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Prenatal exposure to mixtures of xenoestrogens and repetitive element DNA methylation changes in human placenta

Nadia Vilahur[1,2,3,4], **Mariona Bustamante**[1,2,3,4], **Hyang-Min Byun**[5], **Mariana F. Fernandez**[4,6,7], **Loreto Santa Marina**[4,8,9], **Mikel Basterrechea**[4,8,9], **Ferran Ballester**[4,10,11], **Mario Murcia**[4,10,11], **Adonina Tardón**[4,12], **Ana Fernández-Somoano**[4,12], **Xavier Estivill**[2,3,4,13], **Nicolas Olea**[4,6,7], **Jordi Sunyer**[1,2,4,13], and **Andrea A. Baccarelli**[5]

[1]Center for Research in Environmental Epidemiology (CREAL), Barcelona, Catalonia, Spain

[2]Hospital del Mar Medical Research Institute (IMIM), Barcelona, Catalonia, Spain

[3]Genomics and Disease Group, Bioinformatics and Genomics Program, Center for Genomic Regulation (CRG), Barcelona, Catalonia, Spain

[4]Spanish Consortium for Research on Epidemiology and Public Health (CIBERESP), Spain

[5]Laboratory of Environmental Epigenetics, Exposure Epidemiology and Risk Program, Department of Environmental Health, Harvard School of Public Health, Boston, MA, USA

[6]Department of Radiology, Centro de Investigación Biomédica, University of Granada, Granada, Spain

[7]Instituto de Investigación Biosanitaria de Granada, Hospital Universitario San Cecilio, Granada, Spain

[8]Subdirección de Salud Pública de Gipuzkoa, Department of Health of the Basque, Spain

[9]Health Research Institute, BIODONOSTIA, Basque Country, Spain

[10]Centre for Public Health Research (CSISP-FISABIO), Valencia, Spain

[11]University of Valencia, Valencia, Spain

[12]University of Oviedo, Oviedo, Asturias, Spain

[13]Department of Health and Life Sciences, University Pompeu Fabra (UPF), Barcelona, Catalonia, Spain

Abstract

Corresponding Author: Nadia Vilahur, Center for Research in Environmental Epidemiology (CREAL), C/Doctor Aiguader, 83. 08003 Barcelona, Spain., Phone: +34933160177 nvilahur@creal.cat.

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Background—Prenatal exposure to endocrine disrupting compounds (EDCs) has previously shown to alter epigenetic marks.

Objectives—In this work we explore whether prenatal exposure to mixtures of xenoestrogens has the potential to alter the placenta epigenome, by studying DNA methylation in retrotransposons as a surrogate of global DNA methylation.

Methods—The biomarker Total Effective Xenoestrogen Burden (TEXB) was measured in 192 placentas from participants in the longitudinal INMA Project. DNA methylation was quantitatively assessed by bisulfite pyrosequencing on 10 different retrotransposons including 3 different long interspersed nuclear elements (LINEs), 4 short interspersed nuclear elements (SINEs) and 3 human endogenous retrovirus (HERVs). Associations were tested using linear mixed-effects regression models and sex interaction was evaluated.

Results—A significant sex interaction was observed for *AluYb8* (p value for interaction <0.001, significant at Bonferroni corrected p-value threshold of 0.0025). Boys with the highest TEXB-alpha levels of exposure (third tertile) presented on average a decrease of 0.84% in methylation compared to those in the first tertile (p value<0.001), while no significant effects were found in girls (p value= 0.134).

Conclusions—Our findings suggest that boys may be more susceptible to the effect of exposure to xenoestrogens during prenatal development, producing shifts in DNA methylation of certain sensitive genomic repetitive sequences in a tissue important for fetal growth and development.

Keywords

xenoestrogens; placenta; sex; repetitive elements; global DNA methylation; biomarker; TEXB

1. INTRODUCTION

Endocrine disrupting compounds (EDCs) are exogenous compounds or mixtures that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, with the highest concerns being raised for reproductive and neurobehavioral abnormalities, immune function and cancer risk (WHO 2002). Among EDCs, xenoestrogens are a subgroup of hormonally active compounds that interfere with the estrogenic signaling pathway. They can either affect the levels of endogenous hormone (Prins et al. 2008; Tabb and Blumberg 2006), or mimic estrogen action and bind with different affinities to the estrogen receptors ERα or ERβ, that function as ligand-activated transcriptional factors, and modify the pattern of expression of specific target genes (Bulzomi and Marino 2011). Xenoestrogens have been extensively used not only in agriculture but also in the manufacture of materials, products and goods like resins, textiles, plastics, cosmetics, flame retardants or insulators. They may also arise as by-products of industrial processes. A variety of adverse health effects have been described in relation to environmental exposure to single xenoestrogens (Arrebola et al. 2013b; Ibarluzea Jm et al. 2004; Valvi et al. 2013). Many xenoestrogens are persistent organic pollutants (POPs) and bioaccumulate in lipids. Therefore, exposure in human populations, even at low-doses, is most likely ubiquitous, persistent and occurs in complex mixtures (Arrebola et al. 2013a; Fucic et al. 2012), with effects that may not be predictable when analyzing single

compounds independently (Webster 2013). As stated by the latest World Health Organization (WHO) report on endocrine disrupting chemicals *"…there is emerging evidence that many chemicals may act additively and, each at levels without individual effect, could act together to cause health problems"* (WHO 2013).

Several studies have evidenced the transplacental transfer of xenoestrogens including POPs, flame retardants and arsenic, which only weakly or fail to bind to α-fetoprotein, the fetal form of serum albumin (Frederiksen et al. 2010; Pilsner et al. 2012; Tan et al. 2009). Exposure *in utero* has been related to a number of adverse health endpoints in children, including a higher risk for overweight, alterations in psychomotor and cognitive development or urogenital abnormalities in male newborns (Forns et al. 2012; Palmer et al. 2009; Puertas et al. 2010; Valvi et al. 2013), and several studies have reported sex specific effects occurring by mechanisms that remain poorly understood (Fernandez et al. 2007; Papadopoulou et al. 2013; Vafeiadi et al. 2013; Valvi et al. 2012).

A number of *in vivo* and *in vitro* studies and a few human investigations have shown effects of EDCs, mainly pesticides, on different epigenetic marks including DNA methylation as reviewed by Collotta *et al.* (2013) (Collotta et al. 2013), and there is some evidence in animal models of transgenerational inheritance of epigenetic alterations and disease-associated states due to EDCs exposure (Guerrero-Bosagna et al. 2012; Guerrero-Bosagna et al. 2013; Manikkam et al. 2013; Skinner et al. 2013).

Repetitive elements are genomic DNA sequences that account approximately for half of the human genome and have often been studied as surrogates of global DNA methylation (Klose and Bird 2006; Lander et al. 2001; Tajuddin et al. 2013; Wilhelm et al. 2010). A group of these sequences, called retrotransposons, propagate themselves by RNA mediated transposition (Rogers 1983) and constitute an interesting source of human genomic variation, genomic instability and occasionally, disease (Callinan and Batzer 2006; Wolff et al. 2010). Among retrotransposons, the best studied families are the long interspersed nuclear elements (LINEs), the short interspersed nuclear elements (SINEs), and the human endogenous retrovirus (HERV). Most epidemiological studies in humans have analyzed methylation in LINE1 and *AluSx* subfamilies in relation to environmental exposures (Kile et al. 2012; Kim et al. 2010; Rusiecki et al. 2008; Wright et al. 2010). Additional repetitive element subfamilies have been described in humans differing in their nucleotide sequence and estimated evolutionary age of insertion in the human genome. These subfamilies present heterogeneous methylation patterns, which may lead to varying degrees of regulation and susceptibility to environmental stressors like, for example, airborne pollutants or tobacco smoke during pregnancy (Breton et al. 2009; Byun et al. 2013).

The purpose of the present study is to explore whether prenatal exposure to mixtures of xenoestrogens can affect DNA methylation of several retrotransposons in human placental tissue and if effects differ among boys and girls.

2. MATERIAL AND METHODS

2.1 Study population

The INMA (Infancia y Medio Ambiente) Project, is a population-based longitudinal birth cohort, including mother and children pairs from different Spanish regions, aiming at understanding the effects of exposure to prenatal and early postnatal environmental factors on different health outcomes in children including growth trajectories, allergies, asthma and neurodevelopment. Detailed information on recruitment has been published elsewhere (Guxens et al. 2012). This study was based on INMA individuals from the cohorts of Asturias, Gipuzkoa Sabadell and Valencia. DNA methylation was analyzed on a total of 192 placentas randomly selected among those of Caucasian origin, having TEXB biomarker measurement available as well as with maximal completeness of information on covariates during pregnancy, and ensuring a similar male to female ratio (97 males *vs* 95 females).

Mothers were orally informed and prior to delivery provided written informed consent of their participation in the study, which was approved by the Institutional Review Board of all the Institutions involved.

2.2 Total Effective Xenoestrogen Burden

The Total Effective Xenoestrogen Burden is a biomarker of the combined estrogenic effect of environmental chemicals which has previously been studied in relation to the risk for several human diseases including anomalies of sexual maturation in males, breast cancer and Type 2 diabetes and common phenotypes such as birth weight (Arrebola et al. 2013b; Fernandez et al. 2007; Ibarluzea Jm et al. 2004; Vilahur et al. 2013b).

INMA placentas were randomly collected at the time of delivery in the different hospitals of recruitment and after physical examination they were weighted without deciduas basalis and chorionic plate and frozen at −20ºC or −80ºC. No information was available about the time-to-storage after delivery for each placenta, but a maximum of 6 hours was estimated. Then, they were transferred in dry ice to the Biobank at the San Cecilio University Hospital (HUSC) in Granada and stored at −80ºC.

Before performing the TEXB biomarker assay, each placenta was slightly defrosted and cut in half, and one of the halves was placed in the glass container of a mixer (Büchi Mixer B-400 Büchi Laboratories AG, Flawil, Switzerland) and homogenized to ensure representativity of the whole placenta in the aliquoting process. The remaining half of the placenta was stored again at −80ºC. The methodology has been published previously (Fernandez et al. 2007). Briefly, 0.4 g of placenta homogenate was extracted with hexane and eluted in a glass column filled with Alumine. The process was performed per quadruplicate for each placenta sample (total weight: 1.6 g of placenta). The pooled concentrated eluate was injected into the preparative high-pressure liquid chromatography (HPLC). The HPLC method was developed to efficiently separate according to their polarity organohalogenated lipophilic xenoestrogens (HPLC-alpha fraction) from endogenous hormones (HPLC-beta fraction), using a normal-phase column. The combined estrogenic effect of both HPLC fractions was then tested in the E-Screen bioassay for estrogenicity; a proliferative *in vitro* assay using MCF-7 human breast cancer cells. Each sample was

assayed in triplicate with a negative (vehicle) and positive (estradiol) control, as described in detail by Fernandez *et al.* (Fernandez et al. 2007). The proliferative effect (PE) was calculated as the ratio between the highest cell yield obtained with 100 pM of estradiol and the proliferation of hormone-free control cells. The PE of alpha and beta fractions was referred to the maximal effect obtained with estradiol and transformed into estradiol equivalent units (Eeq) by reading from a dose–response curve prepared using estradiol (concentration range 0.1 pM to 10 nM) and was expressed as TEXB-alpha and TEXB-beta values in Eeq pM per milliliter (pM Eeq/ml) and Eeq pM per gram of tissue (pM Eeq/g) (Fernandez et al. 2007).

The limit of detection (LOD) was defined as the concentration needed to produce a significantly different proliferative effect from that observed in control cells (0.1 pM Eeq/ml). A concentration equal to 0.05 pM Eeq/ml was assumed for samples with TEXB values below the LOD (n=31, 16.15%).

The estrogenicity of the alpha-fraction, which contains no endogenous sex-hormones or polar xenoestrogens (beta-fraction), can be considered a marker of the TEXB of persistent environmental organohalogenated estrogens (Fernandez et al. 2007).

2.3 Placental biopsying and DNA extraction

Half placentas were kept frozen at −80ºC at the Hospital Universitario San Cecilio (HUSC) Biobank in Granada. In order to obtain biopsies for DNA extraction, placentas were physically examined to determine the fetal side, which was not always easily recognizable. To avoid basal plate contamination (maternal side) and exclude the chorionic plate (fetal membranes amnios and chorion) and to ensure as homogeneous sampling procedures across samples as possible, biopsies of 1 to 2 cm^3 were obtained from the inner region (in relation to the dorsal and ventral sides) from the middle of the placenta (in terms of distance from the outer part to the center), similarly to what has been proposed by other authors (Janssen et al. 2012). During the whole procedure samples were handled on ice to prevent complete defrosting of the tissue and biopsies were immediately transferred in labeled tubes kept in dry ice and stored at −80ºC.

Placental biopsies were rinsed twice in 0.8 mL PBS 0.5X during 5 minutes prior to DNA extraction to remove traces of maternal blood. On average, around 20 μg of good quality genomic DNA was obtained from 25 mg of starting material using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA). Sample concentrations and purity ratios (260/230 and 260/280) were measured using a NanoDrop spectrophotometer device (Thermo Scientific, Waltham, MA, USA). Additionally, 100 ng of DNA were loaded on 1.3% agarose gels with SYBR safe staining (Invitrogen, Carlsbad, CA, USA) to visually check for DNA degradation (smear bands or bands below 10,000 bp). The isolated genomic DNA presented a good DNA quality and was stored at −20°C for further quantification of DNA methylation.

2.4 Global DNA methylation

We measured placental genome-wide levels of DNA methylation in repetitive elements as a proxy of global DNA methylation. Bisulfite pyrosequencing was used to quantitatively measure DNA methylation levels of 4 LINEs (L1PA5, L1PA2, L1HS and L1Ta), 3 SINEs (*AluSx, AluYb8 and Alud6*) and 3 HERV (MLT1D, ERV1 and ERV9) in 192 placenta samples.

First, 1 μg of genomic DNA was bisulfite-converted using the Zymo Gold EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA) and eluted in a final volume of 25 μl. Then, 1 μl of bisulphite-converted DNA was used for PCR amplification using the GoTaq Hot Start Polymerase (Promega, San Luis, CA, USA) and specific primers designed for bisulfite converted DNA sequences for each of the elements analyzed. PCR conditions and pyrosequencing primer sequences for L1HS, *AluSx*, and *AluYb8* have been previously published by Yang et al. and by Choi et al. (Choi et al. 2009; Yang et al. 2004), while additional assays specific for L1PA5, L1PA2, and L1Ta, for the *AluYd6* subfamily, and for the three HERV subfamilies (MLT1D, ERV1 and ERV9) were developed by Byun *et al*. (Byun et al. 2013). Primer sequences and PCR cycling conditions for each assay as well as number of CpG sites analyzed in each repetitive element are shown in Supplemental Material, Table S1. Finally, CpG methylation was evaluated by pyrosequencing. Samples were run in duplicate on the same day, and non-CpG cytosines in the analyzed sequence were used as controls to determine the efficiency of bisulfite-conversion. To control repeatability of the assay, we filled a well in each plate with a known fully methylated DNA sequence and another with a fully unmethylated DNA sequence. The percentage of methylation was expressed for each CpG site as the %5-mC divided by the sum of methylated and unmethylated cytosines.

2.5 Statistical Analysis

Correlations between adjacent CpG sites within each element were tested by Spearman rank correlations. Average correlations of CpG sites within the same repetitive element ranged from 0.15 to 0.76 (Supplemental Material, Table S2) and therefore we decided to use CpG position as a random effect rather than averaging methylation across sites, similarly to what has been suggested by other authors (Burris et al. 2012).

To explore sex differences in DNA methylation two-sample t-tests with unequal variances or Mann-Whitney U tests were performed accordingly.

TEXB-alpha presented a non-normal right skewed distribution (see Supplemental Material, Figure S1) with 31 samples below the LOD (0.01 pM Eeq/ml) which were assigned values of LOD/2. We then created sex-specific TEXB-alpha tertiles which allowed us to explore possible nonlinear dose-response effects at different levels of exposure, as described for several EDCs (Vandenberg et al. 2012).

A linear mixed-effects regression model was used to estimate the association between tertiles of TEXB-alpha and absolute changes in percentage of DNA methylation in each repetitive element. Restricted Maximum Likelihood estimates were used to test random effect parameters using the Wald z-test. Significant individual random effects included in

the final models were specified to take into account the correlation between technical replicates from the same individual (duplicates) and the different means of methylation between sites in the same element (CpG position).

TEXB-alpha tertiles and *a priori* selected covariates (child sex and cohort) were included as fixed coefficients. The fully adjusted model additionally included maternal age, pre-pregnancy body mass index, and smoking during pregnancy, selected because of previous knowledge to impact both DNA methylation and TEXB levels (Morales et al. 2012; Vilahur et al. 2013b).

We used the following model:

$$Y_{\mathrm{i,j,k}}=\beta_0+\beta_{1\mathrm{TEXB}}+\beta_2\,\mathrm{x}_2+\cdots+\beta_{\mathrm{p}}\,x_{\mathrm{p}}+\upsilon_{0\mathrm{i}}+\upsilon_{1\mathrm{j}}+\upsilon_{2\mathrm{k}}+\varepsilon_{\mathrm{ijk}}$$

$Y_{i,j,k}$ represents the mean methylation level of all the CpG sites at any of the repetitive elements investigated for the i-th subject, the j-th duplicate run and the k-th CpG position (i=1,…,192; j=1,2 and k=1,…, 5).

To explore possible effect modification by sex we introduced an infant sex interaction term to the multivariable mixed-effect regression models.

Considering the number of associations explored, a total of 20, the threshold for significant effects was set at a p-value below 0.0025 after applying Bonferroni correction for multiple testing.

Analyses were conducted using STATA 10.1 statistical software (Stata Corporation, College Station, TX).

3. RESULTS

3.1 Demographic characteristics, TEXB and DNA methylation levels

Main characteristics of mother and children of the study population are presented in Table 1. Mothers showed medium to high educational level, low prevalence of smoking habits during pregnancy and a mean age of around 32 years, similar to what we observe in the rest of the mothers from the INMA cohort for whom placenta was not available (n=2017) (data not shown). There was an equal distribution of sex among the newborns included in our study (49.48 % boys).

Overall and sex-specific levels of TEXB-alpha tertiles, expressed in estrogen equivalent units per gram of placenta (pM Eeq/g), are shown in Table 2. Mean placental DNA methylation levels for each repetitive element analyzed and estimated evolutionary age are shown in Supplemental Material, Table S1. Older retrotransposons had lower average DNA methylation levels than younger ones except for the MLT1D element, DNA methylation levels for LINE1 and *AluYb8* were similar to what has previously been described in placenta (Armstrong et al. 2014; Wilhelm-Benartzi et al. 2012) and lower than in whole blood as shown by other authors (Armstrong et al. 2014; Byun et al. 2013). We explored differences in DNA methylation between boys and girls, comparing the mean methylation across all the

CpG sites analyzed for each element. Boys presented on average significantly higher methylation levels compared to girls for 5 of the 10 repetitive elements studied, namely the three *Alus* and two of the HERV elements (ERV1 and ERV9) (Table 3).

3.2 Association of TEXB-alpha levels and LINE, *Alu* and HERV DNA methylation

We used a linear mixed-effects regression model to estimate associations between repetitive element DNA methylation and TEXB-alpha exposure, taking the first tertile (T1) as the reference (Table 4).

After correcting for multiple testing (p value=0.0025) no significant associations were found between exposure to TEXB-alpha and any of the repetitive elements analyzed when considering boys and girls together. However, methylation of two LINEs showed associations with TEXB-alpha exposure that did not pass multiple testing threshold: TEXB-alpha exposure in the second tertile resulted in a 0.81% decrease in L1HS DNA methylation (p value=0.003), while exposure in the third tertile produced on average an increase in L1PA5 DNA methylation of 0.99% (p value=0.003) in children when compared with TEXB-alpha in the first tertile. When we explored effect modification by sex, we observed a significant male-specific negative association between *AluYb8* methylation and TEXB-alpha (p value interaction<0.001) (Table 5). Boys with higher levels of TEXB-alpha (third tertile) had on average a decrease in methylation of 0.84% compared to boys in the first tertile (p value=0.001), while no significant effects were found in girls (β=0.30, p-value=0.08). Mean methylation level for each CpG site analyzed of the *AluYb8* element is shown by TEXB-alpha tertile in boys and girls separately in Supplemental Material, Table S3. We did not find statistically significant sex interactions for the other *Alu* elements analyzed (*AluSx* and *AluYd6*), but marginally significant sex interactions according to our p value threshold were observed for two LINE elements, L1PA2 and L1Ta. In both cases, methylation levels tended to decrease in boys and increase in girls upon exposure to TEXB-alpha (Supplemental Material, Table S4).

4. DISCUSSION

This is the first epidemiological study analyzing the association between a biomarker of prenatal exposure to mixtures of xenoestrogens and DNA genome-wide methylation of retrotransposons in placenta including a large number of retrotransposons and exploring effect modification by sex. We found a statistically significant association between exposure to TEXB-alpha and *AluYb8* repetitive element DNA methylation: boys in the highest tertile of TEXB-alpha presented on average a decrease of 0.84% in methylation compared to boys in the first tertile, while no effects were found in girls.

Two previous epidemiological studies in adults with smaller sample sizes have analyzed the effects on blood LINE-1 and *Alu* DNA methylation of exposure to POPs such as organochlorine (OC) pesticides and several of its by-products, which are lipophilic compounds accounted by the TEXB-alpha biomarker. In the first study, Rusiecky *et al.* found a significant negative association between POPs concentrations and *AluSx* DNA methylation in a population of highly exposed adult Greenlandic Inuit (n=70, 87% males) (Rusiecki et al. 2008). In the second study, a cross sectional design exploring the effect of

low-dose OC pesticide exposure in a healthy adult Korean population (n=86), authors also found that environmental exposure was inversely associated with DNA methylation in the *AluSx* element (Kim et al. 2010). Although both studies find hypomethylation of an *Alu* element in relation to OC exposure, neither has specifically analyzed DNA methylation of the *AluYb8* subfamily, nor explored sex interactions.

A balanced neonatal androgen:estrogen ratio is critical during prenatal period for male sexual differentiation and for the establishment of brain sexual differences during early development (Arnold 2009; Fernandez et al. 2007; Lenz et al. 2012). A growing body of evidence in humans suggests that sex differences may arise in epigenetic responses to prenatal environmental exposures (Gabory et al. 2009), and animal studies have suggested that effects may persist over generations (Anway et al. 2005).

Although probably EDCs will produce some of their adverse effects on health by interfering with the binding of endogenous steroid hormones to nuclear receptors in order to regulate gene expression (Bulzomi and Marino 2011), genome-wide methylation reshaping as a result of increased oxidative stress has been postulated as an alternative mechanism. Some environmental toxins, including POPs and other endocrine disruptors like Bisphenol A, can affect mitochondrial function and cause pro-oxidative conditions (D'Cruz et al. 2012; Lim et al. 2010; Yang et al. 2009). During pro-oxidant states, homocysteine is diverted away from the methionine cycle and toward the production of glutathione, leading to a deficiency in methyl donors and genome-wide DNA hypomethylation (Hitchler and Domann 2009; Madrigano et al. 2011). Supporting this hypothesis, there is a study showing a decrease in *AluYb8* DNA methylation from buccal cells in prenatally exposed children to tobacco smoke, a known source of oxidative stress (Breton et al. 2009). A fine regulation of global methylation states is important in the development and function of extraembryonic tissues (Nelissen et al. 2011), and experimentally induced alterations in global DNA methylation affect placental weight in rats and disrupt trophoblast proliferation and migration (Rahnama et al. 2006; Serman et al. 2007).

Additionally, hypomethylation of repetitive elements contributes to loss of genomic stability, which is believed to result in somatic retrotranposition and insertional mutations (Romanish et al. 2010; Wilson et al. 2007). Disease events due to retrotransposition are mainly caused by the younger *Alu* elements, mostly the *AluYa5, AluYb8 and AluYs* subfamilies (Hancks and Kazazian 2012), which are the most active retrotransposons in humans with >1 million copies. We found that *AluYb8*, the repetitive element associated with TEXB-alpha exposure in boys in the present study, had higher levels of DNA methylation in placenta than other elements, similarly to what others have reported (Armstrong et al. 2014; Wilhelm-Benartzi et al. 2012) and likely explained by its younger evolutionary age and higher CpG content (Choi et al. 2009). There is evidence that retrotransposons can serve as alternative promotors in several genes (van de Lagemaat et al. 2003; Waterland and Jirtle 2003). Retrotransposon transcription has a key influence upon the transcriptional output of the mammalian genome (Faulkner et al. 2009) and hypomethylation of repetitive elements has been associated with alterations of expression of particular genes (Morgan et al. 1999; Wolff et al. 2010).

The methylation assay used in this study provides a cumulative measure of DNA methylation of repetitive element sequences dispersed throughout the genome (Yang et al. 2004). Thus, the change in DNA methylation observed for *AluYb8* in boys might represent a very small genome-wide change in methylation levels or alternatively a more pronounced change in one particular region. Although small, the effect size is similar to what other studies have reported analyzing either methylation of LINE-1 and several *Alu* retrotransposons or the global content of genomic 5′-methyl-deoxycytidine in relation to aging, prenatal tobacco smoke exposure and air pollution in blood, buccal cells and placenta (Bollati et al. 2009; Breton et al. 2009; Janssen et al. 2013; Madrigano et al. 2011).

We found that boys had significantly higher levels of methylation in all the *Alu* elements and in two of the endogenous retroviruses analyzed, similarly to previous literature that reported increased *AluSx* and *AluYb8* DNA methylation in boys compared to girls in total blood and in placenta tissue, with methylation differences in the same range of what we have reported (around 1% increase) (El-Maarri et al. 2007; Rusiecki et al. 2008; Wilhelm-Benartzi et al. 2012). Although the origin of these sex differences in repetitive elements methylation remains unsolved, the origin may reside in the X chromosomes. Some have postulated that there may be a factor in the X chromosome inducing hypomethylation that escapes proper inactivation in the female Xi (X inactive) chromosome, or alternatively that the epigenetic “resources” necessary to maintain a whole chromosome inactive in females may result in less resources to properly methylate autosomal loci (El-Maarri et al. 2007).

Limitations

An important source of confounding in epigenetic studies may arise from the difference in cell composition of the tissue studied across samples (Jacoby et al. 2012). This is particularly relevant in the context of blood, composed of a variety of different cell subtypes with unique DNA methylation profiles (Houseman et al. 2012; Reinius et al. 2012), but it may be less problematic for tissues like the parenchymal villous of the human placenta, which is mainly dominated by mesenchymal-derived fibroblasts and trophoblasts. There are no epidemiological studies so far that have dissected different placental cell types and studied their specific DNA methylation patterns. However, cell heterogeneity may have impacted the effect size observed in our results (Liu et al. 2013).

Although in our study placentas were not frozen at −80ºC immediately after delivery in a standardized way, which is a common situation in the context of human epidemiological studies, we have previously shown that retrotransposon DNA methylation analyzed by bisulfite pyrosequencing remains stable in relation to time to storage delay at room temperature for up to 24 hours (Vilahur et al. 2013a), unlike what happens in more labile biological marks such as RNA or protein levels (Adibi et al. 2009; Ferrer et al. 2007).

5. CONCLUSIONS

The present study shows an association between increasing levels of *in utero* exposure to xenoestrogens and lower placental *AluYb8* DNA methylation in boys, both analyzed in the same tissue, which may provide some clues regarding the molecular effects triggered by exposure to mixtures of hormonally active compounds commonly found in the environment

and the differential susceptibility among males and females. Additionally, how xenoestrogens may modify the epigenome of specific genes deserves further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

INMA	Infancia y Medio Ambiente Project
TEXB	Total Effective Xenoestrogen Burden
LOD	Limit of Detection
LINE	Long Interspersed Nuclear Element
SINE	Short Interspersed Nuclear Element
HERV	Human Endogenous Retrovirus
POPs	Persistent Organic Pollutants
EDCs	Endocrine Disrupting Compounds

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Highlights

- Few epidemiologic studies have analyzed effects of mixtures of hormonally active compounds
- We measured a biomarker of prenatal cumulative exposure to xenoestrogens
- We examined its relationship with DNA methylation of repetitive elements in placenta
- Higher levels of xenoestrogens were associated with lower *AluYb8* methylation in boys

Table 1

Maternal and children demographic characteristics

	N	% or mean (SD)
Mothers		
Active smoking during pregnacny		
no	159	84.13
<=5 cigarettes/day	16	8.47
>5 cigarettes/day	14	7.41
Parity		
primiparous	110	56.21
multiparous	82	43.79
Cohort		
Asturias	33	17.19
Gipuzkoa	64	33.33
Sabadell	70	36.46
Valencia	25	13.02
Education		
< secondary school	34	17.71
secondary school	89	46.35
university degree	69	35.94
Age (y)	192	31.83 (3.94)
Body mass index (BMI)	192	23.39 (4.07)
Children		
Sex		
male	97	49.48
female	95	50.52
Birth weight (g)	191	3301 (411.03)
Gestational age (weeks)	189	39.82 (1.34)

Table 2

Total Effective Xenoestrogen Burden (TEXB-alpha) levels in placenta samples (n=192)

	1st Tertile					2nd Tertile					3rd Tertile					
	N	**Median**	**IQR**	**Min**	**Max**	**N**	**Median**	**IQR**	**Min**	**Max**	**N**	**Median**	**IQR**	**Min**	**Max**	**N total**
All	64	0.09	0.03–0.28	0.03	0.4	64	0.75	0.68–0.90	0.41	0.98	64	1.82	1.26–2.70	0.99	51.94	192
Boys	33	0.12	0.03–0.28	0.02	0.4	32	0.75	0.69–0.93	0.4	1.21	32	2.23	1.48–2.70	1.21	31.53	97
Girls	32	0.09	0.03–0.24	0.02	0.41	32	0.76	0.68–0.90	0.43	0.95	31	1.48	1.14–3.55	0.97	51.94	95

TEXB-alpha levels are expressed in estrogen equivalent units per gram of placenta (pM Eeq/g) and categorized in tertiles. IQR: Inter Quantile Range. ¡

Table 3

Differences in average mean methylation levels between boys and girls for the ten repetitive elements analyzed

	Repetitive Element	Mean % methylation (SD)		p-value
		Girls (n=97)	Boys (n=95)	
LINEs	L1PA5[1]	23.28 (2.40)	23.03 (2.71)	0.349
	L1PA2[1]	44.37 (2.57)	43.98 (2.77)	0.159
	L1Hs	55.42 (2.92)	55.35 (2.99)	0.639
	L1Ta	50.32 (3.09)	50.37 (3.26)	0.862
Alus	AluSx[1]	25.89 (1.08)	26.40 (0.98)	<0.001*
	AluYb8[1]	82.91 (1.80)	83.50 (1.91)	0.001*
	AluYd6	65.14 (3.1)	66.25 (3.00)	0.004*
HERVs	MLT1D	86.22 (3.39)	86.44 (3.55)	0.524
	ERV1[1]	17.22 (2.34)	17.56 (1.97)	0.004*
	ERV9[1]	49.48 (5.74)	49.83 (2.64)	<0.001*

[1] Mann Whintey U- tests were used since these repetitive elements presented a non-normal distribution.

* Significant below p-value ≤ 0.001

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Table 4

Adjusted association between TEXB-alpha tertiles and repetitive element DNA methylation.

	N	TEXB-alpha	β[1]	(95 % CI)	p-value	Interaction p-value[2]
LINEs						
L1PA5	189	T1	Ref			
		T2	−0.042	(−0.755 0.6713)	0.908	
		T3	0.992	(0.304 1.679)	**0.005**	0.836
L1PA2	189	T1	Ref			
		T2	−0.023	(−0.828 0.782)	0.955	
		T3	1.15	(0.317 1.982)	**0.007**	**0.007**
L1HS	189	T1	Ref			
		T2	−0.807	(−1.384 −0.229)	**0.006**	
		T3	0.1647	(−0.392 0.721)	0.562	**0.018**
L1Ta	189	T1	Ref			
		T2	−0.219	(−0.831 0.394)	0.484	
		T3	0.211	(−0.379 0.801)	0.483	**0.007**
Alus						
AluSx	189	T1	Ref			
		T2	−0.073	(−0.343 0.198)	0.599	
		T3	0.113	(−0.148 0.375)	0.396	0.16
AluYb8	189	T1	Ref			
		T2	0.012	(−0.274 0.297)	0.936	
		T3	−0.265	(−0.540 0.010)	0.059	**<0,001**[*]
AluYd6	189	T1	Ref			
		T2	−0.343	(−1.017 0.331)	0.319	
		T3	0.316	(−0.333 0.965)	0.341	0.066
HERVs						

	N	TEXB-alpha	β[1]	(95 % CI)	p-value	Interaction p-value[2]
MLT1D	189	T1	Ref			
		T2	−0.045	(−1.087 0.997)	0.933	
		T3	−0.823	(−1.827 0.181)	0.108	**0.018**
ERV1	189	T1	Ref			
		T2	0.023	(−0.497 0.542)	0.931	
		T3	−0.003	(−0.503 0.497)	0.991	0.426
LTR12	188	T1	Ref			
		T2	0.354	(−1.0312 1.739)	0.617	
		T3	−0.550	(−1.886 0.786)	0.420	0.151

Models adjusted by cohort, sex, maternal pre-pregnancy BMI, active smoking and age during pregnancy TEXB-alpha is expressed in estrogen equivalent units per gram of placenta (pM Eeq/g) and categorized in tertiles.

[1] β represents the absolute change in DNA methylation levels (expressed in percentage) in individuals in the second or in the third tertile of TEXB-alpha compared to those in the first tertile (reference).

[2] p-value for the interaction with newborns sex

* significant Bonferroni-adjusted p-value (< 0.0025)

Table 5

Adjusted association between tertiles of TEXB-alpha (pM Eeq/g) and *AluYb8* DNA methylation stratified by sex

AluYb8	N	TEXB-alpha[1]	β	(95 % CI)	p-value
Girls	93	T1	Ref		
		T2	−0.023	(−0.370 0.323)	0.893
		T3	0.300	(0.043 0.643)	0.086
Boys	96	T1	Ref		
		T2	−0.111	(−0.568 0.347)	0.635
		T3	−0.835	(−1.279 −0.390)	<0.000*

Models adjusted by cohort, maternal pre-pregnancy BMI, active smoking, age at pregnancy

*significant Bonferroni-adjusted p-value (< 0.0025).

[1]Sex-specific TEXB-alpha tertiles.