et al., 2017b). In this study, iAs exposure was estimated with sum of urinary iAs, MMA, and DMA. We have previously reported lack of correlation between fish/seafood consumption and urinary iAs, MMA, and DMA concentrations (Signes-Pastor et al., 2017b). However, urinary DMA from biotransformation of organosenicals from marine product consumption (i.e. arsenosugars and arsenolipids) may still overestimate iAs. Thus, we adjusted for fish and seafood consumption and performed several sensitivity analyses (Jones et al., 2016; Molin et al., 2015, 2014, 2012; Signes-Pastor et al., 2017b). Indeed, to address the potential for overestimation of exposure from fish/seafood consumption (Signes-Pastor et al., 2017b), we calibrated children's urinary arsenic species concentrations using a residual-based method (Jones et al., 2016). Nevertheless, our analyses using adjusted or calibrated sum of

urinary arsenic concentrations for fish/seafood consumption generally did not result in appreciable changes in our findings. Similar results were observed when adjusting for children's hair mercury concentrations as a biomarker of fish/seafood intake (Elhamri et al., 2007). Also, similar findings were obtained when we restricted our analysis to only children without fish/seafood consumption (i.e. urinary AsB < 1 μg/L), which despite the small sample size (n = 49) produced an inverse trend between exposure to iAs and children's scores in global and fine motor function. Rice contains iAs but also DMA and potentially traces of MMA (Meharg and Zhao, 2012) that may be excreted in the urine unchanged raising concerns of potential iAs exposure misclassification, and therefore we adjusted the regression models for rice intake. Cadmium exposure has been associated with impaired child development (Forns et al., 2014; Freire et al., 2018; Kippler et al., 2012), and thus we analyzed cadmium concentrations in rice from Spain as a potential exposure source; however, we found levels almost undetectable owing to its cultivation under flooded conditions (Arao et al., 2009; Signes-Pastor et al., 2016). Information on children's cadmium level of exposure in our study population is not available yet; however, we would expect levels to be lower than those of children from an industrial and mining region in southwestern Spain and possibly more similar to that reported in children of 6-8 years in Germany or 6-11 years in the U.S. (Rodríguez-Barranco et al., 2014). A preliminary analysis of 5-year-old children from the New Hampshire Birth Cohort Study do not suggest a strong correlation between the children's urinary iAs and cadmium concentrations (n = 389; Spearman r = 0.2) (personal communication). In order to address residual confounding from mercury exposure as a risk factor (Freire et al., 2018), we adjusted our core models for children's hair mercury concentrations. Children's diet differed by sub-cohort location (Supplemental Material, Table S3) along with their urinary AsB, MMA, and iAs concentrations, but not DMA (Signes-Pastor et al., 2017a). However, they did not differ in their sum of urinary arsenic concentrations (Supplemental Material, Table S3). In order to account for geographical differences in metal exposure (Freire et al., 2018), we adjusted for sub-cohort location excluding children from Valencia because of collinearity between sub-cohort location and age at MSCA test and the results followed the trend of our main findings, but the strength of the associations were attenuated. We did not consider exposure to lead and manganese as risk factors (Freire et al., 2018), and that is a limitation of our study since they could result in residual confounding if they were strongly associated with iAs exposures; however, we do not expect that to be the case.

Our sex-stratified analyses are based on relatively small sample sizes, and therefore caution must be taken in the interpretation of the results. Our findings suggest that boys may be more susceptible to iAs neurotoxicity compared to girls particularly for cognitive tasks related to numerical function, and temporarily storing and managing information. For each interquartile range increase in exposure, we found a decrease of 2.6 points in the scores for the quantitative index and working memory among boys. In contrast, in a study from Bangladesh, pre- and post-natal exposure to iAs was inversely associated with verbal and full-scale IQ in girls of 5 years of age (Hamadani et al., 2011). In an industrial polluted area in Mexico, an inverse association was identified between urinary arsenic concentrations and problem solving, vocabulary and attention scores among boys, and with memory among girls at the age ranging

from 6 to 8 years (Rosado et al., 2007). Sex- related differences in susceptibility to metals toxicity have been associated with differences in patterns of exposure, gastrointestinal absorption, metabolism, and detoxification (Llop et al., 2013; Tseng, 2009); however, information regarding early-life gender differences in susceptibility to iAs neurotoxicity is scarce and will require further investigation (Llop et al., 2013).

This study is among the first to assess the association between iAs exposure, mainly from diet, and neuropsychological development of children taking part in a well-designed cohort (Gascon et al., 2017), and despite the relatively small size of the study population and relatively low level of iAs exposure, we observed associations between children's iAs exposure and the scores in various neuropsychological function scales. Our results should be interpreted cautiously given the cross-sectional design of the study that precludes us from determining temporality and thus limits any inferences about causality. We adjusted for several potential confounding factors, but the effect of unknown factors such as other environmental/dietary factors or residual confounding remains a possibility. A particularly small sample size was used in the sex-stratified analyses with limited statistical power. Children's daily rice and fish/seafood consumption were measured in personal interviews with parents using a validated FFQ (Vioque et al., 2016). The FFQ is considered a reliable method to assess usual diet in epidemiologic studies (Willett, 2012). In this study, the validity of the FFQ was examined by comparing the nutrient values from FFQ with the average nutrient values of three 24-hour dietary recalls, and with the concentrations in blood specimens for several vitamins (i.e. carotenoids, folate, vitamin B12, vitamin C and **α**-tocopherol) (Vioque et al., 2016). A mathematical method independent to the data recorded on the FFQ was applied to calibrate children's sum of urinary arsenic concentrations for fish/seafood intake. Further, we carried out analysis adjusting for children's hair mercury concentrations, sub-cohort location, and analysis including only children without fish/seafood consumption. In general, sensitivity analyses supported our primary findings, with some attenuation with adjustment for sub-cohort location possibly due the reduced statistical power. Further, multiple testing could have led to false positive results, and therefore our finding should be interpreted with caution and be explored if they persist in further follow-up assessments (Blakesley et al., 2009; Rothman, 1990).

In conclusion, our study focused on a population with low arsenic in drinking water but who consume iAs in their diet, exposure to iAs was related to certain domains of neuropsychological function scores, in particular motor development. Our findings, along with others, support the reduction of iAs exposure particularly during critical developmental windows early in life.

Competing financial interests

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

Conflict of interest

The authors do not have conflicts of interest to declare.

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Table 1: Selected characteristics of the study population for the entire dataset and stratified by sex (minimum; interquartile rage; maximum) for continuous and *n* (%) for categorical variables.

Selected characteristics of the study population	All (*n* = 361)		Girls (*n* = 185)	Boys (*n* = 176)	*p*-value
Children:					
Sum of urinary arsenic concentrations (µg/L)[1]	4.85 (0.12; 2.74 -7.54; 84.46)		4.76 (0.21; 2.36 - 7.48; 84.46)	4.96 (0.12; 3.09 - 7.60; 47.65)	0.393
Urinary AsB (%)2	67.0 (3.4; 41.4 - 86.8; 100)		67.8 (5.9; 44.4 - 86.8; 100)	66.5 (3.4; 37.4 - 86.8; 100)	0.493
Rice consumption (g/day)	27.2 (0.9; 27.2 – 39.9; 155.2)		26.1 (5.2; 16.2 – 37.8; 142.2)	28.5 (0.9; 18.9 – 40.9; 155.2)	0.373
Fish/Seafood consumption (g/day)	39.9 (10.5; 31.7 - 48.8; 102.3)		40.6 (10.5; 32.5 - 49.3; 102.3)	38.4 (11.2; 29.9 - 48.6; 91.1)	0.078
Sub-cohort (n)					
Asturias	96 (27)		48 (26)	48 (27)	0.932
Gipuzkoa	90 (25)		47 (25)	43 (24)	
Sabadell	76 (21)		41 (22)	35 (20)	
Valencia	99 (27)		49 (26)	50 (28)	
BMI (kg/m2)	16.0 (11.5; 15.2 – 17.2; 25.0)		16.0 (12.9; 15.2 – 17.1; 23.5)	15.9 (11.5; 15.3 – 17.3; 25.0)	0.578
Maternal:					
Age at enrollment (years)	31 (21; 29 – 34; 43)		31 (21; 29 – 34; 43)	31 (21; 29 – 34; 42)	0.277
Social class					
Upper - I+II	83 (23)		43 (23)	40 (23)	0.483
Middle - III	106 (29)		59 (32)	47 (27)	
Lower - IV+V	172 (48)		83 (45)	89 (50)	
Highest attained level of education					
Primary	70 (19)		35 (18)	35 (20)	
Secondary	148 (41)		75 (41)	73 (41) 0.929	
University	143 (40)		75 (41)	68 (39)	
Number of previous live births					
0	198 (55)		103 (56)	95 (54)	0.278
1	141 (39)		68 (37)	73 (41)	
2	21 (6)		14 (7)	7 (4)	
3	1 (0)		0 (0)	1 (1)	

For test of differences by sex, we used Welch's t-test or Wilconxon's rank test for continuos variables, and Chi-square or Fisher's exact test for categorical variables. BMI = Body mass index.

[1]DMA + MMA + iAs.

[2]AsB (%) = (AsB/(iAs + MMA + DMA + AsB)) *100.

Table 2: Selected characteristics of the study population stratified by the median of the sum of urinary arsenic species concentration (4.85 µg/L) (minimum; interquartile rage; maximum) for continuous and *n* (%) for categorical variables.

Selected characteristics of the study population		< 4.85 µg/L (*n* = 180)	≥4.85 µg/L (*n* = 181)	*p*-value
Children:				
Sex	Girls	96 (53)	89 (49)	0.493
	Boys	84 (47)	92 (51)	
Rice consumption (g/day)		26.7 (0.1; 18.2–36.4; 155.2)	27.9 (0.9; 18.7–42.3; 96.8)	0.587
Fish/Seafood consumption (g/day)		39 (10.5; 29.6–48.1; 88.5)	40.1 (14.9; 33.5–50.0; 102.3)	0.090
Urinary arsenobetaine (µg/L)		5.41 (0.05; 1.24–17.47; 3569)	15.95 (0.29; 5.90–59.00; 1098)	< 0.001
Sub-cohort	Asturias (*n*)	49 (27)	47 (26)	0.863
	Gipuzkoa (*n*)	45 (25)	45 (25)	
	Sabadell (*n*)	40 (22)	36 (20)	
	Valencia (*n*)	46 (26)	53 (29)	
BMI (kg/m^2)		15.9 (11.5; 15.2–16.9; 25.0)	16.1 (12.9; 15.2–17.5; 21.0)	0.546
Maternal:				
Enrollment	Age (years)	31.0 (21.0; 29.0–34.2; 43.0)	31.0 (24.0; 29.0–34.0; 42.0)	0.624
Social class	Upper - I+II	42 (23)	41 (23)	0.807
	Middle - III	50 (28)	56 (31)	
	Lower - IV+V	88 (49)	84 (46)	
Highest attained level of education	Primary	37 (21)	33 (18)	0.583
	Secondary	69 (38)	79 (44)	
	University	74 (41)	69 (38)	
Number of previous live births	0	91 (51)	107 (59)	0.150
	1	74 (41)	67 (37)	
	2	14 (8)	7 (4)	
	3	1 (1)	0 (0)	

For test of differences by sex, we used Welch's t-test or Wilconxon's rank test for continuous variables, and Chi-square or Fisher's exact test for categorical variables. BMI = Body mass index.

Table 3: Association between children's sum of urinary arsenic concentrations (ln-transformed) and the McCarthy Scales of Children's Ability scores standardized to a mean of 100 points with a standard deviation of 15 according to child sex.

McCarthy Scales of Children's Abilities		Model 1 (n = 361)[a]				Girls (n = 185)[c]				Boys (n = 176)[c]				
		β	95% CI		*p-value*	β	95% CI		*p-value*	β	95% CI		*p-value*	*p-value*
Original functions	General cognition	−0.86	−2.43	0.71	0.281	−0.08	−2.00	1.84	0.937	−1.87	−4.58	0.84	0.176	0.213
	Verbal	−0.20	−1.88	1.49	0.819	0.71	−1.37	2.79	0.502	−1.54	−4.43	1.34	0.293	0.208
	Perceptual-performance	−1.30	−2.79	0.2	0.09	−0.94	−2.78	0.9	0.313	−1.56	−4.14	1.03	0.236	0.539
	Quantitative index	−0.91	−2.58	0.77	0.288	0.28	−1.84	2.39	0.796	−2.59	−5.36	0.17	0.066	0.065
	Memory	−0.75	−2.39	0.88	0.367	0	−2.16	2.17	0.997	−1.63	−4.20	0.94	0.212	0.224
	Global motor	−2.29	−3.95	−0.63	0.007	−1.85	−3.84	0.15	0.069	−3.00	−5.93	−0.07	0.045	0.533
New functions	Executive	−0.28	−1.86	1.3	0.727	0.54	−1.33	2.41	0.57	−1.56	−4.35	1.23	0.27	0.188
	Visual executive	−0.53	−2.10	1.04	0.508	−0.56	−2.49	1.38	0.571	−0.43	−3.08	2.22	0.751	0.971
	Verbal executive	−0.16	−1.82	1.5	0.85	1	−0.92	2.92	0.307	−2.00	−5.00	0.99	0.189	0.085
	Visual and verbal span	−0.50	−2.16	1.16	0.557	−0.36	−2.63	1.92	0.757	−0.64	−3.11	1.84	0.611	0.754
	Working memory	−0.67	−2.37	1.04	0.442	0.61	−1.57	2.79	0.581	−2.56	−5.36	0.24	0.073	0.052
	Verbal memory	−0.58	−2.26	1.11	0.501	0	−2.11	2.12	0.999	−1.03	−3.85	1.79	0.471	0.446
	Gross motor	−1.92	−3.52	−0.31	0.02	−1.86	−3.67	−0.04	0.045	−2.27	−5.24	0.69	0.132	0.931
	Fine motor	−1.54	−3.06	−0.03	0.046	−0.98	−2.95	0.98	0.326	−2.18	−4.66	0.3	0.085	0.394
	Cognitive function of posterior cortex	−1.18	−2.80	0.45	0.156	−0.24	−2.28	1.79	0.813	−2.23	−4.97	0.52	0.111	0.177

[a,b]Multiple linear regression models adjusted for maternal highest attained level of education (i.e. primary, secondary, or university), and children's sex (i.e. girls or boys), BMI (kg/m^2), age at MSCA (years) and calorie adjusted consumption of rice and fish/seafood (g/day). [c]Multiple linear regression models adjusted for maternal highest attained level of education (i.e. primary, secondary, or university), and children's BMI (kg/m^2), age at MSCA (years) and calorie adjusted consumption of rice and fish/seafood (g/day). [d]Interaction between children's sum of urinary arsenic species concentrations (ln-transformed) and sex.

Supplemental Material

Table S1: Association between children's sum of urinary arsenic concentrations (ln-transformed) and the McCarthy Scales of Children's Ability scores

McCarthy Scales of Children's Abilities		Model 0 (*n* = 361)				Model 1 (*n* = 361)				Model 2* (*n* = 361)				Model 3# (*n* = 234)				Model 4 (*n* = 49)			
		β	95% CI		*p-value*	β	95% CI		*p*-value	β	95% CI		*p*-value	β	95% CI		*p*-value	β	95% CI		*p*-value
Original functions	General cognitive	-0.74	-2.37	0.90	0.376	-0.86	-2.43	0.71	0.281	-0.80	-2.46	0.85	0.340	0.48	-1.46	2.41	0.629	-1.87	-7.41	3.67	0.499
	Verbal	-0.07	-1.79	1.64	0.933	-0.20	-1.88	1.49	0.819	-0.01	-1.79	1.77	0.991	1.45	-0.68	3.57	0.182	-0.66	-6.02	4.71	0.806
	Perceptual performance	-1.25	-2.80	0.30	0.113	-1.30	-2.79	0.20	0.090	-1.31	-2.88	0.27	0.104	-1.01	-2.87	0.85	0.285	-3.32	-8.15	1.51	0.173
	Quantitative index	-0.79	-2.48	0.91	0.362	-0.91	-2.58	0.77	0.288	-0.99	-2.75	0.77	0.270	0.63	-1.33	2.59	0.527	-0.28	-6.12	5.56	0.922
	Memory	-0.54	-2.21	1.13	0.523	-0.75	-2.39	0.88	0.367	-0.88	-2.61	0.85	0.316	0.48	-1.53	2.49	0.640	2.37	-3.07	7.81	0.383
	Global motor	-2.16	-3.82	-0.50	0.011	-2.29	-3.95	-0.63	0.007	-2.11	-3.86	-0.36	0.018	-2.12	-4.22	-0.02	0.048	-4.77	-10.03	0.49	0.074
New functions	Executive	-0.12	-1.74	1.49	0.882	-0.28	-1.86	1.30	0.727	-0.10	-1.76	1.56	0.903	1.06	-0.90	3.02	0.286	-2.13	-7.77	3.50	0.449
	Visual executive	-0.42	-2.01	1.17	0.601	-0.53	-2.10	1.04	0.508	-0.39	-2.04	1.26	0.641	0.06	-1.92	2.05	0.950	-0.51	-6.06	5.03	0.852
	Verbal executive	-0.01	-1.69	1.67	0.991	-0.16	-1.82	1.50	0.850	0.00	-1.74	1.75	0.997	1.29	-0.81	3.39	0.227	-2.39	-7.98	3.20	0.393
	Visual and verbal span	-0.35	-2.02	1.33	0.685	-0.50	-2.16	1.16	0.557	-0.49	-2.24	1.26	0.585	0.52	-1.47	2.51	0.609	3.75	-2.02	9.51	0.197
	Working memory	-0.48	-2.19	1.23	0.582	-0.67	-2.37	1.04	0.442	-0.69	-2.48	1.11	0.454	0.43	-1.63	2.48	0.683	-0.21	-6.79	6.36	0.948
	Verbal memory	-0.42	-2.12	1.28	0.625	-0.58	-2.26	1.11	0.501	-0.75	-2.53	1.03	0.410	0.42	-1.64	2.49	0.686	0.43	-4.85	5.70	0.871
	Gross motor	-1.68	-3.32	-0.04	0.045	-1.92	-3.52	-0.31	0.020	-1.38	-3.08	0.32	0.112	-1.31	-3.33	0.70	0.201	-2.52	-7.71	2.67	0.332
	Fine motor	-1.59	-3.16	-0.02	0.047	-1.54	-3.06	-0.03	0.046	-1.82	-3.41	-0.22	0.026	-1.79	-3.67	0.09	0.062	-4.77	-10.20	0.67	0.084
	Cognitive function of posterior cortex	-1.14	-2.82	0.55	0.186	-1.18	-2.80	0.45	0.156	-1.18	-2.90	0.53	0.176	0.09	-1.93	2.11	0.928	-1.40	-6.68	3.89	0.596

Model 0: Univariant models. **Model 1**: Models adjusted for maternal highest attained level of education (i.e. primary, secondary, or university), children's sex (i.e. girls or boys), BMI (kg/m^2), age at MSCA test (years) and calorie adjusted consumption of rice and fish/seafood (g/day). **Model 2**: The sum of urinary arsenic species concentrations were calibrated following a mathematical method previously described (Jones *et al.* 2016) and the confounders were those included in Model 1 excluding consumption of fish/seafood. **Model 3**: In addition to the potential confounding factors included in Model 1 we added children's hair mercury concentrations analyzed at 4 years. **Model 4**: Analyses were restricted to children with low urinary AsB (i.e. < 1 µg/L) as an indicator of exclusion of fish/seafood consumption and the models were adjusted for the potential confounding factors included in Model 1.

*We calibrated children's urinary arsenic species concentrations following a methodology previously described (Jones et al. 2016). This methodology takes advantage of the fact that urinary arsenobetaine (AsB), a putative non-toxic form of arsenic excreted unchanged rapidly in urine, is an adequate biomarker of fish/seafood intake. To proceed with the calibration, the original sum of urinary arsenic concentrations (i.e. iAs + MMA + DMA) were regressed by the urinary AsB and the model residuals were extracted. Then, we added the mean level of the urinary arsenic species concentrations of participants with low AsB (<1 μg/L; n = 49) to the residuals, assuming that iAs exposure levels not derived from fish and seafood are similar in participants with low and high AsB concentrations (Jones et al. 2016). Finally, the calibrated children's urinary arsenic concentrations were included as an independent variable in the multiple linear regression core models adjusted also for potential confounding factors to assess the association with children's neuropsychological scores.

[#]Among children included in our models only 234 had their hair mercury concentrations analyzed.

Table S2: Association between children's sum of urinary arsenic concentrations (ln-transformed) and the McCarthy Scales of Children's Ability scores adjusted by sub-cohort location.

McCarthy Scales of Children's Abilities		Asturias, Gipuzkoa, and Sabadell (n = 262)[a]			
		β	95% CI		*p-value*
Original functions	General cognition	-0.63	-2.42	1.16	0.487
	Verbal	-0.13	-2.04	1.77	0.891
	Perceptual-performance	-0.51	-2.22	1.20	0.558
	Quantitative index	-1.35	-3.28	0.57	0.168
	Memory	-1.02	-2.89	0.85	0.285
	Global motor	-1.75	-3.61	0.10	0.064
New functions	Executive	-0.10	-1.88	1.68	0.911
	Visual executive	0.08	-1.75	1.91	0.929
	Verbal executive	-0.21	-2.04	1.62	0.818
	Visual and verbal span	-0.52	-2.43	1.40	0.595
	Working memory	-1.07	-3.00	0.86	0.275
	Verbal memory	-0.81	-2.79	1.17	0.420
	Gross motor	-1.48	-3.30	0.34	0.110
	Fine motor	-1.16	-2.86	0.55	0.182
	Cognitive function of posterior cortex	-1.06	-2.91	0.79	0.261

Models adjusted for maternal highest attained level of education (i.e. primary, secondary, or university), children's sex (i.e. girls or boys), BMI (kg/m^2), age at MSCA test (years), sub-cohort location (i.e. Asturias, Gipuzkoa, or Sabadell), and calorie adjusted consumption of rice and fish/seafood (g/day).

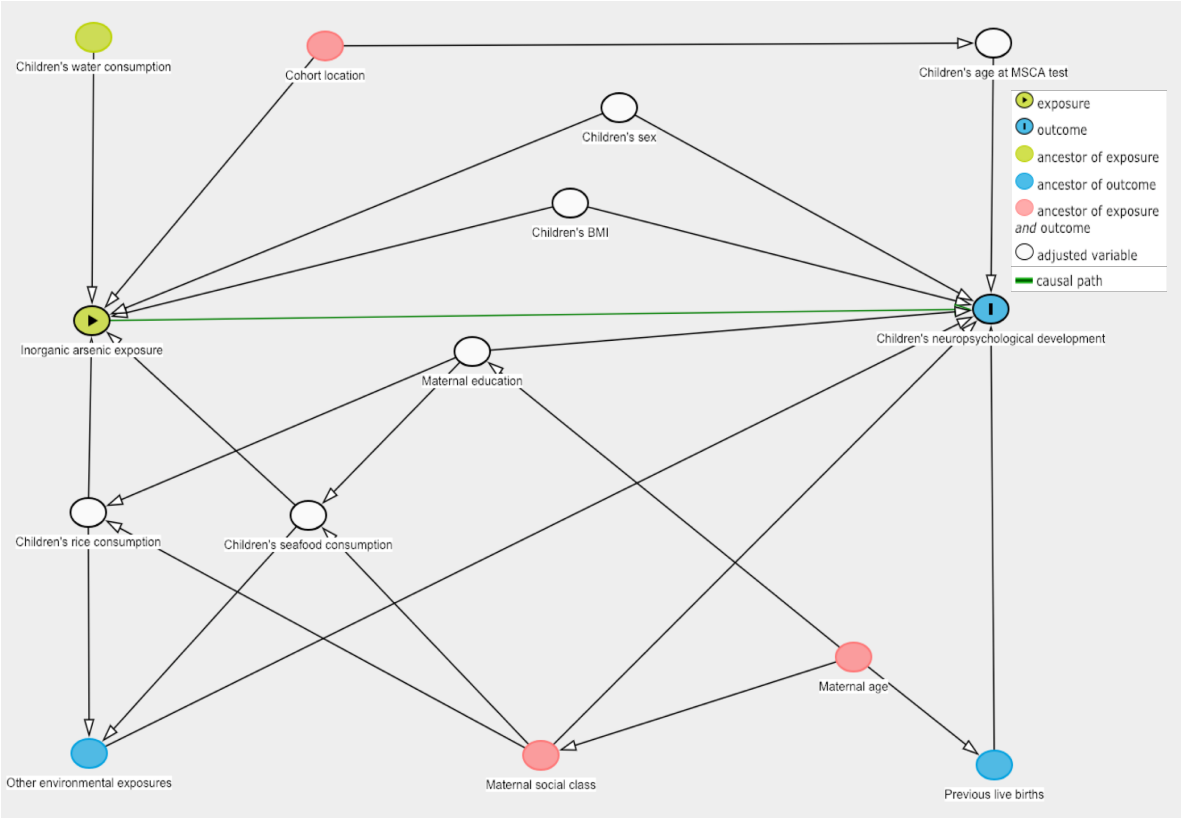
Table S3: Selected characteristics of the study population stratified by sub-cohort location (minimum; interquartile rage; maximum) for continuous and *n* (%) for categorical variables.

Selected characteristics of the study population		Asturias (*n* = 96)	Gipuzkoa (*n* = 90)	Sabadell (*n* = 76)	Valencia (*n* = 99)	*p*-value
Children:						
Sex	Girls	48 (50)	47 (52)	41 (54)	49 (49)	0.932
	Boys	48 (50)	43 (48)	35 (46)	50 (51)	
Rice consumption (g/day)		26.5 (0.9; 11.2 – 37.5; 72.5)	22.2 (3.7; 9.9 – 34.1; 83.3)	30.9 (7.2; 21.9 – 48.0; 155.2)	28.2 (0.9; 22.2 – 39.3; 142.2)	0.000
Fish/Seafood consumption (g/day)		40.1 (14.7; 32.5 - 50.2; 93.1)	37.1 (18.2; 30.4 - 45.1; 102.3)	43.8 (21.1; 36.9 - 56.0; 82.7)	38.0 (10.5; 26.9 - 47.2; 75.0)	0.000
Sum of urinary arsenic concentrations (μg/L)[1]		4.81 (0.31; 3.04 - 7.19; 84.46)	4.85 (0.12; 2.70 - 8.49; 69.60)	4.76 (0.22; 2.14 - 6.48; 49.1)	5.23 (0.37; 2.95 - 7.80; 28.49)	0.615
BMI (kg/m^2)		16.1 (11.5; 15.3 – 17.5; 21.0)	16.1 (13.2; 15.3 – 17.2; 22.8)	15.6 (12.9; 15.0 - 17.0; 25.0)	15.9 (12.6; 15.2 - 16.9; 21.0)	0.371
Maternal:						
Enrollment	Age (years)	32.0 (21.0; 29.0 - 35.0; 42.0)	32.0 (25.0; 29.0 - 35.0; 43.0)	30.5 (22.0; 28.8 - 34.0; 40.0)	30.3 (21.0; 27.0 - 33.0; 42.0)	0.004
Social class	Upper - I+II	18 (19)	33 (37)	14 (18)	18 (18)	0.007
	Middle - III	24 (25)	27 (30)	27 (36)	28 (28)	
	Lower - IV+V	54 (56)	30 (33)	35 (46)	53 (54)	
Highest attained level of education	Primary	19 (20)	7 (8)	22 (29)	22 (22)	0.000
	Secondary	38 (40)	31 (34)	31 (41)	48 (48)	
	University	39 (41)	52 (58)	23 (30)	29 (29)	
Number of previous live births	0	59 (61)	46 (51)	40 (53)	53 (54)	0.632
	1	30 (31)	39 (43)	31 (41)	41 (41)	
	2	7 (7)	4 (4)	5 (7)	5 (5)	
	3	0 (0)	1 (1)	0 (0)	0 (0)	

For test of differences by sex, we used Kruskal-Walis rank test for continuos variables, and Chi-square exact test for categorical variables. BMI = Body mass index.

[1]DMA + MMA + iAs.

Figure S1: Directed acyclic graph showing the minimal sufficient adjustment set (Textor et al. 2017).



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