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# Maternal urinary concentrations of bisphenol A during pregnancy are associated with global DNA methylation in cord blood of newborns in the "NELA" birth cohort

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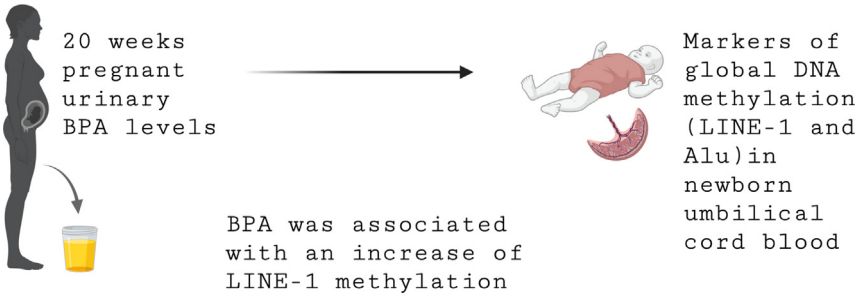
## HIGHLIGHTS

- Early exposure to bisphenols may have potential toxicoepigenetic effects.
- Global DNA methylation of LINE-1 and Alu in cord blood samples were analyzed.
- Maternal urinary Bisphenol A was associated with an increase of LINE-1 methylation.
- No associations between bisphenols and percentage of Alu methylation were found.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Endocrine disrupting chemicals (EDCs) set a public health risk through disruption of normal physiological processes. The toxicoepigenetic mechanisms of developmental exposure to common EDCs, such as bisphenol A (BPA), are poorly known. The present study aimed to evaluate associations between perinatal maternal urinary concentrations of BPA, bisphenol S (BPS) and bisphenol F (BPF) and *LINE-1* (long interspersed nuclear elements) and *Alu* (short interspersed nuclear elements, SINEs) DNA methylation levels in newborns, as surrogate markers of global DNA methylation. Data come from 318 mother-child pairs of the `Nutrition in Early Life and Asthma´ (NELA) birth cohort. Urinary bisphenol concentration was measured by dispersive liquid–liquid microextraction and ultrahigh performance liquid chromatography with tandem mass spectrometry detection. DNA methylation was quantitatively assessed by bisulphite pyrosequencing on 3 LINEs and 5 SINEs. Unadjusted linear regression analyses showed that higher concentration of maternal urinary BPA in 24th week's pregnancy was associated with an increase in *LINE-1* methylation in all newborns ($p$ = 0.01) and, particularly, in male newborns ($p$ = 0.03). These associations remained in full adjusted models [beta = 0.09 (95 % CI = 0.03; 0.14) for all newborns; and beta = 0.10 (95 % CI = 0.03; 0.17) for males], including a non-

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linear association for female newborns as well (p-trend = 0.003). No associations were found between maternal concentrations of bisphenol and *Alu* sequences. Our results suggest that exposure to environmental levels of BPA may be associated with a modest increase in *LINE-1* methylation -as a relevant marker of epigenomic stability- during human fetal development. However, any effects on global DNA methylation are likely to be small, and of uncertain biological significance.

## 1. Introduction

Bisphenol A (BPA) is commonly used in commerce and industry for manufacturing polycarbonate plastics (e.g., water storage tanks, water bottles,) and food packaging materials (containers or food cans), among other consumer goods (CDC (Centers for Disease Control and Prevention), 2021). BPA, a known endocrine-disrupting chemical (EDC), can leach from some of these polymers into food products or water and, consequently, the main exposure routes occur through dermal absorption and dietary ingestion (Calafat et al., 2015; Wong and Durrani, 2017). Worldwide biomonitoring studies have reported that BPA is usually found among the general population, being detectable in >90 % of studied subjects (CDC (Centers for Disease Control and Prevention), 2021; Koc t al., 2012; Konieczna et al., 2015).

BPA is rapidly and almost totally excreted in urine in humans (CDC (Centers for Disease Control and Prevention), 2021), but there is a repeated and chronic exposure to this compound (Koch et al., 2012). Urine is an optimal matrix for quantifying bisphenol levels due to their short half-lives (<24 h) and non-persistent nature (Calafat et al., 2015; Koch et al., 2012). As a result of its estrogenic and anti-androgenic activity, BPA may contribute to hormone and endocrine dysfunction in adults (Gore et al., 2015). In this context, bisphenol S (BPS) and bisphenol F (BPF) are proposed to be used as BPA safety alternatives since these new chemicals have undergone some structural changes. Nevertheless, recent investigations have shown adverse hormonal and reproductive effects with the exposure to these BPA substituents as well (Ahsan et al., 2018 in rats; da Silva et al., 2019 in rats; Eladak et al., 2015 in human fetal testis explants; Reina-Pérez et al., 2021 in adipose-derived stem cells; Wan et al., 2018 human gestational age).

Exposure to bisphenol at any life stage may be relevant; however, the fetal development is a period of high vulnerability to EDCs exposure, as this is a critical interval of rapid growth and development (Basak et al., 2020; Caporossi and Papaleo, 2017; Grignard et al., 2012; Konieczna et al., 2015). Thus, any alteration during gestation may have long-lasting consequences on future disease susceptibility (Wadhwa et al., 2009). Indeed, several birth cohort studies have reported that prenatal exposures to EDCs are associated with adverse growth-related outcomes (Buckley et al., 2016; Valvi et al., 2015).

The early stage of prenatal development is also a period of high susceptibility for epigenomic programming of the fetus. Epigenetic alterations, including changes in DNA methylation, can lead to modifications in gene expression without changes in the DNA sequence. Therefore, epigenetic alterations can be considered as markers of genome instability (Latchney et al., 2018). Epigenetic modifications during prenatal life may influence fetal outcomes and are the epicentre of disorders occurring in the medium- and long-term, being an independent risk factor for the occurrence of some chronic diseases in adult life (Basak et al., 2020; Merid et al., 2020). Then, epigenetic mechanisms play a key role in embryonic and fetal development and, more importantly, can be potentially affected by some environmental exposures (Basak et al., 2020).

Global DNA hypomethylation is a common phenotype of aging and cancer and, conversely, high levels of global methylation have been associated with Down's syndrome, gestational diabetes, or preeclampsia (Martin and Fry, 2018). *LINE-1* (long interspersed nuclear elements) and *Alu* (short interspersed nuclear elements, SINEs) are the most common and well-characterized DNA transposable sequences regarding characterization of human DNA methylation. These transposable sequences are robust and repetitive portions of the genome showing their utility as global DNA methylation proxies (Perera et al., 2020). In fact, measurements of *Alu* and *LINE-1* methylation have been previously used to estimate global genomic DNA methylation content (Baccarelli and Bollati, 2009; Perera et al., 2020; Yang et al., 2004). However, only a few studies have assessed how the exposure to EDCs influences embryonic or fetal programming through epigenetic modifications in humans (Basak et al., 2020; Lu et al., 2020; Ouidir et al., 2020; Zhao et al., 2019). Furthermore, changes in methylation of *Alu* or *LINE-1* have been previously related to EDCs exposure (Huang et al., 2018; Huen et al., 2016; Montrose et al., 2018; Yang et al., 2017, 2020). For example, prenatal phthalate exposure was inversely associated with *Alu* methylation in female newborns from a birth cohort study in Wuhan (China) (Huang et al., 2018). Likewise, comparable associations were reported for *Alu* or *LINE-1* in newborns from a Mexican-American cohort (Huen et al., 2016) and in a birth cohort form Michigan (USA) (Montrose et al., 2018).

Our current study assessed the relationship between in utero exposure to bisphenol and global DNA methylation, as a biomarker of genome instability, in a European birth cohort. Therefore, our aim was to evaluate associations between perinatal maternal urinary concentrations of BPA, BPS and BPF and global DNA methylation of *LINE-1* and *Alu* in cord blood samples of Spanish newborns from the `Nutrition in Early Life and Asthma´ (NELA) birth cohort.

## 2. Material and methods

### 2.1. Study population

Data come from participants embedded in the Nutrition in Early Life and Asthma (NELA) study (https://nela.imib.es), a prospective population-based birth cohort set up in Murcia, a south-eastern Mediterranean region of Spain. The main goal of NELA was to unravel the developmental origins and mechanisms of asthma and allergy. Rationale, study design and comprehensive methodology have been previously described (Morales et al., 2022).

Pregnant women who fulfill the inclusion criteria were invited to participate in the study at the time of the 20-weeks of gestation control visit at the Maternal-Fetal Unit of the "Virgen de la Arrixaca" University Hospital, over a 36-month period, from March 2015 to April 2018. The inclusion criteria were women from Health Area I and certain districts of Health Areas VI and VII of the Region of Murcia; planning to live in the area of study during at least 2 years; intention to give birth at the reference hospital; Spanish Caucasian origin; 18–45 years of age; singleton pregnancy; non-assisted conception; and normal echography at 20 weeks of gestation (no major malformations). The exclusion criteria included: an existing chronic disease; pregnancy complications (except gestational diabetes and hypertensive disorders); and not intending to deliver in the reference hospital.

Fig. 1 shows the number of participants and the inclusion criteria. Among the 1350 women invited to participate, 738 (54 %) were finally enrolled in the study. Of these, 365 (49 %) were excluded because cord blood DNA samples were not collected and urinary bisphenol concentrations were not determined in 55 mothers. The remaining 318 mother-child pairs with completed information were enrolled in the current study. Compared with excluded subjects, parents of those who were included in the present analysis tended to be weightier; mothers presented slightly lower consumption of alcohol and smoking; and there were higher percentage of male neonates with relatively higher gestational age and birth weight. Other main baseline characteristics did not differ significantly.

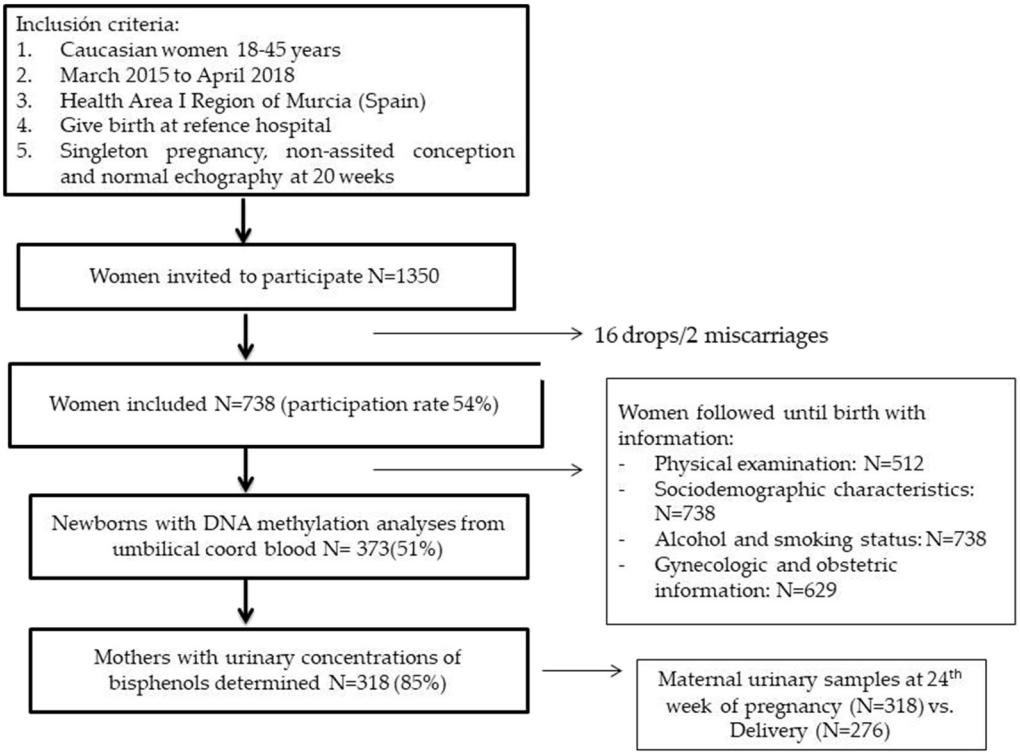


**Fig. 1.** Flow diagram for the current study population.

This study was approved by the Ethics and Clinical Research Committee (CEIC) of the Virgen de la Arrixaca Clinical University Hospital in accordance with the guidelines of The Declaration of Helsinki. Written informed consents were obtained from all participants.

### 2.2. Urinary and blood samples

Maternal urinary samples were collected in a subsequent visit (24th week) to that of the recruitment one and at delivery, in addition to umbilical cord blood samples. The same standardized protocol was used for collection and storage of samples. Urinary samples were frozen at −80 °C in aliquots of 1 mL. Umbilical cord blood was collected in EDTA tubes at time at birth, and blood buffy coat was isolated after centrifugation and frozen at −80 °C. All samples were stored in the Biobank of Biomedical Research Institute of Murcia (Spain) (BIOBANC-MUR, IMIB-Arrixaca).

### 2.3. Maternal urinary concentration of bisphenol

Samples were sent to the Environmental Medicine Research Laboratory of University of Granada (Spain) on dry ice and stored at −20 °C until analyses were performed. Three bisphenols compounds, bisphenol A (BPA), bisphenol S (BPS) and bisphenol F (BPF), were quantified in urine samples from each participant, using a methodology previously validated and described (Adoamnei et al., 2018; Jiménez-Díaz et al., 2016; Vela-Soria et al., 2014). Briefly, 0.5 mL urine was analyzed by dispersive liquid-liquid micro-extraction (DLLME) and ultra-high performance liquid chromatography with tandem mass spectrometry detection (UHPLC-MS/MS). Samples were thawed at room temperature and extracted for analysis. In order to determine total urinary bisphenol concentrations (free plus conjugated), each sample was incubated with 50 μL of an enzyme solution of β-glucuronidase/sulfatase [prepared by dissolving 10 mg of β-glucuronidase/sulfatase (3·106 U g solid-1) in 15 mL of 1 M ammonium acetate/acetic acid buffer solution (pH 5.0)] at 37 °C for 24 h. The enzymatically treated samples were transferred to 15 mL screw-cap glass tube and spiked with 10 μL of the surrogate standard solution (20 μg/L), plus 10 mL of 10 % NaCl aqueous solution (*w/v*) (pH 2.0, adjusted with 0.5 M HCl). Then, 1.5 mL of acetone/trichlorometane (1:2, *v*/v) mixture was also injected into the aqueous phase with a syringe, and samples were finally shaken manually for 30 s and centrifuged at 4000 × *g* for 10 min. Next, the organic phase was carefully collected, placed in 2 mL glass vials and evaporated under a nitrogen stream. The direct residue was then resuspended with 100 μL of an acetonitrile/water mixture [0.1 % ammonia, 70:30 (*v*/v)], and finally 10 μL was injected into the UHPLC-MS/MS system; the ACQUITY UPLC™ h-Class equipment. A Xevo TQ-S tandem quadrupole mass spectrometer equipped with and orthogonal Zspray™ electrospray ionization source was employed for EDC detection. The compounds were separated using a gradient mobile phase consisting of 0.1 % (v/v) ammonia aqueous solution (solvent A) and 0.1 % (v/v) ammonia in methanol (solvent B). The column temperature was maintained at 40 °C. Total run time was 10 min.

The limit of detection (LOD) was determined as the minimum detectable amount of analyte with a signal-to-noise ratio ≥ 3. For all of the three selected chemical compounds a limit of detection of 0.1 ng/mL and a limit of quantification (LOQ) of 0.3 ng/mL was established. Urinary samples with non-detectable bisphenol concentrations were assigned the value of LOD divided by the square root of 2, as recommended when the data are not highly skewed (e.g., standard deviation <3) (Hornung and Reed, 1990).

Quality control, sample collection, storage, and processing of biospecimens were performed under controlled conditions and taking into account any potential external contamination (sample containers, equipment and/or labware, etc.). Blanks of the collection vessels, matrix blanks and blanks of the chromatography equipment were included, and the final concentration of study samples adjusted by subtracting the contribution from the blanks, if any. In addition, urinary samples were analyzed in several batches, and all of them included standards for calibration curves (injected at beginning and end of each batch), 15–20 studied samples, 2 blanks, 2 urine pool controls, and 2 urine pool controls with mixture of tested chemicals at low and high concentration levels. The intra-class correlation (ICC) coefficients of bisphenol concentrations between the two maternal

urinary samples (24th week of pregnancy and delivery) were calculated (data not shown). BPA levels were correlated in both timepoints, but BPS and BPF were not.

Urinary creatinine concentration (mg/dL) was determined using an automated colorimetric determination based on the Jaffe assay, in the same urine samples in which bisphenol was assessed. Because of the relatively constant excretion rate of creatinine into the urine (which makes urinary creatinine concentration inversely proportional to urine flow rate), creatinine adjustment is widely used to normalize analyte concentrations in spot samples for environmental exposure monitoring in humans (Barr et al., 2005). Bisphenol concentrations (μg/L) were divided by creatinine levels (g/L) to yield creatinine adjusted bisphenol concentrations expressed in μg/g-creatinine.

### 2.4. DNA methylation of LINE-1 and Alu in cord blood of newborns

Umbilical venous cord blood samples were collected at birth and stored at −80 °C until processing. DNA was extracted from cord blood buffy coat using the Maxwell DNA Blood SEV kit with the automated extraction equipment Maxwell 3000, according to the manufacturer's protocol. DNA methylation was quantified by bisulfite-PCR and pyrosequencing, according to the methods developed by Royo et al. (2007) and Yang et al. (2004), in the Genomics Core Facility of the Biomedical Research Institute of Murcia (IMIB). DNA (500 ng) was converted with bisulfite using the EZ DNA Methylation kit (Zymo Research), following the manufacturer's protocol. PCR amplification of DNA methylation of *LINE-1* and *Alu* elements was performed according to designs previously described (Bollati et al., 2007) in a PyroMark Q24 System (Qiagen). Specific details of the pyrosequencing methods such as the PCR primers and conditions, sequencing primers and nucleotide dispensation are shown in Supplementary Table 1. PCR amplification of *LINE-1* and *Alu* elements was performed using 25 ng of bisulfite-converted DNA, 2 μM of each primer and the PyroMark PCR Master mix of the PyroMark PCR kit (Qiagen), in a final volume of 25 μL. In both methods, the size of the resulting PCR products was approximately 150 bp, as assessed by electrophoresis of 2 μL of each PCR reaction on 2 % agarose gels. A no-template control was included in each PCR plate and checked for negativity on agarose gels. Each PCR reaction was split into two pyrosequencing reactions. Thus, the PCR products (2 × 10 μL) were bound to Streptavidin Sepharose High Performance (GE Healthcare), then denatured and purified using PyroMark buffers (Binding, Annealing, Denaturation, and Wash) in a PyroMark Q24 MDx Vacuum Workstation. Finally, sequencing was performed using PyroMark Gold Q24 Reagents in a PyroMark Q24 MDx instrument. The pyrosequencing runs were analyzed using the PyroMark Q24 software. The second nucleotide dispensed in the *LINE-1* runs (dCTP, Supplementary Table 1) represents an internal control for bisulfite treatment. Human Methylated & Non-Methylated DNA Set (Zymo Research) were also included in each PCR and pyrosequencing plate as controls, positive and negative, respectively, for the pyrosequencing assays. In addition, all plates contained randomly coded samples from different age groups to reduce the risk of plate bias. Methylation of each CpG site was calculated twice. Coefficient of variation (CV) for duplicate measurements was calculated and if the average %CV was >5 % the plate was repeated. Global DNA methylation variables were the result to average duplicate measurements on different CpG sites. Firstly, we obtained a mean value of each CpG site (mean of duplicated measurements) and, then, the mean values of different CpGs for each element, that is, mean of 3 CpGs for *LINE-1* elements and 5 CpGs for *Alu* elements. Intra-class correlation coefficients between duplicated values of *LINE-1* and *Alu* methylation were 0.81 (95%CI = 0.76; 0.85 and 0.90 (95%CI = 0.87; 0.92), respectively.

### 2.5. Covariates

Maternal sociodemographic information was collected through questionnaires at 20th and 32nd week visits, such as age, educational level (basic education, incomplete secondary, complete secondary and university) and current occupation (defined as maternal occupation during pregnancy based on the highest social class by using the Spanish adaptation of the international ISCOSS coding system: I/II, managers/technicians/graduated; III, skilled; IV/V, semi-skilled/unskilled; and unemployed) (Domingo-Salvany et al., 2000). Further information about gynecologic and obstetric history of the current and previous pregnancies was obtained from clinical records: previous gestations, miscarriages, deliveries and preterm deliveries (<37 weeks); gestational age of newborns at birth; and gestational thyroid dysfunction, gestational diabetes mellitus, hypertension or pre-eclampsia in the current pregnancy (yes/no). The height (cm) and weight (kg) of pregnant women was measured at both the 20th and 32nd visits. Additionally, women self-reported their weight before pregnancy; the paternal height and weight; and alcohol consumption and smoking habits during pregnancy (yes/no). Maternal and paternal BMI were calculated [weight (kg)/height$^2$ (m)]. Weight (gr) and height (cm) of newborns were collected during the physical examination at birth.

### 2.6. Statistical analysis

Descriptive statistics are shown using crude data. Continuous variables were summarized by arithmetic mean, standard deviation (SD), median and 5th–95th percentiles, and categorical variables as number and percentage (%). A descriptive analysis of the main study variables (bisphenol and DNA methylation of *LINE-1* and *Alu*) was carried out for all newborns and also stratified by sex. Raw bisphenol (ng/mL) and corrected by creatinine (μg/g-creatinine) concentrations were reported at 24th week of pregnancy and at the time of delivery. BPS concentration at 24th week of pregnancy and at time of delivery and BPF at time of delivery were not used in bivariate and multivariate analyses due to their high percentage of data below the LOD. *LINE-1* and *Alu* methylation was calculated as previously detailed. Differences in these variables between males and females were assessed using Mann Whitney *U* test. Assessment for normality was performed using a Kolmogorov-Smirnov test and Pearson or Spearman coefficients were employed to evaluate potential correlations between variables.

Multiple linear regression models were used to evaluate the associations between urinary bisphenol concentrations (BPA and BPF) of mothers and global DNA methylation in transposable sequences of *LINE-1* and *Alu* in cord blood of newborns. Due to the low percentage of detection for BPF, creatinine corrected values were dichotomized at below/above LOD values. Results are reported by sex of the newborns (males/females) and for all newborns together; parameter estimates were reported with beta and 95 % confidence intervals (CI). Several potential covariates and confounders were evaluated and, when the inclusion of a potential covariate resulted in a change in the beta coefficient of <10 %, the variable was not retained in final models. These variables included factors previously related to DNA methylation of *LINE-1* and *Alu* or maternal exposure to bisphenol during pregnancy, regardless of whether they had been previously described as predictors of reproductive health in early stages. Paternal BMI, weight, height and gestational age of newborns, maternal weight at 32nd week of pregnancy, previous miscarriages and creatinine levels at 24th week of gestation or at time of delivery were retained in the final models.

Further analyses were performed to estimate mean values of maternal urinary bisphenol concentrations (mean bisphenol concentrations in the 24th week of pregnancy plus delivery samples) according to sex of newborns and all together. Associations between percentage of *Alu* or *LINE-1* methylation and mean values of BPA concentrations were assessed through linear regression adjusting by the same covariates mentioned previously. Additionally, values of BPA and BPF concentrations from both samples were divided into quartiles to explore potential non-monotonic dose response relationships with percentage of *Alu* or *LINE-1* methylation in females, males and all newborns at any time point. ANOVA and ANCOVA tests were employed to assess these relationships. For the statistical analyses, the software package IBM SPSS 20.0 was employed (IBM Corporation, ARMONK, NY, USA), assuming a 0.05 level for all tests.

## 3. Results

Background and baseline characteristics of pregnant women and their newborns are shown in Table 1. Mean (SD) age of participating pregnant women ($n$ = 318) was 32.9 (4.3) years and mean (SD) gestational age of newborns 39.8 (1.4) weeks. 52.2 % of newborns ($n$ = 166) were males and 47.8 % ($n$ = 152) females (Table 1).

Differences in maternal concentrations of bisphenol (BPA, BPS and BPF) at 24th week of pregnancy and delivery (corrected by creatinine) and percentage DNA methylation (*LINE-1* and *Alu* sequences) in cord blood of newborns by sex are summarized in Table 2. The mean (SD) maternal urinary creatinine concentrations were 102.5 (49.0) and 120.1 (60.4) mg/dL for 24th week and delivery, respectively. Percentage of data measurement above the LOD for maternal BPS concentrations (24th week of gestation and delivery samples) and BPF at delivery were fairly small (5.0 %, 2.9 % and 6.9 %, respectively) and thus were not further included in the statistical analysis. The proportion of DNA methylation of *LINE-1* was significantly higher in males (76.99 ± 1.52 %) than in female (76.24 ± 1.60 %; $p < 0.001$) newborns in the bivariate analyses (Table 2). Raw values of bisphenol concentrations without creatinine adjustment (ng/mL) are shown in Supplementary Table 2. Furthermore, mean values of maternal urinary bisphenol concentrations of 24th week of pregnancy plus at time of delivery samples can be seen in Supplementary Table 3.

In unadjusted linear regression analyses (Table 3), maternal BPA concentrations at 24$^{th}$ week of pregnancy were positively associated with percentage of *LINE-1* methylation in the whole population of newborns (beta = 0.05; 95 % CI = 0.01; 0.09). Although differences in both sexes were not statistically significant (p for interaction sex*BPA = 0.60), the

**Table 1**
Comparison of the main characteristics between included and excluded participants.

| Maternal characteristics | Women included in the study ($N$ = 318) | | Women excluded from the study ($N$ = 420) | | p-Value[a] |
|---|---|---|---|---|---|
| | Mean (SD) | Median ($P_5$-$P_{95}$) | Mean (SD) | Median ($P_5$-$P_{95}$) | |
| Age (years) | 32.9 (4.3) | 33.0 (25.0–40.0) | 32.3 (4.9) | 33.0 (24.0–40.0) | 0.06 |
| Maternal height (cm) | 163.7 (5.9) | 164.0 (153.9–173.0) | 164.1 (5.7) | 164.0 (154.0–173.0) | 0.33 |
| Maternal weight before pregnancy (kg) | 64.9 (12.8) | 62.0 (49.8–94.6) | 63.8 (13.1) | 61.5 (47.9–90.1) | 0.13 |
| Maternal weight (20th week) (kg) | 71.3 (12.5) | 69.4 (55.8–100.4) | 70.6 (12.7) | 68.3 (53.9–95.7) | 0.35 |
| Maternal BMI (20th week) (kg/m$^2$) | 26.6 (4.3) | 25.8 (21.5–35.7) | 26.2 (4.3) | 25.6 (20.4–34.5) | 0.23 |
| Maternal weight (32nd week) (kg) | 76.2 (12.9) | 74.6 (59.7–106.7) | 75.3 (12.6) | 73.8 (58.9–98.3) | 0.52 |
| Maternal weight gain during pregnancy (kg) | 12.2 (5.0) | 12.0 (5.0–20.0) | 12.2 (5.0) | 12.0 (5.0–20.0) | 0.56 |
| Previous gestations (n) | 1.0 (1.2) | 1.0 (0–3.1) | 1.0 (1.1) | 1.0 (0–3.0) | 0.97 |
| Previous miscarriages (n) | 0.36 (0.82) | 0 (0–2.0) | 0.37 (0.67) | 0 (0–2.0) | 0.12 |
| Previous deliveries (n) | 0.64 (0.77) | 1.0 (0–2.0) | 0.62 (0.76) | 0 (0–2.0) | 0.68 |
| Previous preterm deliveries (<37 weeks) (n) | 0.05 (0.22) | 0 (0–1.0) | 0.04 (0.20) | 0 (0–0) | 0.52 |
| Paternal height (cm) | 177.8 (6.9) | 178.0 (167.0–190.0) | 177.7 (7.2) | 178.0 (167.0–190.0) | 0.84 |
| Paternal weight (kg) | 84.7 (13.5) | 83.0 (65.0–110.0) | 82.4 (12.15) | 80.0 (65.0–104.2) | 0.04 |
| Paternal BMI (kg/m$^2$) | 26.8 (4.2) | 26.1 (21.6–35.2) | 26.1 (3.3) | 25.7 (21.4–32.5) | 0.05 |
| | N (%) | | | | |
| Educational level: | | | | | 0.48 |
| Basic education | 24 (7.5 %) | | 33 (7.9 %) | | |
| Incomplete secondary education | 35 (11.0 %) | | 54 (12.9 %) | | |
| Secondary education | 76 (23.9 %) | | 115 (27.4 %) | | |
| University education | 183 (57.5 %) | | 218 (51.9 %) | | |
| Social class: | | | | | 0.30 |
| Managers | 51 (16.0 %) | | 63 (15.0 %) | | |
| Graduated | 73 (23.0 %) | | 77 (18.3 %) | | |
| Technicians | 78 (24.5 %) | | 89 (21.2 %) | | |
| Skilled manual worker | 12 (3.8 %) | | 20 (4.8 %) | | |
| Semi-skilled manual worker | 34 (10.7 %) | | 57 (13.56 %) | | |
| Unskilled manual worker | 9 (2.8 %) | | 12 (2.9 %) | | |
| Unemployed | 61 (19.2 %) | | 102 (24.3 %) | | |
| Maternal alcohol intake: | | | | | 0.04 |
| Yes | 19 (6.3 %) | | 24 (6.4 %) | | |
| No | 281 (93.7 %) | | 350 (93.6 %) | | |
| Smoking status during the pregnancy: | | | | | 0.04 |
| Yes | 50 (16.7 %) | | 64 (17.1 %) | | |
| No | 250 (83.3 %) | | 310 (82.9 %) | | |
| Gestational thyroid dysfunction: | | | | | 0.15 |
| Yes | 98 (32.7 %) | | 103 (27.5 %) | | |
| No | 202 (67.3 %) | | 271 (72.5 %) | | |
| Gestational Diabetes Mellitus: | | | | | 0.68 |
| Yes | 24 (7.5 %) | | 34 (8.7 %) | | |
| No | 294 (92.5 %) | | 358 (91.3 %) | | |
| Hypertension: | | | | | 0.64 |
| Yes | 7 (2.2 %) | | 11 (2.8 %) | | |
| No | 310 (97.8 %) | | 378 (97.2 %) | | |
| Pre-eclampsia: | | | | | 0.72 |
| Yes | 4 (1.3 %) | | 3 (0.9 %) | | |
| No | 307 (98.7 %) | | 315 (99.1 %) | | |
| Newborn characteristics | | | | | |
| Sex: | | | | | <0.001 |
| Males | 166 (52.2 %) | | 191 (47.5 %) | | |
| Females | 152 (47.8 %) | | 211 (52.5 %) | | |
| Gestational age at birth (weeks) | 39.8 (1.4) | 40.0 (41.3–41.4) | 39.4 (1.6) | 39.7 (36.4–41.4) | 0.01 |
| Height at birth (cm) | 50.8 (1.9) | 51.0 (47.0–54.0) | 50.4 (2.44) | 50.0 (46.0–54.0) | 0.14 |
| Weight at birth (gr) | 3294.3 (426.9) | 3295.0 (2580.0–3980.3) | 3200.2 (506.7) | 3205.0 (2372.5–4016.2) | 0.01 |

SD: standard deviation; $P_5$-$P_{95}$: percentiles 5th–95th; N: number, %: percentage.
[a] Differences between included (N = 318) and not included (N = 420) women. Mann Whitney U test or Chi-squared test.

**Table 2**
Maternal urinary concentrations of bisphenol (μg/g-creatinine) and percentage of DNA methylation in *LINE-1* and *Alu* sequences in umbilical cord blood of newborns.

| Variables | % > LOD | ALL PARTICIPANTS (*N* = 318) | | | | MALE NEWBORNS (N = 166) | | | | FEMALE NEWBORNS (N = 152) | | | | p-Value[a] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | n | Mean (SD) | Median ($P_5$-$P_{95}$) | Geometric mean | n | Mean (SD) | Median ($P_5$-$P_{95}$) | Geometric mean | n | Mean (SD) | Median ($P_5$-$P_{95}$) | Geometric mean | |
| Maternal urinary bisphenol concentrations using dilution-corrected by creatinine (μg/g-creatinine) at 24th week of pregnancy (*n* = 318) | | | | | | | | | | | | | | |
| BPA | 88.1 | 318 | 5.53 (7.0) | 3.41 (0.07–21.35) | 2.38 | 166 | 5.39 (6.96) | 3.20 (0.08–21.37) | 2.38 | 152 | 5.68 (7.08) | 3.55 (0.06–21.41) | 2.38 | 0.61 |
| BPS | 5.0 | 22 | 2.12 (1.98) | 1.48 (0.05–3.25) | 1.00 | 7 | 2.96 (1.55) | 2.68 (1.35–2.76) | 2.64 | 15 | 1.73 (2.08) | 0.82 (0.04–2.88) | 0.64 | 0.07 |
| BPF | 30.8 | 131 | 1.43 (2.81) | 0.73 (0.05–4.51) | 0.61 | 70 | 1.19 (1.39) | 0.66 (0.05–3.82) | 0.55 | 61 | 1.71 (3.84) | 3.84 (0.07–5.91) | 0.68 | 0.56 |
| Maternal urinary bisphenol concentrations using dilution-corrected by creatinine (μg/g-creatinine) at delivery (*n* = 276) | | | | | | | | | | | | | | |
| BPA | 97.8 | 276 | 6.56 (10.01) | 4.16 (1.0–19.57) | 4.12 | 148 | 7.21 (11.90) | 4.36 (1.07–22.98) | 4.26 | 128 | 5.81 (7.2) | 4.04 (0.82–15.81) | 3.96 | 0.58 |
| BPS | 2.9 | 34 | 0.75 (1.98) | 0.07 (0.03–5.97) | 0.14 | 15 | 1.33 (2.89) | 0.07 (0.03–6.92) | 0.18 | 19 | 0.28 (0.45) | 0.07 (0.04–1.16) | 0.12 | 0.51 |
| BPF | 6.9 | 88 | 0.52 (1.75) | 0.08 (0.03–2.32) | 0.12 | 52 | 0.45 (1.17) | 0.07 (0.03–3.89) | 0.12 | 36 | 0.61 (2.37) | 0.08 (0.04–3.35) | 0.12 | 0.67 |
| % DNA methylation in cord blood of newborns | | | | | | | | | | | | | | |
| *LINE-1* | | 318 | 76.63 (1.60) | 76.71 (73.85–78.96) | 76.61 | 166 | 76.99 (1.52) | 77.11 (74.24–79.31) | 76.97 | 152 | 76.24 (1.60) | 76.24 (73.50–78.75) | 76.22 | <0.001 |
| *Alu* | | 318 | 22.67 (0.61) | 22.67 (21.64–23.59) | 22.66 | 166 | 22.69 (0.56) | 22.70 (21.74–23.63) | 22.68 | 152 | 22.65 (0.66) | 22.63 (21.50–23.50) | 22.64 | 0.65 |

SD: standard deviation; $P_5$-$P_{95}$: percentiles 5th–95th.
LOD = limit of detection; *LINE-1* = long interspersed nucleotide elements; BPA = bisphenol A; BPS = bisphenol S; BPF = bisphenol F.
[a] Differences between male and female newborns. Student`s *t*-test or Mann Whitney U test.

positive association between maternal BPA concentrations and percentage of *LINE-1* remained among male newborns (beta = 0.06; 95 % CI = 0.01; 0.12) and was borderline among female newborns (beta = 0.08; 95 % CI = −0.001; 0.15). These associations remained in full adjusted models. In this case, for example, a 10 unit increase in BPA was associated with a 0.9 percentage point increase in *LINE-1* for all newborns. We also calculated the expected change in *LINE-1* methylation associated with an interquartile increase (25th–75th) in urinary BPA concentrations for a typical mother-baby boy pair in the 24th week of pregnancy. When urinary BPA was 1.55 ng/mL, the 25th percentile of the distribution, the expected *LINE-1* methylation, using our final regression model, was 76,74 %. When urinary BPA was 5.87 ng/mL (the 75th percentile), the expected *LINE-1* methylation was 77.16 %, whereas the predicted value for the 50th percentile of urinary BPA was 76,89 %. Consequently, an interquartile increase in urinary BPA was associated with an increase in *LINE-1* methylation that was 0,5 % of the median, based on the best-fitting model. Comparable increases were

**Table 3**
Unadjusted and adjusted linear regression models of % DNA methylation according to the maternal urinary levels of bisphenol.

| % DNA Methylation in cord blood of newborns | Maternal urinary concentrations of bisphenol (ng/mL) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 24th week of pregnancy samples (*n* = 318) | | | | | | | Delivery samples (*n* = 276) | | |
| | BPA | | | | BPF[b] | | | BPA | | |
| | | n | Parameter estimate[a] | *p*-Value | n (Exposed; not-exposed) | Parameter estimate[a] | *p*-Value | n | Parameter estimate[a] | *p*-Value |
| All newborns (n = 318) | CRUDE MODEL[c] | | | | | | | | | |
| | *LINE-1* | 318 | 0.05 (0.01; 0.09) | **0.01** | 131;187 | 0.10 (−0.25; 0.46) | 0.56 | 276 | 0.02 (−0.02; 0.05) | 0.30 |
| | *Alu* | | −0.005 (−0.02; 0.01) | 0.54 | | −0.003 (−0.14; 0.13) | 0.97 | | 0.003 (−0.01; 0.02) | 0.67 |
| | ADJUSTED MODEL[d] | | | | | | | | | |
| | *LINE-1* | 236 | 0.09 (0.03; 0.14) | **0.001** | 101;135 | 0.13 (−0.32; 0.58) | 0.57 | 206 | 0.01 (−0.05; 0.06) | 0.82 |
| | *Alu* | | 0.004 (−0.01; 0.02) | 0.68 | | 0.02 (−0.14; 0.18) | 0.79 | | 0.01 (−0.01; 0.03) | 0.25 |
| Males (n = 166) | CRUDE MODEL[c] | | | | | | | | | |
| | *LINE-1* | 166 | 0.06 (0.01; 0.12) | **0.03** | 70;96 | 0.03 (−0.44; 0.51) | 0.89 | 148 | 0.02 (−0.01; 0.06) | 0.23 |
| | *Alu* | | −0.01 (−0.03; 0.01) | 0.56 | | 0.01 (−0.16; 0.19) | 0.89 | | 0.003 (−0.01; 0.02) | 0.69 |
| | ADJUSTED MODEL[d] | | | | | | | | | |
| | *LINE-1* | 126 | 0.10 (0.03; 0.17) | **0.01** | 57;69 | 0.12 (−0.46; 0.71) | 0.67 | 114 | 0.02 (−0.06; 0.10) | 0.56 |
| | *Alu* | | −0.005 (−0.03; 0.02) | 0.68 | | 0.05 (−0.15; 0.25) | 0.63 | | 0.02 (−0.01; 0.04) | 0.20 |
| Females (n = 152) | CRUDE MODEL[c] | | | | | | | | | |
| | *LINE-1* | 152 | 0.05 (−0.01; 0.10) | 0.14 | 61;91 | 0.15 (−0.37; 0.68) | 0.56 | 128 | −0.01 (−0.07; 0.05) | 0.85 |
| | *Alu* | | −0.004 (−0.03; 0.02) | 0.77 | | −0.02 (−0.24; 0.2) | 0.85 | | 0.002 (−0.02; 0.03) | 0.88 |
| | ADJUSTED MODEL[d] | | | | | | | | | |
| | *LINE-1* | 110 | 0.08 (−0.001; 0.15) | 0.05 | 44;66 | 0.07 (−0.62; 0.76) | 0.84 | 92 | −0.004 (−0.08; 0.07) | 0.90 |
| | *Alu* | | 0.01 (−0.02; 0.04) | 0.39 | | −0.002 (−0.26; 0.26) | 0.99 | | 0.01 (−0.02; 0.03) | 0.66 |

*LINE-1* = long interspersed nucleotide elements; BPA = bisphenol A; BPF = bisphenol F. Bolded: statistically significant.
[a] Parameter estimate = beta (95 % CI).
[b] Categorized as "exposed" (*n* = 131) and "not exposed" (*n* = 187).
[c] Unadjusted lineal regression models.
[d] Lineal regression models adjusted by paternal BMI; height, weight and gestational age of newborns; maternal weight at 32nd week of pregnancy; previous miscarriages; and urinary creatinine concentration (mg/dL).

seen when including the whole study population. BPF concentrations at 24th week of gestation and BPA at delivery were not related to *LINE-1* methylation in the offspring. Lastly, no associations were found between maternal concentrations of bisphenol and methylation of *Alu* sequences in cord blood of newborns (Table 3).

Some additional statistical models were also performed to explore potential non-linear associations. Adjusted values of percentage DNA methylation (*LINE-1* and *Alu*) in female newborns according to quartiles of maternal BPA concentrations (ng/mL) at 24th week of pregnancy and at delivery are shown in Table 4. Female newborns of mothers with high BPA levels (third quartile) at 24th week of gestation showed higher percentage of *LINE-1* methylation than those in the first quartile of BPA concentrations [77.03 % (95 % CI = 76.36; 77.69) vs. 75.45 % (95 % CI = 74.86; 76.05)] (p-trend = 0.003). Hence, a previous borderline significance in linear models revealed a non-monotonic dose response relationship between maternal urinary BPA concentrations measured at 24th week of pregnancy and percentage of *LINE-1* methylation in female newborns. No such associations were detected for percentage of *Alu* methylation according to maternal levels of urinary BPA concentrations by quartiles in female (Table 4), male or all newborns at any time point (data not shown). Equally, percentages of *LINE-1* or *Alu* were not related either to maternal levels of urinary BPF concentrations by quartiles in females, males or all newborns (data not shown).

Lastly, statistical models examining associations between mean values of maternal urinary BPA concentrations for the 24th week of pregnancy plus time of delivery samples and percentage of DNA methylation (*LINE-1* and *Alu*) were carried out. A consistent positive association between maternal urinary BPA levels and percentage of *LINE-1* methylation in all newborns (and males) was observed (Supplementary Table 4).

## 4. Discussion

In the present study we have observed a positive association between maternal urinary BPA concentrations and global DNA methylation levels measured in *LINE-1* transposable sequences of cord blood of newborns only at the 24th week time point but not at time of delivery. No associations were found between prenatal exposure to BPA and methylation of *Alu* elements. Overall, our study assessed the relationship between in utero exposure to BPA, BPF and BPS and markers of global DNA methylation, as a biomarker of genome instability, in a European birth cohort.

Until now, only the 'Michigan Mother-Infant Pairs (MMIP) cohort' study evaluated associations between maternal exposure to BPA during the first trimester of pregnancy (8–14 weeks) and percentage of DNA methylation *LINE-1* from newborn's cord blood (Montrose et al., 2018). No associations were found between maternal BPA levels and *LINE-1* methylation, while hypomethylation of *LINE-1* was related to higher exposure to phthalates. In this population, average maternal urinary BPA concentration measured in 56 pregnant participants was 0.57 ng/mL (SD = 4.72) and the percentage of DNA methylation in *LINE-1* sequence of their offspring was 79.97 % (SD = 3.22; $n$ = 113). Despite the values of *LINE-1* methylation are close to those found in our study, the sample size of the MMIP cohort was relatively small as compared to ours. Moreover, levels of maternal urinary BPA concentrations were only measured in the first trimester of pregnancy and were relatively low [0.57 (SD = 4.72) ng/mL] (Montrose et al., 2018) compared to the levels observed in our study [2.14 (SD = 4.22) ng/mL] at 24th weeks of pregnancy. In this case, the relatively small sample size and low urinary BPA concentrations might have reduced the power to detect significant differences.

Several birth cohorts have evaluated the relationship between maternal phthalates urinary concentrations and global DNA methylation levels (Huang et al., 2018; Huen et al., 2016; Montrose et al., 2018). More specifically, Montrose et al. (2018) found that maternal urinary mono-3-carboxypropyl phthalate (MCPP) concentration was inversely associated with *LINE-1* methylation in the first trimester of pregnancy in the MMIP cohort. Besides, a recent Chinese birth cohort (Wuhan, China) found associations between maternal urinary mono-n-butyl phthalate (MBP) or monobenzyl phthalate (MBzP) and methylation levels *Alu* and *LINE-1* elements. Maternal urinary MPB was inversely related to methylation of *Alu* elements in cord blood of male newborns. Additionally, an inverse association between maternal urinary MBzP and methylation of *Alu* or *LINE-1* elements among female offspring was observed (Huang et al., 2018). Likewise, a consistent inverse association between prenatal concentration of monoethyl phthalate (MEP) and cord blood methylation of *Alu* repeats at 13th and 26th weeks of gestation were reported in the CHAMACOS cohort (Huen et al., 2016). Moreover, statistically significant negative trends were found in the same study population between MBP, MBzP and mono-isobutyl phthalate (MiBP), measured in mothers at 13th weeks of gestation, and *LINE-1* methylation. However, no differences in methylation levels of *LINE-1* sequences were identified associated with maternal urinary levels of phthalates at 26th weeks of gestation (Huen et al., 2016). It is also important to notice that in our study there was a discordance between findings at 24 weeks of pregnancy versus delivery. We might hypothesize that, as reported in previous studies (e.g., Huen et al., 2016), different timepoints throughout pregnancy might represent more or less specific windows of susceptibility.

As previously noted, several studies have reported associations between prenatal maternal exposures to EDCs and hypomethylation of some global DNA markers (Huang et al., 2018; Huen et al., 2016; Lu et al., 2020;

**Table 4**
Adjusted values of % DNA methylation (*LINE-1* and *Alu*) in female newborns according to quartiles of maternal concentrations of bisphenol A (ng/mL) at 24th week of pregnancy and at delivery.

| Variables | FEMALE NEWBORNS (N = 152) | | | | | |
|---|---|---|---|---|---|---|
| | *LINE-1* | | | *Alu* | | |
| | Mean (95%CI) | p-trend [a] | p-trend [b] | Mean (95%CI) | p-trend[a] | p-trend[b] |
| Maternal urinary BPA concentrations (ng/mL) at 24th week of pregnancy | | | | | | |
| Q1 (0.07–1.17) | 75.45 (74.86; 76.05) | 0.02[c] | 0.003[c] | 22.61 (22.37; 22.85) | 0.96 | 0.32 |
| Q2 (1.18–3.02) | 75.81 (75.21; 76.41) | | | 22.61 (22.37; 22.85) | | |
| Q3 (3.03–6.18) | 77.03 (76.36; 77.69) | | | 22.91 (22.65; 23.18) | | |
| Q4 (6.19–23.19) | 76.61 (75.99; 77.24) | | | 22.70 (22.45; 22.95) | | |
| Maternal urinary BPA concentrations (ng/mL) at delivery | | | | | | |
| Q1 (0.07–2.97) | 76.65 (75.89; 77.42) | 0.84 | 0.41 | 22.74 (22.45; 23.03) | 0.10 | 0.54 |
| Q2 (2.98–4.64) | 75.92 (75.16; 76.69) | | | 22.58 (22.29; 22.87) | | |
| Q3 (4.65–6.22) | 76.45 (75.65; 77.25) | | | 22.56 (22.26; 22.86) | | |
| Q4 (6.23–33.90) | 75.91 (75.19; 76.62) | | | 22.81 (22.53; 23.08) | | |

*LINE-1* = long interspersed nucleotide elements; BPA = bisphenol A.

[a] ANOVA test.

[b] ANCOVA test. Models adjusted by paternal BMI; height, weight and gestational age of newborns; maternal weight at 32nd week of pregnancy; previous miscarriages; and urinary creatinine concentration (mg/dL).

[c] Statistically significant differences between 1st vs. 3rd quartiles of maternal urinary bisphenol A concentrations.

McCabe et al., 2020; Montrose et al., 2018). However, our results, and those from other studies, show that BPA exposure may also be related to DNA hypermethylation (Miura et al., 2019; Nahar et al., 2015; Song et al., 2020). In fact, some diseases -particularly cancer- have been associated with global hypomethylation along with loci-specific hypermethylation (Basak et al., 2020; Ehrlich, 2002). However, this is just partially consistent with our results, because only one study showed similar results to ours in placental tissue (Nahar et al., 2015). In this case, in vitro investigations with placental and fetal tissues have demonstrated a positive relationship between total BPA concentrations and placental DNA methylation of *LINE-1*, but not with DNA methylation of other fetal tissues (liver or kidney) (Nahar et al., 2015). Furthermore, prenatal maternal BPA exposure has been associated with cord blood DNA hypermethylation of some relevant genes involved in neurobehavioral (CAPS2), immune (TNFRSF25), or physical development (HKR1) (Song et al., 2020) or encoding myotubularin related protein 6 [intergenic region (IGR) of MTMR6] (Miura et al., 2019).

From the current knowledge, it appears that BPA and phthalate exposure show potential mechanistic differences on DNA methylation, displaying -in general- positive or inverse associations, respectively (Huen et al., 2016; Montrose et al., 2018; Song et al., 2020). Further *in vivo* or *in vitro* experimental studies might be necessary in order to elucidate such dissimilarities.

Regarding potential biological mechanisms, experimental animal models have demonstrated that, for example, *in vivo/in vitro* BPA exposure resulted in decreased expression of MTMR6 mRNA (Mahemuti et al., 2018), increased DNA methylation of MTMR6 gene (Jadhav et al., 2017), affected the expression of SEMA3B mRNA (Ali et al., 2014; Castro et al., 2015), or decreased DNA methylation of SEMA3B promoter (Jorgensen et al., 2016). Moreover, in human studies, epigenome wide association studies (EWAS) have shown that BPA could interfere signal transduction in some specific genes in males (MAPK, AMPK, Par 1, pluripotency of stem cells, mTOR and Phospholipase D); as well as some endocrine system pathways (estrogen and relaxin signaling and parathyroid synthesis, secretion and action) in female newborns (Miura et al., 2019; Yang et al., 2020). Similarly, type I interferon receptor binding and pathways related to its activity have been associated with an increased methylation mediated by BPA exposure in the MMIP cohort (McCabe et al., 2020). In utero BPA exposure has also been linked with sex specific changes in the transcriptome and methylome of human amniocytes. Specifically, these analyses identified gene expression changes in pathways associated with metabolic disease and novel differentially methylated regions with potential distal regulatory functions (Bansal et al., 2020). Besides, exposure to bisphenols has been associated with down-regulation of some key genes implicated in testosterone biosynthesis (Star, Hsd3b1, or Cyp17a1) (Eladak et al., 2015).

DNA methylation levels of *Alu* sequences in our study population were similar to other cohort studies, but for *LINE-1* sequences were a bit more discordant. For example, the mean *Alu* methylation in the Chinese birth cohort (Wuhan, China) was 23.84 % (95 % CI = 20.90; 26.53) and 60.33 % for the *LINE-1* sequence (95 % CI = 55.12; 72.90) (Huang et al., 2018; Yang et al., 2017); and 25.38 % (SD = 0.61) and 79.03 % (SD = 1.35) in the CHAMACOS cohort (Huen et al., 2016), respectively. Similarly, the levels of urinary BPA reported in our study were also comparable or slightly higher to those observed in other studies. For instance, the average maternal urinary BPA concentration in the first trimester of pregnancy (8–14 week) was 0.57 (SD = 4.72) ng/mL in the MMIP cohort (Montrose et al., 2018), a value relatively lower compared to our study [geometric mean BPA concentration at 24$^{th}$ week of pregnancy = 2.14 (SD = 4.22) ng/mL]. On the other hand, somewhat comparable mean maternal urinary BPA concentrations in the `INMA-Sabadell Spanish birth´ cohort (Casas et al., 2016) and the `Swedish Environmental Longitudinal, Mother and child, Asthma and allergy study´ (SELMA) (Derakhshan et al., 2019) [2.3 (95 % CI = 2.1; 2.4) ng/mL throughout pregnancy] were reported. Besides, somewhat lower median levels of urinary BPA concentrations [1.09 (0.49–2.26) μg/L] in U.S. adult women participating in the National Health and Nutrition Examination Survey (NHANES) 2013–2014 were observed (Lehmler et al., 2018). Regarding the different results observed for *LINE-1* and *Alu*, it is known that both measurements of global DNA methylation have some structural differences. For instance, intact *LINE* elements have a length of 6.000 nucleotides and the number of *LINE-1* copies is between 500.000 and 700.000, occupying about 17 % of the human DNA (Sellis et al., 2007; Erichsen et al., 2018). Nonetheless, *Alu* typical sequence is 300 nucleotides long and is found in the human genome in a number of 1.100.000 copies, covering approximately 11 % of its total length (Sellis et al., 2007). Therefore, we might hypothesize this might be one potential biological reason why we obtained significant results for *LINE-1* but not for *Alu*.

Some limitations in our study must be considered. Firstly, urinary BPS and BPF measurements revealed a large amount of data below the LOD, in line with previous epidemiological studies (Lu et al., 2020; Miura et al., 2019; Montrose et al., 2018; Colorado-Yohar et al., 2021). This may at least be partly due to the low replacement of BPA by BPS and BPF in the Spanish population, and therefore in our study population. Secondly, our work did not consider bisphenol exposure during the first trimester of pregnancy since pregnant women were recruited in the 20$^{th}$ week of gestation, which is a particularity that must be taken into account regarding our findings. Thirdly, some baseline variables diverged in the included study sample compared with the excluded, but these differences are not likely to have affected either the internal or external validity of the results. Fourth, it is also worthwhile to point out the limitation of using cord blood global DNA methylation as a surrogate for other tissues. Fifth, due to the number of comparisons performed, we note that the likelihood of chance findings in our evaluation of the effect of early bisphenol exposures on markers of global DNA methylation cannot be ruled out. Some strengths of the current study include its population-based prospective design, and the availability of extensive information on potential confounders and covariates, allowing the use of multivariate models for adjustment. The methodology employed and reported for the analyses of urinary bisphenol concentrations and *LINE-1* and Alu sequences has been tested and widely used before, ensuring the validity of the obtained results. Finally, it would be recommended to perform in future studies mediation analysis to evaluate the role of epigenetic changes in the relationship between prenatal exposure to EDCs and other health outcomes or biomarkers such as perinatal anthropometry of newborns or birth outcomes.

## 5. Conclusions

Overall, our results suggest that exposure to environmental levels of BPA may be associated with a modest increase in *LINE-1* methylation -as a relevant marker of epigenomic stability- during human fetal development. However, any effects on global DNA methylation are likely to be small, and of uncertain biological significance. Consequently, further studies will be needed to elucidate the associations between bisphenol exposure and markers of global DNA methylation.

## CRediT authorship contribution statement

Fuensanta Navarro-Lafuente: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing - original draft. Evdochia Adoamnei: Formal analysis, Visualization, Writing – original draft. Julián J. Arense-Gonzalo: Data curation, Formal analysis, Methodology, Writing – review & editing. María T. Prieto-Sánchez and María L. Sánchez-Ferrer: Investigation, Resources, Writing – review & editing. Antonio Parrado: Validation, Investigation, Resources, Writing – review & editing. Mariana F. Fernandez and Beatriz Suarez: Validation, Investigation, Resources, Writing – review & editing. Antonia López-Acosta: Resources, Writing – review & editing, Supervision. Antonio Sánchez-Guillamón: Resources, Writing – review & editing, Supervision. Luis García-Marcos: Conceptualization, Methodology, Project administration, Investigation, Resources, Writing – review & editing, Supervision, Funding acquisition. Eva Morales: Conceptualization, Methodology, Project administration, Validation, Investigation, Resources, Data curation, Writing – review & editing,

Supervision, Funding acquisition. Jaime Mendiola: Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Validation, Writing - original draft, Funding acquisition. Alberto M. Torres-Cantero: Conceptualization, Methodology, Writing – review & editing, Supervision, Funding acquisition.

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## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.156540.

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