In utero exposure to mixtures of xenoestrogens and child neuropsychological development

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Abstract

Background: To date, no epidemiological studies have explored the impact and persistence of *in utero* exposure to mixtures of xenoestrogens on the developing brain. We aimed to assess whether the cumulative effect of xenoestrogens in the placenta is associated with altered infant neuropsychological functioning at two and at four years of age, and if associations differ among boys and girls.

Methods: Cumulative prenatal exposure to xenoestrogens was quantified in the placenta using the biomarker Total Effective Xenoestrogen Burden (TEXB-alpha) in 489 participants from the INMA (Childhood and the Environment) Project. TEXB-alpha was split in tertiles to test its association with the mental and psychomotor scores of the Bayley Scales of Infant Development (BSID) at 1-2 years of age, and with the McCarthy Scales of Children's Abilities (MSCA) general cognitive index and motor scale assessed at 4-5 years of age. Interactions with sex were investigated.

Results: After adjustment for potential confounders, no association was observed between TEXB-alpha and mental scores at 1-2 years of age. We found a significant interactions with sex for the association between TEXB-alpha and infant psychomotor development (interaction p-value =0.029). Boys in the third tertile of exposure scored on average 5.2 points less than those in the first tertile on tests of motor development at 1-2 years of age (p-value=0.052), while no associations were observed in girls. However, this association disappeared in children at 4-5 years of age and no association between TEXB-alpha and children's cognition was found.

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Conclusions: Our results suggest that boys' early motor development might be more vulnerable to prenatal exposure to mixtures of xenoestrogens, but associations do not persist in preschool children.

Keywords: xenoestrogens, mixtures, placenta, sex, TEXB, neuropsychological development, prenatal.

Introduction

Xenoestrogens represent a heterogeneous group of endocrine disrupting chemicals (EDCs), including both naturally-occurring (e.g. phyto- and mycoestrogens) and man-made compounds (e.g. many pesticides and other persistent organic pollutants (POPs)), that mimic estrogen action, affect hormone levels, and/or bind to estrogen receptors (Fucic et al., 2012). Some have been shown to be hormonally active at extremely low concentrations, similarly to endogenous blood-born hormones (Alyea and Watson, 2009; Kochukov et al., 2009; Wozniak et al., 2005).

Humans are exposed daily to xenoestrogens through different sources, including environmental contamination of the food chain, and via different pathways, such as inhalation or dermal exposure resulting from direct contact with contaminated household dust (Frye et al., 2012; Johnson et al., 2010; Llop et al., 2010). Many xenoestrogens bioaccumulate in lipids, and significant quantities of persistent congeners banned in the USA and the EU are still detectable in the environment and in the food chain (Dickerson and Gore, 2007; Law et al., 2014). Human exposure is therefore ubiquitous, and occurs in mixtures at low doses, operationally defined as doses below those traditionally tested in toxicological studies within the normal range of human exposure (Vandenberg et al., 2012).

During pregnancy, many xenoestrogens are able to cross the placental barrier and enter fetal circulation (Dorea et al., 2001; Frederiksen et al., 2010; Leino et al., 2013; Vizcaino et al., 2014). Moreover, compounds of small molecular mass (<400–500 Da) with high lipid solubility are also capable of crossing the blood-brain barrier (Pardridge, 2003), which is still underdeveloped at birth (Reed and Fenton, 2013).

Many pesticides, including DDT (dichlorodiphenyltrichloroethane), endosulfan-α, or lindane, which have xenoestrogenic properties, are designed to be toxic to the nervous systems of insects and other target species (Bjorling-Poulsen et al., 2008). The human brain is a sexually dimorphic organ, with morphological differences shaped during prenatal development under the regulation of gonadal steroid hormones, especially estrogen and aromatizable androgens (Dickerson and Gore, 2007; Matsumoto, 1991). Exposure during this critical period to low, environmentally relevant concentrations of xenoestrogens has been shown to produce sex-specific structural and behavioral effects in rodents, likely attributed to an alteration of prenatal androgen/estrogen balance. (Kubo et al., 2003; Wang et al., 2002).

A number of epidemiological studies have found negative associations between prenatal exposure to several xenoestrogens including dichlorodiphenyldichloroethylene (DDE), polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), or phthalates on neuropsychological development in children in the first two years of life (Forns et al., 2012; Gascon et al., 2012; Herbstman et al., 2010; Koopman-Esseboom et al., 1996; Park et

al., 2010; Ribas-Fito et al., 2003; Torres-Sanchez et al., 2007), and later in childhood on mental and psychomotor development, fine motor abilities, cognition and full scale and verbal IQ (Eskenazi et al., 2013; Herbstman et al., 2010; Puertas et al., 2010).

Although synergistic effects of co-exposure to mixtures of EDCs on neurodevelopment have been demonstrated both *in vivo* and *in vitro* (He et al., 2009; Pellacani et al., 2012), the short- and long-term risks from early exposure to environmentally relevant doses of complex mixtures in humans are unclear and represent an area of increasing concern.

The Total Effective Xenoestrogen Burden (TEXB) is a standardized biomarker of the cumulative estrogenic effect resulting from mixtures of environmental compounds based on their proliferative effect on human breast cancer cells (Fernandez et al., 2008; Fernandez et al., 2004). TEXB has previously been studied in relation to the risk of developing several human diseases including anomalies of sexual maturation in males, breast cancer and Type 2 diabetes (Arrebola et al., 2013; Fernandez et al., 2007b; Ibarluzea Jm et al., 2004; Vilahur et al., 2013). In addition, an association has been found between higher prenatal TEXB levels and increased birth weight in males belonging to the same population as the present study (Vilahur et al., 2013).

We aimed to examine the association of prenatal exposure to mixtures of xenoestrogens on neuropsychological development in children at 1-2 years of age and then at 4-5 years of age and to investigate possible effect modification by sex.

Material and Methods

2.1 Study Population

The study population originated from the INMA (INfancia y Medio Ambiente [Childhood and the Environment]) Project, a Spanish multicenter birth cohort study, and included children from the cohorts located in the geographic areas of Asturias, Gipuzkoa, Sabadell and Valencia, which were enrolled between November 2003 and February 2008 during the first trimester of pregnancy either at the health care center or hospital of reference (depending on the region). Inclusion criteria were that mothers had at least 16 years of age, intended to deliver at the reference hospital, had no serious communication problem, and presented a singleton pregnancy without assisted conception (Guxens et al., 2012b). 489 placentas were randomly collected among the participants, which represented approximately 1 out of each 5 live births. The placentas were examined and weighed without deciduas basalis and chorionic plate and immediately frozen at −20 °C until transferred to the Biobank at the San Cecilio University Hospital in Granada.

All participants recruited in the study were informed verbally about the aims of the study and they provided informed consent. The research protocol was approved by the ethics committees of all the hospitals and institutions involved.

2.2 Total effective Xenoestrogen Burden

TEXB is a quantitative biomarker of the cumulative effect of xenoestrogens (Soto et al., 1997). For each sample of placenta, estrogenicity is quantified in two different fractions: the TEXB-alpha fraction, which represents the environmental fraction containing lipophilic xenoestrogens (mainly persistent organohalogenated compounds) and the TEXB-beta fraction where endogenous sex steroids (e.g. progestins, androgens, estradiol esters, steroidal estrogens) as well as phytoestrogens, mycoestrogens and bisphenols can be found (Fernandez et al., 2008).

Half of each placenta was mechanically homogenized to ensure representativeness of the sample. 0.4 g of placenta homogenate was extracted with hexane and eluted in a

glass column filled with Alumine. After drying at 600ºC for 4h, each sample was rehydrated by adding 5% water. This process was performed in quadruplicate for each placenta sample (total weight: 1.6 g of placenta). Extraction of bioaccumulated compounds was then performed using high-pressure liquid chromatography (HPLC), which separated out compounds according to their polarity with the most lipophilic compounds eluting in the shortest time (Fernandez et al., 2008; Fernandez et al., 2004; Lopez-Espinosa et al., 2009).

The combined estrogenic effect of each TEXB fraction—in terms of proliferative effect on MCF-7 human breast cancer cells—was then analyzed using the E-Screen bioassay. For each sample, the alpha and beta fractions obtained using HPLC were tested separately in triplicate including one negative control (vehicle only) and one positive control (estradiol 100pM) per plate. Cell proliferation was read at 492 nm using Titertek Multiscan apparatus (Flow, Irvine, CA, USA).

The proliferative effect of TEXB-alpha and TEXB-beta in each sample was referred to the maximal effect obtained using 100 pM of estradiol by reading from a reference dose-response curve (concentration range 0.1 pM to 10 nM), and transformed into estradiol equivalent units per gram of placenta tissue (Eeq/g placenta) (Fernandez et al., 2007a).

At concentrations below 1 pM estradiol (equivalent to 1 fmol in 1mL of culture medium), mean cell numbers did not differ significantly from those in the steroid-free control, and this was defined as the limit of detection (LOD) of the assay. Samples below this value were assigned a value equal to 0.05 pM Eeq/ml (LOD/2) (N=69, 14.11%). The TEXB biomarker (both TEXB-alpha and TEXB-beta) was measured in 489 placenta samples.

2.3 Neuropsychological testing

Child neuropsychological development was measured at ~14 months of age (range: 11 to 22) using the Bayley Scales of Infant Development (BSID) (Bayley, 1977) by eight experienced and specially trained psychologists in the presence of the mother at the primary care center. The BSID consists of two scales, the mental scale and the psychomotor scale. The 163 items of the mental scale evaluate age-appropriate cognitive development in areas such as performance ability, memory and first verbal learning. The 81 items of the psychomotor scale evaluate fine and gross psychomotor coordination. All assessments were carried out according to a strict fieldwork protocol, and included inter-observer reliability tests estimated by intra-class correlation tests (0.90 for the mental scale and 0.91 for the psychomotor scale). Raw scores were standardized for each child's age in days at the time of test administration using a parametric method for the estimation of age-specific reference intervals (Royston, 1998). The parameters of the distribution are modelled as a fractional polynomial function of age and estimated by maximum likelihood. Standardized residuals were then centered to a mean of 100 with a standard deviation (SD) of 15 points.

Of the 489 children for whom we had placental TEXB measurements, 450 were assessed using the BSID. Additionally, 27 participants for which neuropsychological tests were of poor quality due to neurodevelopmental disabilities (Down syndrome, autistic traits) or less-than-optimal cooperation of the child (due to tiredness, bad mood, illness) were flagged by the psychologists during evaluation and excluded from our analysis. Complete data on BSID were available for a total of 423 children.

At 4 years of age (range 4.04-6.37) the same cohort of children was interviewed by 6 trained psychologists using a standardized Spanish version of the McCarthy Scales of

Children's Abilities (MSCA) (McCarthy, 2009), which provides information on cognitive and motor development in preschool children. The MSCA general cognitive index (GCI) and the motor scale were examined. The GCI is a metric of the child's global intellectual function and is formed by combining scores from three different scales (perceptual-performance, verbal, and quantitative) that do not overlap in content and that assesses number aptitude in addition to verbal and non-verbal reasoning abilities (Puertas et al., 2010).

Raw scores were centered on a mean of 100 with an SD of 15. Tests of poor quality, as mentioned above, were excluded from analysis. Complete MSCA data were available for a total of 360 children.

2.4 Statistical Analysis

Children for which placental TEXB measurements were available (n=489) were compared to the rest of the children in the four INMA cohorts (n=2017) using chi-square, ANOVA, t-Student and Wilcoxon-Mann-Whitney tests (See Supplemental Material, Table 1).

We used multivariate linear regression models to examine the association of TEXB-alpha levels with BSID scores (both mental and psychomotor scales) and MSCA scores (both GCI and motor scales). Considering the skewed distribution of the variable (See Supplemental Material, Figure 1) and in order to account for possible non-linear effects tertiles of TEXB-alpha levels were defined, and the lowest tertile was considered as the reference.

BSID scores were age-standardized, while exact age at child examination in years was included as a covariate in the MSCA models. Additionally, all models were adjusted for sex, cohort (i.e. a variable designating the specific geographical area of origin within the four Spanish regions included) and social class. Three occupational categories were used to define social class according to the occupation of highest status of the mother or father during pregnancy on the basis of a widely used Spanish adaptation of the British classification system (Domingo-Salvany, 2000). We initially considered potential confounders known to be associated with neurodevelopment or TEXB-alpha in the literature and included gestational age, type of delivery (cesarian or vaginal), previous abortions, gestational diabetes mellitus or impaired glucose tolerance, marital status (mother cohabiting with father´s child or not), maternal age, height, body mass index (BMI), gestational weight gain (Kg/ week), parity (primiparous vs. multiparous), smoking (active smoking recorded at week 32 of pregnancy and passive smoking either at home, at work or at leisure), alcohol consumption and predominant breastfeeding (allowing for supplementation with non-milk products and categorized in 4 groups). Other variables included were season of birth, maternal residence (rural vs urban), maternal country of birth (Spanish vs foreign) and educational level, paternal characteristics such as paternal height and weight and the Apgar score of the baby at 5 minutes after birth (<8 was considered non-optimal). Backward stepwise regression was used to build adjusted models for those variables associated with both exposure (tertiles of TEXB-alpha) and outcome with p-values of <0.2. Only those covariates whose inclusion modified the coefficient of the association of either tertile of TEXB-alpha and the neurospsychological test scores by >10%, or presented a marginally significant association (p value<0.1) with the outcome of interest were retained in the final adjusted models.

Multivariable-adjusted models were further adjusted for log-transformed TEXB-beta to minimize the likelihood of residual confounding attributable to the role of

natural endogenous estrogens together with non-halogenated chemicals.

Sex interaction terms were included in both models, and likelihood ratios used to test for effect modification. Significant interactions were considered when p-values were <0.1. Sex-specific TEXB-alpha tertiles were used in the models stratified by sex.

Several sensitivity analyses were carried out to test the results for robustness, whereby the following subgroups were excluded: preterm babies (<37 weeks of gestation; N=10), children with low birth weights (<2500g, N=14) and those who were never breastfed (N=60) since they may present different patterns of neuropsychological development, as well as mothers of non-Spanish origin (N=38). Additionally, 7 samples (3 from females and 4 from males) were identified with very high values of TEXB-alpha (above 40 pM Eeq/g of placenta) and analyses were repeated excluding these observations but since results did not change substantially and there was no objective reason for excluding them (i.e. error in biomarker measurement) they were ultimately retained in the study.

All statistical analyses were conducted with Stata 10.0 statistical software (Stata Corporation, College Station, TX).

Results

Children for whom we measured TEXB were representative of the rest of INMA children in terms of pregnancy and parental characteristics, although they presented on average lower prevalence of preterm births (2.06 % vs 4.96%), as well as more cases of mothers with impaired glucose tolerance or gestational diabetes mellitus. No statistically significant differences were observed between both groups for the neuropsychological outcomes studied, but BSID psychomotor scores were somewhat higher in the study sub-population (p-value=0.054) (Supplementary Table 1).

Girls had higher scores in the BSID mental scale and in the MSCA GCI than males, similarly to what has been shown previously for the whole INMA cohort in the region of Sabadell (N=825) (Vrijheid et al., 2012). No differences were observed for the psychomotor scores according to sex (Table 1).

Descriptive statistics of potential confounding variables by tertiles of TEXB-alpha are presented in Supplementary Table 2. Overall TEXB-alpha levels did not differ according to fetal sex (p-value=0.64), but levels in the third tertile of exposure were somewhat higher in boys than in girls. TEXB-alpha values overall and in the sex specific tertiles are shown in Table 2.

Table 1. BSID and MSCA test scores by sex at ages 1-2 and 4-5.

	Boys	Girls	p-val
	Mean (SD)	*Mean (SD)*	
BSID	**N=211**	**N=212**	
Mental Index	96.83 (14.47)	102.89 (16.45)	<0.00
Psychomotor Index	100.24 (15.77)	100.54 (16.84)	0.85
MSCA	**N=184**	**N=176**	
General Cognitive Index	98.56 (15.50)	102.54 (13.98)	0.03
Motor Scale	98.87 (15.26)	101.27 (13.72)	0.12

Association between TEXB-alpha exposure and the BSID scale scores

Multivariate adjusted regression models between TEXB-alpha tertiles and children's mental and psychomotor test scores of BSID are presented in Table 3. No significant associations were observed for either scale.

However, we found a statistically significant interaction by sex between TEXB-alpha and psychomotor test scores (interaction p-value=0.029) after adjusting by maternal BMI, breastfeeding, marital status, parental social class, maternal height and the TEXB-beta fraction. In boys, TEXB-alpha in the third tertile was marginally associated with a decrease of 5.19 points on average in psychomotor scores when comparing to TEXB-alpha in the first tertile (p-value=0.052), while no significant associations were observed in girls, but a trend towards higher psychomotor scores at intermediate levels of TEXB-alpha (second tertile). Our results did not change if we restricted the analysis only to the children with BSID that had MSCA data later at age 4 (63 children were lost between age 2 and 4) (not shown).

Association between TEXB exposure and the MSCA test scores

After adjustment for confounders, we found no statistically significant associations between TEXB-alpha levels and either the MSCA general cognitive index or the motor scale in preschoolers (Table 4). Additionally, no interactions by sex were observed for the outcomes analyzed.

Discussion

The main goal of our study was to investigate associations of combined exposure *in utero* to xenoestrogens with neuropsychological development in young children and later in preschool children, and to test whether associations differed between boys and girls. This is the first epidemiological study we are aware of using a biomarker of cumulative effect of

Table 2. Overall and sex-specific levels of the biomarker Total Effective Xenoestrogen Burden (TEXB-alpha*) by tertiles in the study population.

	1st Tertile					2nd Tertile					3rd Tertile					
	N	Median	iqr	min	max	N	Median	iqr	min	max	N	Median	iqr	min	max	N total
All	163	0.13	0.02-0.27	0.02	0.46	163	0.76	0.69-0.92	0.46	1.16	163	2.06	1.41-3.98	1.16	60.62	489
Boys	85	0.13	0.02-0.27	0.02	0.46	84	0.76	0.70-0.93	0.47	1.21	84	2.14	1.54-3.27	1.22	60.62	253
Girls	79	0.12	0.02-0.26	0.02	0.46	78	0.76	0.69-0.91	0.47	1.01	78	1.93	1.28-4.27	1.02	60.15	235

*TEXB-alpha represents the estrogenicity due to environmental organohalogenated compounds. TEXB-alpha values are expressed in estrogen equivalents units per gram of placenta tissue (pM Eeq/g) . iqr: interquartile range (percentile 25- percentile 75 of TEXB-alpha exposure).

Table 3. Adjusted association between tertiles of prenatal TEXB-alpha (pM Eeq/g) and test scores of the BSID Mental and Psychomotor Indexes in children at age 1-2.

Mental Developmental Index (MDI)

	*Overall (N=411)**		*Boys (N=206)*		*Girls (N=205)*		
TEXB-alpha	**Coef (SE)**	***p -val***	**Coef (SE)**	***p -val***	**Coef (SE)**	***p -val***	***p-val interaction***
T1	Ref		Ref				
T2	-0.75 (2.04)	0.711	-0.74 (2.80)	0.791	-1.19 (3.12)	0.704	0.337
T3	0.48 (2.23)	0.830	1.83 (3.06)	0.550	0.38 (3.22)	0.905	

Psychomotor Developmental Index (PDI)

	Overall (N=413)ᵛ		*Boys (N=207)*		*Girls (N=206)*		
TEXB-alpha	**Coef (SE)**	***p -val***	**Coef (SE)**	***p -val***	**Coef (SE)**	***p -val***	***p-val interaction***
T1	Ref		Ref		Ref		
T2	1.00 (1.85)	0.591	-4.38 (2.57)	0.090	4. 96 (2.82)	0.080	0.029*
T3	-1.62 (1.89)	0.599	-5.19 (2.65)	0.052	2.78 (2.81)	0.324	

*Adjusted by cohort, sex, parental social class, maternal age, cesarean birth, maternal height, maternal weight gain during pregnancy, passive smoking and log transformed TEXB-beta.
ᵛ Adjusted by cohort, sex, maternal body mass index, breastfeeding, parental social class, maternal height, marital status and log transformed TEXB-beta.

xenoestrogens in the human placenta to investigate its consequences on children's neurodevelopment at two different time points.

We found a significant interaction by sex between TEXB-alpha and the BSID psychomotor test scores (interaction p-value =0.029) in children at 1-2 years of age. The association of placental TEXB-alpha on neuropsychological development was qualitatively different according to sex: boys in the third tertile of TEXB-alpha showed a decrease in psychomotor scores when compared to those in the first tertile, and no significant associations were seen in girls, with regression coefficients in the opposite direction. Although the sample size of the study is relatively small, results were found to be robust even when we excluded various subgroups of the population in sensitivity analyses i.e. preterm and low birth weight babies, those born from diabetic mothers or who were not breastfed and children of mothers of non-Spanish origin. We observed no significant associations or interactions by sex between prenatal TEXB-alpha and children cognition or motor skills in children between 4 and 6 years of age.

In this study of the general population, boys' mean TEXB-alpha levels in the upper tertile were 3.34 pM Eeq/g of placenta. This level of exposure has previously been associated with an increase in boys' birth weight in the same population (Vilahur et al., 2013) and with lower male psychomotor scores at 2 years of age in the current study. This level of exposure is similar to the mean TEXB-alpha value of 2.92 pM Eeq/g observed in placentas of a Spanish population of male newborns presenting reproductive abnormalities (i.e. cryptorchidism and/or hypospadias), and relatively higher than the mean value of 1.45 pM Eeq/g of placenta found in controls in the same study (Fernandez et al., 2007a).

Although several authors have found negative associations between prenatal exposure to single xenoestrogens like DDE, PCBs and phthalates and both mental and psychomotor test scores in children during the first two years of life (Kim et al., 2011; Park et al., 2010; Ribas-Fito et al., 2003),

Table 4. Adjusted associations between tertiles of prenatal TEXB-alpha (pM Eeq/g) and test scores of the McCarthy General Cognitive Index and Motor Scale at 4-5 years of age.

	General Cognitive Index (GCI)						
	*Overall (N=356)**		*Boys (N=184)*		*Girls (N=172)*		
TEXB-alpha	**Coef (SE)**	***p -val***	**Coef (SE)**	***p -val***	**Coef (SE)**	***p -val***	***p-val interaction***
T1	Ref		Ref		Ref		
T2	-0.09 (1.90)	0.960	0.42 (2.86)	0.884	-1.12 (2.65)	0.674	0.639
T3	0.75 (2.08)	0.719	3.27 (3.01)	0.279	-1.35 (2.81)	0.631	
	Motor Scale [v]						
	Overall (N=344)[v]		*Boys (N=175)*		*Girls (N=169)*		
TEXB-alpha	**Coef (SE)**	***p -val***	**Coef (SE)**	***p -val***	**Coef (SE)**	***p -val***	***p-val interaction***
T1	Ref		Ref		Ref		
T2	2.03 (1.92)	0.292	0.54 (2.81)	0.847	2.75 (2.72)	0.313	0.244
T3	2.56 (2.12)	0.229	3.98 (3.03)	0.191	1.17 (2.86)	0.681	

* Adjusted by exact age at evaluation, cohort, sex, parental social class, maternal country of origin, maternal smoking during pregnancy, season of birth and log transformed TEXB-beta.

[v] Adjusted by exact age at evaluation, cohort, sex, breastfeeding, parental social class, maternal smoking during pregnancy, maternal alcohol consumption during pregnancy, maternal country of origin, maternal height,and log transformed TEXB-beta.

others have reported specific effects on psychomotor test scores only (Forns et al., 2012; Herbstman et al., 2010; Torres-Sanchez et al., 2007). Results of existing cohort studies on prenatal exposure to xenoestrogens (pesticides and/or other estrogenic compounds like flame retardants or metals) and neuropsychological development in children aged 4 years or more are heterogeneous, ranging from no evidence of any association (Forns et al., 2014; Torres-Sanchez et al., 2009) to negative associations for fine motor coordination and cognition (Eskenazi et al., 2013; Forns et al., 2014; Puertas et al., 2010; Torres-Sanchez et al., 2009).

Sex specific effects have rarely been analyzed in this field despite the evidence of histological, structural and behavioral sexual dimorphism existing in the CNS in healthy humans and the sex-related differences in the susceptibility to endocrine disrupting neurotoxicants (Llop et al., 2013; Raz et al., 2001). Low-level prenatal exposure to lead, a heavy metal known to interact with estrogen action (Tchernitchin et al., 2003), has been associated with adverse cognitive effects in 3 years old male children and to motor impairment in young male mice (Jedrychowski et al., 2009; Leasure et al., 2008). Kim et al. reported that the association of *in utero* exposure to phthalates with decreases in both mental and psychomotor BSID test scores was stronger in 6 month-old Korean males compared to females, while the association of prenatal exposure to DDT with decreases in cognitive skills at 4 years of age in two Spanish cohorts was significantly stronger in girls than in boys (Kim et al., 2011; Ribas-Fito et al., 2006).

It has been postulated that the motor impairment seen in relation to prenatal exposure to weak xenoestrogens like PCBs could involve the cerebellum (Ma and Sassoon, 2006), a brain area with a relatively long developmental time span which plays an essential role in motor control, motor learning and cognition (Forns et al., 2012; Nguon et al., 2005), and which actively expresses estrogen receptor β (ERβ) in the neurons and in the glia during development in humans (Belcher,

2008; Rissman et al., 2002; Wang et al., 2003). Finally, a study in rodents analyzing the perinatal effects of exposure to PCBs found greater adverse effects on behavior, including fine motor coordination and motor learning in male than female pups. In addition, they observed a significant reduction in cerebellar mass and changes in protein expression in this group (Nguon et al., 2005). To know whether TEXB-alpha exposure has an impact in cerebellar structure and functioning in boys or not would certainly be of great interest.

We did not find any significant association between TEXB-alpha and the BSID mental scores at age 1-2. We think that it is not probable that the lack of a significant association at an early age in our data is explained by the relative immaturity of the prefrontal cortex at this age, as some authors have suggested, since we did not see any significant association between TEXB-alpha and cognition at 4-5 years of age. We would speculate, however, that early post-natal factors such as quality of the early home environment or other lifestyle factors not measured in the present study, may have balanced the adverse effects of prenatal xenoestrogen exposure on cognition, as reported in other studies (Guxens et al., 2012a; Torres-Sanchez et al., 2009), or that the deleterious effects of hormonal imbalance *in utero* may arise in later developmental stages, as suggested by Bosch-Domènech *et al*. in their article reporting an association between the second-to-fourth digit ratio, a proxy for the prenatal ratio of exposure to testosterone versus estrogen, and cognitive abilities in college students aged 19 (Bosch-Domènech et al., 2014).

The major strengths of this study are its prospective nature and the abundance of information on possible confounders, the fact that all neuropsychological assessments were performed by psychologists who had received extensive, study-specific training, and the use of quality controls which ensured high quality of data. In addition, the use of a placental biomarker such as TEXB to assess prenatal exposure to mixtures added relevant biological information to the real world human experience, in which co-exposure to multiple chemicals occurs. Several studies have shown that there is no relationship between single EDCs quantified in extracted samples and the biological effect measured by the TEXB-alpha (Andersen et al., 2007; Rasmussen et al., 2003), suggesting that it represents the overall combined effect taking into account interactions between chemicals. Additionally, the TEXB biomarker is able to account for estrogenicity resulting both from genomic and non-genomic mechanisms since the membrane-associated ERα are present in MCF7 cells (Zivadinovic et al., 2005), while other assays, like transcriptional assays, may lack to provide information on non-genomic actions (Francois et al., 2003). A limitation to the assay we used is that some xenoestrogens, such as methoxychlor or PBDEs (among others), may simultaneously exhibit more than one mechanism of action in disrupting endocrine function (ERα agonist, ERβ antagonist and AR agonist) (Berger et al., 2014; Gaido et al., 1999; Maness et al., 1998), and also that weak environmental estrogens with more polar properties, for example bisphenol A, do not elute in the alpha fraction due to their polar properties and therefore are not accounted for in the environmental TEXB-alpha fraction.

In conclusion, our results suggest that male newborns may be at increased risk of neuropsychological alterations following *in-utero* exposure to mixtures of xenoestrogens compared to their female counterparts, although the association with adverse motor development observed at 1-2 years of age does not persist. More studies in larger cohorts using valid and standardized biomarkers of cumulative

effect such as the TEXB are needed in order to replicate our findings and further follow-up of this cohort is important, since it may offer the opportunity to test for developmental alterations that may not arise until later in development.

Abbreviations:

INMA- INfancia y Medio Ambiente Project
TEXB- Total Effective Xenoestrogen Burden
BSID- Bayley Scales of Infant Development
MSCA - McCarthy Scales of Children's Abilities
LOD- Limit of Detection
POPs- Persistent Organic Pollutants
EDCs- Endocrine Disrupting Compounds
DDT- Dichlorodiphenyltrichloroethane
DDE- Dichlorodiphenyldichloroethylene
PCBs- Polychlorinated Biphenyl
PBDEs- Polybrominated Diphenyl Ethers
GCI-General Cognitive Index
SD- Standard Deviation
BMI- Body Mass Index

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Supplemental Material

In utero exposure to mixtures of xenoestrogens and child neuropsychological development

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Supplementary Table 1. Comparison of characteristics of neurodevelopmental outcomes and main covariates between included children (with TEXB-alpha) and the remaining of the INMA children (without TEXB-alpha).

		Children with TEXB values N=489			Children without TEXB values N=2017			
		N	% mean (sd) median (iqr)	NA	N	% mean (sd) median (iqr)	NA	P val
OUTCOMES								
Bayley Scales*								
Mental Development Index		450	99 (16.67)	39	1763	98.09 (16.28)	254	0.295
Psychomotor Development Index		450	100.27 (16.65)	39	1763	98.67 (15.45)	254	0.054
Mc Carthy Scales **								
General Cognitive Index		360	100.26 (14.84)	129	1447	100.23 (14.81)	570	0.979
Motor Index		360	99.98 (14.58)	129	1447	100.24 (15.00)	570	0.768
COVARIATES								
Sex	Boys	253	51.84	1	1034	51.26	9	0.890
	Girls	235	48.16		974	48.74		
Gestational age		484	40.00 (39.00-40.71)	5	1990	39.86 (38.86-40.86)	27	0.708
Low birth weight (<2500)		19	3.9	2	107	5.35	18	0.190
Preterm (<37 weeks)		10	2.06	4	99	4.96	21	0.005
Apgar score at 5 minutes (≥8, optimal)		484	99.38	5	1887	99.21	115	0.339
Parity	Primip	280	57.26	0	1129	56.03	2	0.623

	Multip (2+)	209	42.74		886	43.97		
Season of Birth	Winter	140	28.69	1	513	25.51	6	0.403
	Spring	124	25.41		496	24.66		
	Summer	110	22.54		481	23.92		
	Autumn	114	23.36		521	25.91		
Breastfeeding (weeks)	0	60	13.10	31	287	15.72	191	0.120
	0-16	134	29.26		444	24.32		
	16-24	67	14.63		297	16.27		
	>24	197	43.01		798	43.70		
Mother's age (y)		488	31.64 (4.03)	1	2010	31.33 (4.37)	7	0.156
Gestational diabetes mellitus (current pregnancy)	No	327	80.94	87	1544	86.94	250	0.007
	IGT	49	12.13		151	8.50		
	GDM or pior DM	28	6.93		81	4.56		
Maternal height (cm)		489	162.90 (5.99)	0	2016	162.64 (6.21)	1	0.324
Pre-pregnancy maternal BMI		489	22.66 (20.76-25.28)	0	2016	22.59 (20.70-25.22)	1	0.918
Gestational weight gain (kg/week)		481	0.41 (0.32-0.51)	8	1933	0.42 (0.32-0.51)	84	0.431
Maternal country of origin (Foreign)		38	7.79	1	165	8.19		0.768
Maternal smoking at week 32 (cigarettes)	no	407	84.44	7	1614	82.60	63	0.395
	<=5	46	9.54		187	9.57		
	>5	29	6.02		153	7.83		
Passive smoking pregnancy		294	61.38	10	1220	62.72	72	0.585
Alcohol during pregnancy		104	21.27	0	404	20.03	0	0.541
Marital Status	Not	8	1.64	0	35	1.74	1	0.878

	cohabiting							
Paternal height		484	175.90 (6.84)	5	2001	175.99 (7.02)	16	0.796
Paternal weight		481	80 (72-86)	8	1986	80 (72-87)	31	0.525
Social class (mix)	I+II	166	33.95	0	620	30.75	1	0.155
	III	129	26.38		500	24.80		
	IV+V	194	39.67		896	44.44		
Place of Residence (Rural)	Rural	20	4.11	2	127	6.32	6	0.063
Cohort	Asturias	52	10.63	0	433	21.47	0	<0.001
	Guipuzkoa	168	34.36		444	22.01		
	Sabadell	174	35.58		448	22.21		
	Valencia	95	19.43		692	34.31		

* Age-standardized; ** The mean score for the McCarthy general cognitive and the motor scales is 100, with a Standard Deviation (SD) of 15 points.

Supplementary Table 2. Distribution of covariates across tertiles of TEXB-alpha (pM Eeq/g) levels.

		TEXB-alpha ***1st Tertile***	**TEXB-alpha** ***2nd Tertile***	**TEXB-alpha** ***3rd Tertile***
		mean (SD) or %	*mean (SD) or %*	*mean (SD) or %*
Sex	Boys	52.15	48.47	54.94
Gestational age		39.93 (1.25)	39.66 (1.41)	39.93 (1.28)
Low birth weight (<2500)	Yes	1.2	1.2	1.6
Preterm (<37 weeks)	Yes	1.85	3.07	1.25
Apgar score at 5 minutes (≥8, optimal)	8	1.25	0.61	0
	9	5	3.68	11.8
	10	93.75	95.71	88.2
Parity	Primip	63.19	52.76	55.83
	Multip (2+)	36.81	47.24	44.17
Season of Birth	Winter	33.74	24.54	27.78

	Spring	22.09	33.74	20.37
	Summer	23.31	23.93	20.37
	Autumn	20.86	17.79	31.48
Breastfeeding (weeks)	0	16.67	20.27	27.52
	0-16	44	33.11	36.91
	16-24	31.33	36.49	23.49
	>24	8	10.14	12.08
Mother's age (years)		31.43 (4.10)	31.93 (3.92)	31.56 (4.08)
Gestational diabetes mellitus (current pregnancy)	No	85.31	77.34	79.7
	IGT	9.09	17.97	9.77
	GDM or pior DM	5.59	4.69	10.53
Maternal height (cm)		162.53 (5.87)	163.48 (5.65)	162.68 (6.43)
Pre-pregnancy maternal BMI		23.34 (4.33)	23.49 (4.09)	23.87 (4.40)

Gestational weight gain (Kg/week)		0.43 (0.15)	0.42 (0.16)	0.40 (0.16)
Maternal country of origin	Spain	93.25	94.44	88.96
Maternal smoking at week 32 (cigarettes)	no	82.5	85.19	85.63
	<=5	10	9.26	9.38
	>5	7.5	5.56	5
Passive smoking pregnancy	yes	64.78	56.25	63.13
Alcohol during pregnancy	yes	18.4	23.93	21.47
Marital Status	Not cohabiting	1.23	0	3.68
Paternal height (cm)		176.52 (6.52)	175.94 (6.68)	175.24 (7.30)
Paternal weight (Kg)		80.52 (11.50)	80.24 (11.68)	79.30 (12.67)
Parental social class	I+II	34.36	37.42	30.06
	III	20.86	30.06	28.22
	IV+V	44.79	32.52	41.72

Place of Residence	Rural	6.21	2.45	3.68
Cohort	Asturias	16.56	5.52	9.82
	Guipuzkoa	46.01	41.1	15.95
	Sabadell	19.63	47.85	39.26
	Valencia	17.79	5.52	34.97

Supplementary Figure 1. Distribution of TEXB-alpha (pM Eeq/g) by sex