Prenatal exposure to mixtures of xenoestrogens and genome-wide DNA methylation in human placenta.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved and/or their legal tutors.

Keywords:

Xenoestrogens, placenta, DNA methylation, prenatal, programming, endocrine disruptors, TEXB, epigenome.

Abstract

Background: *In utero* exposure to xenostrogens may modify the epigenome. We explored the association of prenatal exposure to mixtures of xenoestrogens and genome-wide placental DNA methylation.

Materials & methods: Sex-specific associations between methylation changes in placental DNA by doubling the concentration of TEXB-alpha exposure were evaluated by robust multiple linear regression. Two CpG sites were selected for validation and replication in additional male born placentas.

Results: No significant associations were found, although the top significant CpGs in boys were located in the LRPAP1, HAGH, PPARGC1B, KCNQ1 and KCNQ1DN genes, previously associated to birth weight, type 2 diabetes, obesity or steroid hormone signaling. Neither technical validation nor biological replication of the results was found in boys for LRPAP and PPARGC1B.

Conclusions: Some suggestive genes were differentially methylated in boys in relation to prenatal xenoestrogen exposure, but our initial findings could not be validated or replicated.

Background

Xenoestrogens are a group of endocrine disrupting chemicals (EDCs) that specifically interfere with the endogenous estrogen hormone signaling pathways and/or metabolism [1]. Exposure to xenoestrogens during susceptible developmental stages like the prenatal period has been related to a number of adverse health outcomes in the offspring, both in humans and in animals, including alterations in birth weight, growth and body mass index, male and female reproductive abnormalities, infant neurodevelopment or increased risk for diabetes and several types of cancer among others [2-10]; with evidences for sex-specific associations [10-15].

The environmental epigenetic hypothesis suggests that the fetal epigenome may be affected by *in utero* environmental exposures, and this may play a role in later disease phenotypes [16]. The adverse effects of environmental exposures are especially relevant in the context of early exposure to EDCs, since endogenous hormones, active at extremely low concentrations, play critical developmental roles during the prenatal period [17, 18]. Mice models have revealed that *in utero* exposure to xenoestrogens may disrupt DNA methylation. Bisphenol A and diethylstilbestrol, compounds with known xenoestrogenic properties, induced higher expression of the Enhancer of Zeste Homolog 2 gene, a histone methyltransferase which resulted in increased mammary histone H3 trimethylation and triggered methylation changes in several estrogen-responsive genes [19, 20]. In another study, dietary exposure to soy phytoestrogens in pregnant rats advanced sexual maturation and induced aberrant promoter methylation of *skeletal a-actin*, *estrogen receptor-a*and *c-fos* genes in the offspring [21, 22]. In addition, the pesticide methoxychlor induced changes in DNA methylation at a number of imprinted genes, accompanied by a substantial decrease in mice sperm count [23].

In humans, significant associations have been reported between prenatal exposure to single xenoestrogens like the persistent organic chemicals dichlorodiphenyltrichloroethane (DDT), dichlorodiphenyldichloroethylene (DDE) or polychlorinated biphenyls (PCBs) and global DNA hypomethylation, measured as DNA methylation in retrotransposon elements (LINEs and SINEs) [14, 24-26], as well as at gene-specific level [27]. On the other hand, a recent study has shown that prenatal exposure to phthalates and phenols was related to methylation changes in placenta in the imprinted *H19* gene and in the *IGF2* differentially methylated region 2 (DMR2) only in boys, two regions known to play a major role in fetal and placental growth, although a further relation with birth weight could not be demonstrated in this study [28]. Epigenetic dysregulation of the placenta, which can be caused by several environmental factors, may lead to abnormal placental development and function [29]. Even if this organ does not form part of an adult, the human placenta plays a key role in ensuring optimal fetal development and growth, with implications for newborns disease predisposition in later life.

Exposure during pregnancy to arsenic and cadmium, metals which have been suggested to interfere with estrogen signaling [30-33], has been associated to methylation changes in cord blood DNA,

both globally and in specific genomic sites as revealed by epigenome-wide association studies (EWAS)[34], with stronger associations often observed in males [15, 35-37].

However, exposure to single chemicals is often an unrealistic scenario, since environmental contamination, including EDCs, is rarely due to a single compound but to mixtures to which populations are exposed that may produce additive or even synergistic effects [38-40]. The use of biomarkers of cumulative exposure, such as the Total Effective Xenoestrogen Burden (TEXB) is therefore a more realistic approach to study the impact of co-exposure to mixtures of chemicals with estrogenic disrupting properties in a real world scenario [41, 42].

We previously reported male specific associations between placenta TEXB-alpha and children birth weight, early growth and motor development at age 1-2 [43, 44], accompanied by DNA hypomethylation in *Alu* retrotransposons in placenta [45]. The aim of the present study is to perform an epigenome-wide association study (EWAS) analyzing boys and girls separately to identify differentially methylated genomic *loci* in placenta in relation to prenatal TEXB-alpha exposure.

Material and methods

Study population

The INMA- INfancia y Medio Ambiente- (Environment and Childhood) Project is a Spanish multi center birth cohort study exploring the role of environmental pollutants on children development and health [46]. All participants involved in the study provided written consent prior to participation, and the research protocol was approved by the Ethical Committees of the Institutions and Centers from the different Spanish regions.

Two subsets of samples were analyzed in the current study, one for the discovery step and the other for the replication step. The discovery study included 181 women of Caucasian origin enrolled from November 2003 to January 2008 from four different areas of Spain: Asturias (18%), Basque Country (34%), Catalonia (37%) and Valencia (11%), who had not followed any program of assisted reproduction, gave singleton birth at the reference hospitals and had placenta collected at delivery. In the replication step, 126 women from the same cohort which had male deliveries were selected, enrolled in the study in the same period and following the same inclusion criteria from Asturias (4%), Basque Country (39%), Catalonia (37%) and Valencia (20%).

Exposure assessment

The Total Effective Xenoestrogen Burden (TEXB) is a biomarker of the combined estrogenic effect of environmental estrogens [47]. The detailed procedure has previously been published elsewhere [42, 48]. Briefly, half of each placenta was homogenized, in order to obtain a sample representative of the maternal-fetal unit. Thereafter, an hexane-based solid-liquid extraction method

was used to separate less lipophilic chemicals including endogenous hormones (beta fraction) from more lipophilic environmental compounds, i.e. persistent organic pollutants with xenoestrogenic potential (alpha fraction). Then, the estrogenicity of the alpha fraction (i.e. TEXB-alpha) for each placenta sample was quantified using the E-Screen bioassay, a cell proliferation assay using MCF7 breast cancer cells, at the Biomedical Research Center from the University of Granada (Spain). TEXB-alpha was expressed in picomolar (pM) estradiol equivalent units (Eeq) per gram of placenta tissue (pM Eeq/g placenta).

DNA isolation and methylation genome-wide data generation

INMA placentas were stored at -80ºC at the IUSC Biobank of the San Cecilio University Hospital (Granada). Later, half of each sample was homogenized for exposure assessment as described previously, and the other half was partially defrosted and biopsies of 5 cm^3 from the inner region of the placenta were conducted, approximately at a distance of 1.0–1.5 cm below the fetal membranes, which were previously removed, and at a distance of ~5 cm from the site of cord insertion, in order to obtain biopsies from the placental villous parenchyma as homogeneous as possible across samples. Twenty five mg of tissue were used for DNA extraction, previously rinsed twice during 5 minutes in 0.8 mL of 0.5X PBS in order to remove traces of maternal blood. Genomic DNA from placenta was isolated using the DNeasy® Blood and Tissue Kit (Qiagen, CA, USA) in narrow time windows and by the same person, in order to minimize technical and operator variations.

DNA quality was evaluated using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) and additionally 100 ng of DNA were run on 1.3% agarose gels to confirm that samples did not present visual signs of degradation (smears or bands below 10,000 bp). Isolated genomic DNA was stored at -20°C until further processing.

Genome-wide DNA methylation was measured in 202 placenta samples (including ten duplicates) using the Illumina Infinium Human Methylation450 BeadChip, a panel which roughly spans 486,000 CpG sites in the human genome. Samples were plated on each chip, experimentally randomized with regard to sex distribution and processed blind to sample identification at the Genome Analysis Facility of the University Medical Center Groningen (UMCG) in Holland, where500 ng of good quality DNA was used to perform bisulfite conversion followed by methylation profiling following Illumina's protocol.

BeadChips were scanned with an Illumina iScan and image data was uploaded into the Methylation Module of Illumina's analysis software GenomeStudio (lllumina, SanDiego, CA USA), and converted in β-values, that range from 0 (unmethylated) to 1 (fully methylated) and represent the fraction of methylation at a given CpG locus.

Methylation data quality control and normalization

Methylation data quality control (QC) was performed in several steps to exclude low quality samples and probes.

First, using the Genome Studio software, we removed samples that did not reach a call rate of 95% at a p-value below 0.05. Then, following Illumina recommendations we verified the intensities of several control probes provided by Illumina in order to: i) assess the quality of the experiment (sample-dependent controls) and ii) identify problems in specific experimental steps (sample-independent controls). If a given sample failed in 3 or more Illumina controls, it was excluded from further analyses. Altogether, 2 samples were excluded that did not meet these two criteria.

Ten biological duplicates, distributed either in the same or in different bisulfite plates and BeadChips, were used to estimate the discrimination threshold of the Infinium450K Array using the total deviation index (TDI) [49], which was 0.059 in duplicates from different bisulfite and hybridation arrays, 0.057 in duplicates from the same bisulfite but different hybridation arrays, and 0.066 in duplicates both from the same bisulfite and hybridation array. Additionally, the 450K BeadChip features 65 control probes which assay highly-polymorphic single nucleotide polymorphisms (SNPs). Consistent results were observed when we performed pairwise correlations of these genotypes in our duplicates (See Supplementary Figure 1). After these steps, one of the biological duplicate samples (randomly selected) as well as the 65 SNP probes were removed from the dataset.

Methylation patterns in chromosome X probes were used to cluster subjects according to sex by principal component analysis, and 8 mismatched samples were detected in relation to the information on sex contained in our database. These samples were also excluded from further analyses.

An additional QC step was performed in R environment using the WateRmelon package [50], and 1 sample presenting more than 1% of sites with a detection p-value greater than 0.05 was excluded, in addition to 1,859 probes, either because they occurred in more than 1% of samples with a p-value greater than 0.05 or because they presented a bead count below 3 in more than >5% of samples.

Finally, probes that ambiguously mapped to the human genome with at least 47 base pairs or more (N=29.233) were excluded, and probes containing a SNP with a minor allele frequency (MAF) in HapMap European population (CEU) > 4%, either at the extension site or in the 10 nucleotides immediately before (N=14.122), as suggested by Chen et al. [51]. Probes corresponding to CpG sites located in chromosomes X and Y were also excluded (n=8.537).

A total of 433.131 CpG sites on autosomes were tested with regard to TEXB-alpha exposure in the remaining 181 samples, representing 93 boys and 88 girls. Raw methylation beta values were then normalized to reduce technical variability and four different normalization methods were compared (dasen, BMIQ, quantile normalization and Swan) using three performance metrics as proposed by

Pidsley et al [52]. Dasen normalization was used for further analyses as it resulted the best ranked method in our data (Additional information in Supplemental Material and Methods).

Epigenome-Wide Association Study

A robust linear regression model was employed using MASS (R package), to test the association between doubling of TEXB-alpha concentration and methylation at each CpG site in boys and in girls separately. Analyses were adjusted for area of study and two technical factors: chip and bisulfite plate. Covariates included in the adjusted model were selected by testing the difference of the correlation of p values before and after correction using a Kolmogorov-Smirnov test. Since crude and adjusted robust regression models produced very similar results, only adjusted models are presented. The False Discovery Rate (FDR) correction for multiple testing was calculated with the Benjamini and Hochberg (B&H) method.

CpG sites annotation

In order to obtain information on the top differentially methylated CpG sites in our study the University of California, Santa Cruz (UCSC) Genome Browser interface was used, which in addition contains ENCODE (Encyclopedia of DNA Elements) detailed information on regulatory elements, including chromatin accessibility and epigenetic marks across the genome both in DNA and in histones [53, 54]. The human gene database GeneCards (http://www.genecards.org/)[55] was used to obtain information on the genomic location and gene (or nearest gene) function and reported disease associations, while toxicological interactions of these genes with chemical compounds were explored with the Comparative Toxicogenomics Database (www.ctdbase.org) [56].

Validation and replication by pyrosequencing

Two CpGs were selected for further DNA methylation validation in the same samples as in the discovery study, and replication was conducted in 126 independent placenta samples (boys only) from the INMA cohort. For that purpose, bisulfite pyrosequencing, a highly quantitative PCR-based analysis was used. A total of 500 ng of extracted DNA was bisulfite converted using the EZ DNA Methylation-Gold™ Kit (Zymo Research, CA, USA), and 1 µl of converted DNA was further PCR amplified and sequenced. Additional information on primer design and PCR assay conditions can be found in Supplementary Material and Methods, Table 1. Samples were run in duplicate on a PyroMark Q96 ID pyrosequencing system (Qiagen) and the pairwise correlation between technical duplicates was 0.94 for cg05342136 and 0.80 for cg15809858 (See Suplementary, Figure 2).

The association between doubling the concentration of TEXB-alpha and changes in DNA methylation was tested using adjusted linear mixed regression models including repeated measurements (pyrosequencing duplicates) as random intercept.

Results

Study population characteristics

Overall, our discovery study population did not differ from the rest of INMA cohort participants for the main pregnancy related and socio-demographic characteristics Women in the discovery study were on average 32 years old, with a pre-pregnancy BMI of 23.45 kg/m^2. Only 3 children were born preterm, which represented a slightly lower (non-significant) percentage than what we observed in the rest of the INMA cohort participants and, concordantly, we saw less small for gestational age (SGA) children in our discovery study group when compared to the rest of INMA cohort (p-value<0.02) (Supplementary Material and Methods, Table 2). Additionally, no significant differences were observed for any of the maternal and infant sociodemographic and pregnancy related characteristics when comparing the discovery and replication populations used in our study, as shown in Table 1.

TEXB-alpha exposure among the 181 participants in the discovery sample did not differ by newborn sex (Kruskal-Wallis Test p-value<0.624), and was also similar between the discovery and the replication samples (discovery: median=0.75, iqr=0.28 to 1.28 pM Eeq/g placenta and replication: median=0.76, iqr: 0.40 to1.41 pM Eeq/g placenta; Kruskal-Wallis Test p-value<0.438).

Table 1. Main characteristics and comparison between discovery and replication study mother-child pairs enrolled in the INMA Project from the four participating INMA cohorts*.

Variables	**Discovery study sample (n=181)** N (%), mean (SD) or Median (IQR)	**Replication study sample (n=126)** N (%), mean (SD) or Median (IQR)	***P-value***
Maternal characteristics			
Maternal age (years)	32 (3.97)	31.70 (4.04)	*0.437*
Pre-pregnancy BMI (kg/m²)	23.45 (4.10)	24.01(5.30)	*0.705*
Type of delivery			
Vaginal	121 (67.22)	80 (63.49)	*0.539*
Instrumental	31 (17.22)	28 (22.40)	
Cesarean	28 (15.56)	17 (13.60)	
Parity			
Primiparous	106 (58.56)	69 (54.76)	*0.497*
Multiparous (2+)	75 (41.44)	57 (45.24)	
Smoking during pregnancy (yes)	49 (27.37)	31 (24.60)	*0.777*
Maternal educational level			
Below Secondary School	34 (19)	40 (31.75)	*0.131*
Secondary School	83 (45.86)	33 (26.19)	
University Degree	64 (35.14)	53 (42.06)	
Infant characteristics			

Sex (male)	93 (51.38)	126 (100)[4]	-
Gestational age (weeks)	39.80 (1.35)	39.85 (1.33)	*0.674*
Preterm (<37 weeks)	3 (1.67)	4 (3.17)	0.574
Birth weight	3299.07 (419.04)	3305.75 (447)	*0.396*
Small for gestational age (SGA)[1]	10 (5.65)	11 (8.73)	*0.385*
Large for gestational age (LGA) [2]	14 (7.91)	12 (9.52)	*0.259*

* Asturias, Gipuzkoa, Sabadell and Valencia. [1]SGA: below the 10th percentile of birth weight, adjusted for sex and gestational age; [2]LGA: above the 90th percentile of birth weight, adjusted for sex and gestational age. [4]Only boys were included in the replication study.

Association of TEXB-alpha and genome-wide DNA methylation in placenta

No epigenome-wide significant associations were found either in boys or in girls between each doubling of TEXB-alpha (pM Eeq/g placenta) and methylation at CpG sites in placenta after correcting for multiple testing. Results for the 12 most significant CpG sites in boys (n=93) and in girls (n=88) are presented in Table 2. Quantile-quantile (Q-Q) plots showing the observed versus expected -log10 (P-values) under the null hypothesis of no association are shown in Supplementary Figure 3. Among the top CpG sites differentially methylated in boys we found some genes previously related to growth and steroid hormone signaling, while none of these genes were observed in girls (See Supplementary Materials and Methods, Table 3 for additional information).

Table 2. Top CpG sites differentially methylated in placenta in relation to prenatal TEXB-alpha exposure, ranked by nominal p-value.

CpG name	*% Mean methylation (SD)*	*Regression β** (% methylation)*	*P-value*	*FDR**	*Chr*	*UCSC GeneName*
Boys (N=93)						
cg05342136	89.07 (1.41)	0.29	4.72 E-07	0.20	4	*LRPAP1*
cg08983490	6.63 (0.71)	-0.12	2.25 E-06	0.49	16	*HAGH*
cg00698124	9.73 (1.07)	0.21	7.84 E-06	0.70	18	*SETBP1*
cg23261491	11.52 (1.10)	0.19	7.88 E-06	0.70	12	*OSBPL8*
cg15809858	10.55 (1.29)	0.23	8.87 E-06	0.70	5	*PPARGC1B*
cg14218861	29.81 (4.91)	-0.77	9.73 E-06	0.70	10	
cg16172549	61.33 (7.01)	-1.25	1.52 E-05	0.72	1	*PCP4L1*
cg10447095	55.83 (8.40)	-1.55	1.75 E-05	0.72	16	
cg19584136	87.49 (2.38)	-0.40	1.82 E-05	0.72	10	*MXI1*
cg00836964	91.08 (1.17)	0.22	2.22 E-05	0.72	5	
cg00957580	84.65 (2.30)	0.37	2.27 E-05	0.72	14	*NDRG2*
cg23903244	55.29 (2.82)	0.48	2.61 E-05	0.72	11	*KCNQ1*
Girls(N=88)						
cg21877656	4.88 (0.45)	0.08	1.66 E-06	0.54	19	*ZNF329*
cg19743820	14.26 (3.82)	-0.87	3.15 E-06	0.54	5	*COX7C*
cg21690627	6.19 (0.56)	-0.13	3.73 E-06	0.54	17	*C17orf59*
cg04919579	7.60 (0.89)	-0.19	9.85 E-06	0.88	3	*RNF168*
cg23313650	12.04 (1.62)	-0.28	1.24 E-05	0.88	7	*WASL*
cg01648887	94.32 (0.84)	-0.16	2.19 E-05	0.88	16	*SPG7*
cg11804334	5.31 (0.48)	-0.10	2.96 E-05	0.88	11	*CCDC34*
cg25248213	88.40 (1.55)	0.30	3.14 E-05	0.88	11	
cg01374565	8.97 (0.95)	-0.20	3.15 E-05	0.88	11	*GDPD5*
cg16705665	8.02 (0.70)	-0.13	3.35 E-05	0.88	11	*RCOR2*
cg27489994	8.48 (1.57)	0.31	3.36 E-05	0.88	13	*TPT1*
cg10424681	83.85 (2.98)	-0.62	3.98 E-05	0.88	6	*C6orf201*

*FDR: False Discovery Rate; all models were adjusted for area of study, bead chip and bisulfite plate. **Estimates per doubling TEXB-alpha concentration.

Validation and replication of selected top CpGs in boys

Two CpGs differentially methylated in relation to TEXB-alpha in boys were selected for further validation by pyrosequencing in the same samples (n=92) and replication was conducted in additional 126 placenta samples from male deliveries.

The first CpG, the top differentially methylated cg05342136 in boys, is located in the exon 1 of the low density lipoprotein receptor-related protein associated protein 1*(LRPAP1*), a lipid-metabolism gene highly expressed in placenta that in turn interacts with TGFb1, an angiogenic factor mediating successful placentation and fetal growth via regulation of trophoblast invasion, cell differentiation, immunosuppression and apoptosis of vascular endothelial cells, which has also been associated with susceptibility to degenerative dementia [57-60]. The second selected CpG site, cg15809858, is located in the first intron of Homo Sapiens peroxisome proliferator-activated receptor gamma, coactivator 1 beta (*PPARGC1B*), a gene expressed in human placenta that stimulates the activity of several transcription factors and nuclear receptors, including estrogen receptor alpha, nuclear respiratory factor 1, and glucocorticoid receptor, shown to be down regulated in pre-diabetic and type 2 diabetes mellitus patients and previously related to an increased risk of developing obesity [61-63].

Results from the discovery EWAS could not be neither validated nor replicated for the two CpG sites analyzed (Table 3), although a trend for technical validation (i.e. we were able to confirm the magnitude and the direction of the effect) was observed for cg05342136 (β=0.29 in the discovery *vs* β=0.25 in the validation study). Scatter plots showing the correlation between DNA methylation values measured using the Illumina 450K array platform and by bisulfite pyrosequencing in the same samples (discovery) are shown for cg05342136 and cg15809858 in Supplementary Material and Methods, Figure 4.

Table 3. Technical validation and biological replication in boys of placenta DNA methylation in selected CpG sites in relation to prenatal TEXB-alpha exposure.

			Technical validation (discovery samples)				**Biological replication (independent samples)**			
CpG name	***UCSC Gene name***	***% Mean methylation (SD)***	***n***	***β*****	***95 % CI***	***P-value***	***n***	***β*****	***95 % CI***	***P-value***
cg05342136	*LRPAP1*	89.77 (2.6)	92	0.25	-0.23 to 0.76	0.299	125	-0.07	-0.28 to 0.15	0.546
cg15809858	*PPARGC1B*	1.87 (1.52)	92	0.06	-0.08 to 0.19	0.405	126	0.01	-0.04 to 0.06	0.652

*Adjusted for area of study, gestational age, maternal age during pregnancy, smoking during pregnancy and bisulfite plate. Pyrosequencing duplicate was included as a random intercept. **Estimates per doubling TEXB-alpha concentration.

Discussion

This is the first genome-wide study analyzing site-specific DNA methylation changes in placenta tissue in relation to a biomarker of exposure to mixtures of environmental estrogens. At the interface between mother and child, the human placenta is an organ involved in the regulation of fetal growth and development and represents a gateway for substances, including xenoestrogens, to enter fetal circulation [64, 65].

In the INMA cohort we previously reported sex-specific associations between the TEXB-alpha biomarker measured in placenta and birth weight changes in boys only [48], along with lower *AluYb8* retrotransposon methylation in placental DNA in the same group, used as a surrogate to study global genomic DNA methylation [45]. In order to gain further mechanistic insight into these associations, we have performed an EWAS study in placenta, stratifying data by sex, to identify differentially methylated genes as a result of prenatal exposure to xenoestrogens. No genome-wide significant associations were found between each doubling of TEXB-alpha and DNA methylation after correcting for multiple testing in either group. However, among the top significant CpG sites differentially methylated in relation to each doubling of TEXB-alpha in boys, several were located in genes that have been related to birth weight regulation, type 2 diabetes and obesity risk or steroid hormone metabolism, and are known to be expressed in human placenta tissue. The top significant one, cg05342136, is located in the first exon of *LRPAP1*), a gene involved in cholesterol metabolism, the primary metabolite of steroid hormone synthesis [66]. We also found an increase in DNA methylation in cg15809858, located in exon 1 of *PPARGC1B*. The protein encoded by this ubiquitously expressed gene stimulates the activity of several transcription factors and nuclear receptors, including estrogen receptor alpha (*ERa*), and may be involved in fat oxidation, non-oxidative glucose metabolism, and the regulation of energy expenditure. This protein is down-expressed in pre-diabetic and type 2 diabetes mellitus patients and certain allelic variations in this gene increase the risk of the development of obesity, type 2 diabetes and breast cancer [61, 67]. Moreover, it has been shown to be downregulated by the hormonally active compound benzo(a)pyrene in mice [68]. Other suggestive genes appeared among the top hits in boys, such as *HAGH*, encoding for an hydroxylase enzyme involved in the pyruvate metabolism, or *KCNQ1,* a paternally imprinted gene that although relatively low expressed in placenta, contains genetic polymorphisms related to birth weight and type 2 diabetes [69] and is located within a cluster of imprinted genes in the chromosomal region 11p15.5, that includes *KCNQ1DN,H19*, *IGF2* and *KCNQ1OT1* among others, previously associated with fetal and placental growth [28]. Mutations and epimutations in these genes have been associated to the Beckwith-Wiedemann Syndrome (BWS), an overgrowth imprinting disorder that causes large body size and large organs in addition to other clinical manifestations present from birth [28, 70, 71].

Overall, although statistically non-significant, these findings seem to go in line with our previous results, showing that higher levels of xenoestrogens (TEXB-alpha) measured in placenta were associated with higher birth weight in boys (on average 148 grams when comparing high versus low exposed children), while no effects were found in girls [48]. However, we could not validate neither replicate our initial findings for two selected CpGs, and only for cg05342136, technical verification showed a coefficient (β value) of the same magnitude and direction as in the discovery study. In our data, we observe a poor reproducibility between DNA methylation values measured using the 450K Illumina array platform and by bisulfite pyrosequencing in the same samples, especially when CpGs are hypomethylated (as for cg15809858). Whether this lack of technical replication and biological validation of the results in our study reflects differences in the reproducibility, specificity and/or measurement sensitivity across different platforms used to measure DNA methylation (i.e. hibridation array *vs* bisulfite pyrosequencing), as previously demonstrated for miRNA quantitative expression data [72], a lack of statistical power to reach statistical significance (especially when the magnitude of changes might be small), or truly negative findings remains to be addressed with additional larger studies.

Lack of statistical power is a problem when analyzing *-omics* data, and we are likely underpowered in our study, where stratified analyses were conducted. Moreover, by using an array based approach such as the Illumina Infinium Human Methylation450 BeadChip, covering with probes roughly a 2% of the ~28 million CpG sites described in the human genome [73], we might have missed potentially important genomic regions in our study.

Our study has two main methodological strengths: first, exposure to mixtures of xenoestrogens was measured in placenta tissue using a biomarker, and second DNA methylation changes were analyzed in the same tissue, which is relevant considering the tissue specificity of epigenetic marks, the role of this organ during prenatal development and its sensitivity to the effects of hormones [74].

The magnitude of the differences in DNA methylation that we observed was small, although similar to what has been previously shown in other EWAS in relation to prenatal exposure to other environmental chemicals, including potential xenoestrogens like cadmium [75]. To some extent, our results may have been confounded by cell type mixtures in placenta samples, or by the possible maternal cell contamination, which in both cases could have led to a possible underestimation of the effects, while we do not know whether the observed changes in DNA methylation have functional effects on gene expression, since RNA was degraded due to placenta collection conditions in our cohort. Only one biopsy for DNA extraction was conducted per sample, which could have introduced additional noise due to regional variations in DNA methylation, although some authors have suggested that this is not a major source of DNA methylation variation in human placenta [76]. Finally, some uncertainty exists on whether the TEXB-alpha biomarker, based

on a lipophilic extraction of compounds (excluding endogenous steroid hormones and more polar xenoestrogens) followed by a quantification of MCF7 cell proliferation assay, is exclusively a biomarker of xenoestrogenicity, or also a biomarker of other lipophilic compounds present in the placenta that activate growth, and that might not necessarily (or uniquely) act through binding or interacting with the estrogen receptor.

Conclusion

We conducted a genome-wide methylation study in placental tissue in relation to prenatal exposure to mixtures of xenoestrogens using the TEXB-alpha biomarker, and although we identified some suggesting genes differentially methylated in boys, we were not able to validate neither replicate our initial findings by pyrosequencing. Future studies are warranted to confirm the observed associations and their potential to mediate the effect of prenatal exposure to mixtures of endocrine disruptors on the offspring's health.

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