



Full length article

Association of in utero exposure to phthalate and DINCH metabolites with placental DNA methylation



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ABSTRACT

Phthalates and DINCH are non-persistent chemicals widely used in consumer products. In utero exposure to these compounds has been linked to adverse reproductive and long-term health outcomes, potentially through epigenetic changes in the placenta. This study investigated associations between maternal phthalate and DINCH metabolite levels and placental DNA methylation in 469 mother–child pairs from the Barcelona Life Study Cohort (BiSC). Fifteen phthalate and two DINCH metabolites were measured in pooled maternal urine samples collected at 19 and 35 weeks of gestation using liquid chromatography–tandem mass spectrometry (LC-MS/MS). Placental DNA methylation was assessed using the Illumina EPIC array. We applied robust linear regression models to test associations between single exposures at 19 weeks, 35 weeks, and whole pregnancy (average of the two time points), with each CpG site. In secondary analyses, quantile g-computation was used to test associations between exposure mixtures and suggestive CpGs (p-value < 1E-05). We identified 38 Bonferroni significant associations in the single exposure models (p-value < 1E-07)— 24 at 19 weeks, 8 at 35 weeks and 6 for the whole pregnancy period. Suggestive CpGs (p-value < 1E-05) were annotated to genes involved in metabolic, immune and vascular pathways, steroid biosynthesis, and sex hormone signaling. Sex-stratified analyses revealed 49 female-specific and 42 male-specific associations, most of which were identified at a single time point. Mixture analyses revealed 20 significant associations, all consistent in direction with the single-metabolite models. These results suggest that prenatal exposure to phthalates and DINCH may contribute to placental epigenetic alterations supporting a role for endocrine disruption, metabolism, and vascular and immune modulation in mediating their effects.

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1. Introduction

Phthalates and 1,2-cyclohexane dicarboxylic acid diisononyl ester (DINCH), non-persistent chemicals, are commonly used plasticizers and stabilizers typically added to plastic materials such as food packaging, cosmetics, toys or drug films and other medical devices (Schettler, 2006; Ao et al., 2024; Genuis et al., 2012). Due to their widespread use, phthalates and DINCH have become ubiquitous in the environment, leading to chronic exposure through ingestion, inhalation, and skin absorption in humans (Schettler, 2006; Diamanti-Kandarakis et al., 2009).

Exposure to phthalates has been linked to a broad range of health disorders (Benjamin et al., 2017; Mariana et al., 2016). In particular, the in utero exposure has been related to an increased risk of pregnancy complications such as miscarriage, preeclampsia, or gestational diabetes (Grindler et al., 2018; Qian et al., 2020; Warner et al., 2021), and also to short and long-term health consequences in the offspring, including intrauterine growth restriction, preterm birth, low birth weight, obesity, immune disorders or neurodevelopmental disorders (Almeida-Toledano et al., 2024; Dutta et al., 2020). Some of these associations have been shown to be sex-specific, such as reduced ovarian weight or altered puberty timing in females, and altered testosterone synthesis or increased risk of ADHD in males, among others (Freire et al., 2024; Huang et al., 2024; Jurewicz and Hanke, 2011; Niemann et al., 2015).

The placenta is a central organ for fetal development, responsible for nutrient and oxygen exchange, immune defense, and endocrine regulation (Herrick and Bordon, 2025). It is permeable to phthalates and DINCH, and several studies have reported comparable levels of these substances in both maternal and cord blood (Chen et al., 2008; Latini et al., 2003; Jensen et al., 2012; Yan et al., 2009; Zhang et al., 2009). These chemicals can disrupt multiple biological processes, particularly through endocrine mechanisms, potentially impairing placental formation and function (Gorini et al., 2025; Saad et al., 2021). As the primary interface between maternal and fetal systems, the placenta integrates exposures and biological signals throughout pregnancy, making it a mechanistically relevant tissue for studying the effects of chemical exposures. Despite this, it remains relatively understudied compared to other perinatal tissues. Importantly, such effects can be captured in the placental epigenome (Nelissen et al., 2011)—the chemical modifications to DNA and associated proteins, including DNA methylation, histone modifications, and non-coding RNA changes, which influence gene expression without altering the DNA sequence (Gibney and Nolan, 2010; Li, 2021).

Several studies have investigated the association of in utero phthalate and DINCH exposure with epigenetic alterations in the offspring. In cord blood, epigenome-wide association studies (EWAS), ranging from 64 to 336 samples, have reported up to 25 CpGs with differential DNA methylation (Chen et al., 2018; Khodasevich et al., 2024; Lee et al., 2023; Miura et al., 2021; Petroff et al., 2022; Sol et al., 2022; Solomon et al., 2017). In the placenta, two EWAS studies with 202 and 387 samples from two French cohorts have identified 1 and 114 differently methylated CpGs, respectively (Jedynak et al., 2022; Jedynak et al., 2024). However, these studies were relatively small, and some of them did not explore the whole genome, or the effects of the exposure window or fetal sex. Moreover, assessment of in utero phthalate and DINCH exposure was based on one or a few spot urine samples in all but one study (Jedynak et al., 2024), potentially producing high levels of measurement error, since exposure to these short-lived chemicals is known to be highly variable.

In this study we aimed to evaluate the association between phthalate and DINCH metabolite levels measured in pooled maternal urine samples collected at 19 and 35 weeks of gestation, and placental genome-wide DNA methylation in 469 mother–child pairs from the Barcelona Life Study Cohort (BiSC).

2. Material and methods

2.1. Study population

This study was nested in the Barcelona Life Study Cohort (BiSC), a population-based birth cohort in Barcelona, Spain (Dadvand et al., 2024). Pregnant women ($n = 1,080$) were recruited during the first trimester of pregnancy between 2018 and 2021 in three hospitals in Barcelona (Hospital Sant Joan de Déu, Hospital de la Santa Creu i Sant Pau, and Hospital Clínic de Barcelona). The detailed information about inclusion criteria, follow-up visits and data collected can be seen elsewhere (Dadvand et al., 2024). Briefly, we included pregnant women between 18 and 45 years old, living in the catchment area of recruiting hospitals and being able to communicate in Catalan, Spanish, or English. We excluded women having a fetus with major congenital anomalies (i. e., malformations incompatible with life). In this study we examined 469 BiSC mother–child pairs with available data of urinary phthalate and DINCH levels in 19 weeks of gestation ($N = 349$) or 35 weeks of gestation ($N = 404$) of pregnancy (overlap 284), placental DNA methylation, gestational age, and covariates (Fig. S1). The goal of selecting these two time points for urine sampling was to include the early-mid and late stages of pregnancy.

All participants signed an informed consent form prior to their enrolment into the cohort. Ethics approvals were obtained from the corresponding authorities in all the participating institutions and hospitals. Study data were collected and managed using REDCap electronic data capture tools hosted at ISGlobal (Harris et al., 2019; Harris et al., 2009).

2.2. Exposure assessment

Pregnant participants' first and last voids of the day were collected during six consecutive days at approximately 19 weeks (mean (standard deviation (SD)) = 18.8 (2.9) weeks, $N = 695$ samples) and 35 weeks (mean (SD) = 34.7 (1), $N = 756$ samples). The samples were kept in freezers of participants' home during the sampling week and then transported to the BiSC biobank and kept in -80°C freezers till the time of the analysis. Urine pools, containing equivalent volumes of each void (with a minimum of 10 voids), were created for each participant at each collection time point.

A total of 15 phthalate metabolites and 2 DINCH metabolites were measured in each urine pool using high-performance liquid chromatography and tandem mass spectrometry (LC-MS/MS) at the Norwegian Institute of Public Health (Sabareddzovic et al., 2015). These included: monoethyl phthalate (MEP), mono-*iso*-butyl phthalate (MiBP), mono-*n*-butyl phthalate (MnBP), mono benzyl phthalate (MBzP), mono-*n*-pentyl phthalate (MnPeP), monocyclohexyl phthalate (MCHP), mono-*n*-octyl phthalate (MnOP), mono-2-ethylhexyl phthalate (MEHP), mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP), mono-2-ethyl-5-oxohexyl phthalate (MEOHP), mono-2-ethyl 5-carboxypentyl phthalate (MECPP), mono-4-methyl-7-hydroxyoctyl phthalate (oh-MiNP), mono-4-methyl-7-oxooctyl phthalate (oxo-MiNP), mono-4-methyl-7-carboxyoctyl phthalate (cx-MiNP), 6-hydroxy monopropylheptylphthalate (oh-MPHP), 2-(((4-hydroxy-4-methyloctyl)oxy)carbonyl)cyclohexanecarboxylic acid (oh-MINCH), 2-(((4-methyl-7-oxooctyl)oxy)carbonyl)cyclohexanecarboxylic acid (oxo-MINCH). Some of the phthalate metabolites (oh-MiNP, oxo-MiNP, cx-MiNP and oh-MPHP) and DINCH metabolites (oh-MINCH and oxo-MINCH) have multiple isomers. Thus, for these metabolites the sum of the isomers was reported. Three blanks and two in-house control samples were analyzed alongside the urine samples in each laboratory batch (about 50 samples per batch). For MBzP, MEHP, MEHHP and MEOHP, a signal was detected in $< 25\%$ of the analyzed blanks with a minimal signal (< 2 times limits of quantification), thus no correction was indicated. For the remaining compounds no blank detection was observed. The coefficient of variation of control samples within batches ranged between 7 %

(MEP) to 33 % (MEHP) (Supplementary Table S1). Phthalate and DINCH levels are expressed as ng/mL urine.

oh-MiNP was discarded from the analysis due to co-elution with a metabolite of a terephthalate that has the same mass and retention time (mono-2-ethyl-5-carboxypentyl terephthalate, MECPTP). Three phthalate metabolites, MnPeP, MCHP, MnOP were detected in < 5 % of samples and were also excluded from the analysis. The limit of detection (LOD), the lower and upper limit of quantification (LLOQ, ULOQ), and the descriptive of the subset of samples examined in this study (19 weeks: N = 349, 35 weeks: N = 404) are shown in Tables TS1A-B. After testing for normal distribution, phthalate levels were log2-transformed to meet the requirement of Gaussian distribution for analysis. For phthalates where no levels were detected due to signals < LOD, the missing values were imputed using the left-censored 'fill in' imputation method described by Helsel (1990) (Helsel, 1990). Additionally, the weighted sum of metabolites (nmol/L) derived from the same parent compound was calculated by summing the metabolite levels (in original scale and with values < LOD imputed) divided by their molecular weight (sum DEHP: MEHP, MEHHP, MEOHP and MECPP, sum DiNP: oxo-MiNP and cx-MiNP, sum DINCH: oh-MINCH and oxo-MINCH). Then, the sums were tested for normal distribution and log2-transformed. For the analysis of whole-pregnancy phthalate exposure in relation to placental DNA methylation, we calculated the average metabolite concentrations when measurements from both trimesters were available, and used trimester-specific concentrations when only one measurement was available (65 unique at 19 weeks and 120 unique at 35 weeks).

The effect of several technical and design variables (laboratory batch, urine time to storage, number of urine samples in the pool), and biological variables (urine creatinine levels and gestational age) on phthalate levels were investigated, and found to be minimal with the exception of creatinine. Creatinine levels (mg/L urine) were measured in the urine pool as described elsewhere (Granerud, 2022). To eliminate this potential noise, we applied a standardization method, based on linear regression that adjust the exposure for creatinine protecting other variables (hospital of delivery, COVID19 confinement, gestational age, maternal smoking, education, BMI and age, child's ethnicity and sex, and parity) described by Mortamais, et al. (2012) (Mortamais et al., 2012). This second dataset corrected for creatinine has been used in the sensitivity analysis (see section 2.5).

2.3. DNA methylation

2.3.1. Placental biopsy collection

Following a harmonized protocol across hospitals, placental biopsies (2.5×1 cm, N = 611) were collected from two opposite quadrants approximately 3–4 cm from the cord insertion site. Biopsies were then halved (2.5×0.5 cm). One half was stored in liquid nitrogen, and the other half treated with RNAlater and dissected into four 0.5×0.5 cm samples representing the chorionic plate/membranes, upper chorionic villi, lower chorionic villi, and decidua. All biopsies were finally stored at -80°C until the time of analysis.

2.3.2. Placental DNA extraction

For DNA extraction, we selected 30–40 mg of tissue from the upper chorionic villi to minimize contamination from maternal tissue and to obtain a homogeneous sample composed predominantly of syncytiotrophoblasts—the multinucleated cells forming the outermost layer of the chorionic villi that mediate maternal–fetal exchange and endocrine signaling. Briefly, the dissected samples were homogenized using a bead mill (bead beater) at 4°C for 26 s. Next, the genomic DNA was isolated using the AllPrep®DNA/RNA/miRNA Universal Kit, (Qiagen, CA, USA). DNA was eluted in 80 and 60 μL and stored in different aliquots at -80°C . DNA quality was evaluated on a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) and additionally 500 ng of DNA was run on 1 % agarose gels to confirm that samples did not present visual signs of degradation.

2.3.3. Placental DNA methylation data acquisition

DNA methylation analysis was performed on 624 DNA samples from 589 individuals including 35 duplicates. Seven hundred and fifty ng of DNA were bisulfite-converted using the EZ 96-DNA methylation kit following the manufacturer's standard protocol. The DNA methylation analysis was conducted using the Infinium Methylation EPIC BeadChip from Illumina, following manufacturer's protocol in the Human Genome facility (HUGE-F) at the Erasmus Medical Centre core facility. The samples were randomly distributed into multiple plates, and the random distribution according to main biological and socioeconomic factors was checked. Twenty-six technical control samples were distributed across multiple plates, allowing us to evaluate the consistency of methylation measurements and detect potential batch effects. These control samples were used in quality assessment prior to normalization and contributed to ensuring data comparability between batches.

2.3.4. Placental DNA methylation data quality control and normalization

The DNA methylation data pre-processing was performed using the PACEAnalysis R package (v.0.1.9) (<https://www.epicenteredresearch.com/>). During the data pre-processing, sample quality control, probe quality control, normalization, batch correction, estimation of cell type proportions, and winsorization of outlier values were conducted. Overall, 59 samples were excluded from the data due to: a) low quality (N = 5), b) sample call rate < 95 % (p-value < 0.05, N = 3), c) sex inconsistencies (N = 9), d) substantial contamination with DNA from other samples (log2 odds < -1) (N = 11) (Heiss and Just, 2018), e) duplicates calculated using the probes to genotype 59 single nucleotide polymorphisms (SNPs) contained in the array (N = 29), and f) siblings (N = 2). The 59 excluded samples correspond to 24 individuals. After sample quality control, 565 unique samples remained. Probes with a call rate < 95 % (p-value < 0.05) were discarded according to the SeSAMe method (Zhou et al., 2018). Moreover, control and non-CpG probes, sex chromosome targeting probes, probes giving inconsistent results between 450 K and EPIC arrays (Fernandez-Jimenez et al., 2019), and problematic probes (probes with hybridizing problems and probes affected by the presence of SNPs: probes containing a SNP at the CpG site itself, probes with a SNP adjacent to the CpG site, probes with a SNP within 5 nucleotides of the CpG site if the minor allele frequency (MAF) was greater than 1 % in global populations) according to Zhou et al. (2017) (Zhou et al., 2018), were excluded. The final dataset consisted of 565 samples (469 with phthalate measurements) and 865,859 probes.

The dye-bias and Noob background correction, and normalization with the functional normalization method were conducted using the minfi R package (Fortin et al., 2017; Triche et al., 2013). Correction for type 2 probe bias was performed applying the beta-mixture quantile normalization (BMIQ) (Teschendorff et al., 2013). Data was explored using Principal Component Analysis (PCA) and associations of the first 12 PCs with main variables (hospital of recruitment, hospital of delivery, sex, ethnic, COVID-19 confinement, gestational age, birth weight, maternal smoking) and technical variables (array, working plate, DNA extraction batch, round of extraction, DNA concentration, quality ratio 260/280 and quality ratio 260/230) were examined using linear regression models. The effect of the array batch variable was eliminated using the ComBat method due to its association with most of the PCs, including PC1, which explained 30.36 % of the variance (Johnson et al., 2007). DNA methylation values are expressed as beta values, where 0 indicates no methylation and 1 indicates complete methylation. The beta values were winsorized based on a percentile of 1 % (0.5 % on each side) estimated from the empirical beta-distribution to reduce the influence of outliers.

Cell type proportions for six populations (trophoblasts, syncytiotrophoblast, nucleated red blood cells (nRBCs), Hofbauer cells, endothelial cells, and stromal cells) were estimated from DNA methylation using the placenta reference panel from the 3rd trimester (term) implemented in the planet R package (Yuan et al., 2021).

2.4. Covariates

Based on evidence from previous studies (Solomon et al., 2022; Choudhary et al., 2024; Elliott et al., 2022; Yeung et al., 2024; Ghildayal et al., 2022; Cosin-Tomas et al., 2022; Kashima et al., 2021; Campagna et al., 2023), the following variables were included as covariates in the models. Maternal age (years), maternal education (primary and secondary school/university), maternal tobacco smoking status during pregnancy (non-smoker/smoker), parity (multiparous/nulliparous), and child's ethnicity (European/other) were obtained from self-reported questionnaires. Maternal body mass index (BMI) at 13 weeks of pregnancy (kg/m^2) was obtained through direct measurements. Gestational age at delivery (weeks) was calculated based on the crown-rump-length, measured using ultrasound examination at approximately 12th gestational week obstetric visit (Altman and Chitty, 1997). Placental cell type proportions were estimated from the DNA methylation data (see above). Child's sex (female/male), and birth weight (grams), the later used in the descriptive of the study, were obtained from medical records. COVID-19 confinement (whether pregnancy took place before confinement/during confinement/after confinement) and hospital of delivery (Hospital Sant Joan de Déu, Hospital de la Santa Creu i Sant Pau, and other) were obtained based on conception and gestational age and the hospital where the delivery took place.

Maternal age, education, smoking status, BMI, parity, and child's ethnicity were included in the models as potential confounders of the association. Gestational age was considered a potential mediator of the association, and tested in the sensitivity models. Child's sex and placental cell type proportions were included as precision variables due to their strong effects on epigenetic profiles. COVID-19 confinement and hospital of delivery were added to account for their potential influence on life-style and/or sample handling and processing (indeed both were related to DNA methylation PCs).

2.5. Statistical analyses

Prior to the main analysis, we calculated the Spearman's correlation between phthalate and DINCH metabolites and the Spearman's correlation with creatinine in each trimester. To compare the variability of phthalate and DINCH metabolite levels between trimesters, we calculated the intra-class correlation (ICC), which quantifies the consistency of measurements within the same individual over time. Bland–Altman plots were used to illustrate the trimester differences for each individual and each metabolite.

In our main analysis, we tested the association of exposure to each of the single phthalate and DINCH metabolites and the sum of metabolites (sum DiNP, sum DEHP and sum DINCH) at different exposure windows (19 weeks, 35 weeks and whole pregnancy) with each CpG site, using robust linear regression models (PACeanalysis R package). The main model was adjusted for hospital of delivery, COVID19 confinement, maternal smoking, maternal education, maternal BMI at 13 weeks of pregnancy, maternal age at delivery, parity, ethnicity and sex of the offspring, and placental cell composition.

To control for multiple-testing the Bonferroni correction was applied considering the total number of CpGs tested for each metabolite and time point (a p -value $< 1\text{E-}07$ was considered statistically significant). The lambda inflation factor was calculated as a ratio between median of observed distribution of p -values and median of expected distribution of p -values (Guintivano et al., 2020). Effect size was expressed as the change in DNA methylation (from 0 to 1) per doubling in phthalate levels (ng/mL).

As a secondary analysis, we performed the analysis of chemical mixtures using quantile g-computation implemented in *qgcomp* R package to examine the potential mixture effect of phthalate and DINCH metabolites on DNA methylation (Keil et al., 2020). The quantile g-computation uses a generalized linear model to estimate the overall and partial effects of mixture exposure on the outcome of interest using

quantiles of metabolite levels, rather than continuous concentrations, while allowing for covariate adjustment. The overall mixture effect represents the change in the outcome per one quantile increase across all mixture components simultaneously, whereas the partial effects indicate the relative contribution of each individual metabolite to this overall effect. This analysis was restricted to statistically suggestive CpGs (p -value $< 1\text{E-}05$) from the single-metabolite analysis. Bonferroni corrected p -value for the mixture analyses was 0.05/493 CpGs at 19 weeks, 0.05/295 at 35 weeks and 0.05/277 for whole pregnancy.

We further stratified the main analyses by sex. Differences in the effect size of Bonferroni significant CpGs between sexes were compared with the Cochran's Q test implemented in *meta* R package (Balduzzi et al., 2019). The p -value of the Q test was adjusted by the number of associations identified in boys and girls (p -value threshold $< 7\text{E-}04$).

Finally, a number of sensitivity analyses were conducted on the main model: a) additionally adjusting for gestational age, which may be a mediator in the associations; b) restricting the analyses to the subset of children of European origin, a more homogeneous population at the genetic level; c) not adjusting for placental cellular composition, to test whether epigenetic associations were driven by true methylation changes rather than shifts in cell proportions; and d) using the phthalate levels corrected for creatinine, in order to account for variability in urine dilution in pools.

2.6. Downstream analyses

The IlluminaHumanMethylationEPICanno.ilm10b4.hg19 R package (Hansen, 2017) was used to annotate the CpGs to genes (within the gene or at $< 1,500$ bp upstream of the gene start site) and to CpG islands. In addition, to annotate the CpGs to enhancers and link them to genes in placenta tissue, we used the EPIraction webpage tool (run 14/08/2024) (Nurtdinov and Guigó, 2025). Furthermore, we searched whether identified CpGs overlapped with placental methylation quantitative trait loci (mQTLs), placental germline differentially methylated regions (gDMRs) (Hamada et al., 2016), placental partially methylated domains (PMDs) and placental chromatin states according to ROADMAP (Schroeder and LaSalle, 2013). Placental fetal *cis*-mQTLs were identified in BiSC (for more information see Supplementary Methods).

To identify potential biological pathways underlying the effects of identified DNA methylation changes (suggestive CpGs at p -value $< 1\text{E-}05$), we conducted several functional enrichment analyses using the missMethyl R package (Phipson et al., 2016). We tested enrichment for: (i) biological pathways (KEGG and Panther,); (ii) transcription factors (ENCODE, ChEA); and (iii) diseases (OMIM Expanded). A minimum of two genes in the pathway and a False Discovery Rate (FDR) < 0.2 was needed to report the pathways in the manuscript. Finally, we searched for previous associations with traits and exposures of significant CpGs in the EWAS Atlas (Li et al., 2019), EWAS catalog (Battam et al., 2022), and the Comparative Toxicogenomics Database (CTD) (Davis et al., 2023).

All analyses were performed using R Statistical Software (v4.1.2, v4.2.2, and v4.4.3) (R Core Team, 2021; R Core Team, 2022; R Core Team, 2025).

3. Results

3.1. Study population

Our study sample included 469 pregnant women and their offspring from the BiSC cohort, 349 with phthalate levels measured at 19 weeks (mean: 18.74, SD: 2.86), and 404 at 35 weeks (mean: 34.74, SD: 1.38), with an overlap of 284. The average maternal age at the enrollment was 34.6 years and the average BMI at 12w was 24.5 kg/m^2 . Overall, most of the women had completed university education (69 %) and did not smoke during pregnancy (92 %). Around 23 % of participants were pregnant during the COVID-19 confinement and half (50 %) delivered in

Hospital Sant Joan de Déu and 46 % in Hospital Sant Pau. The participants gave birth at an average gestational age of 39.8 weeks and 55 % of them were nulliparous. Average birth weight was 3287.4 g, the sex of the babies was uniformly distributed and the majority of the babies were of European origin (70 %). Detailed information of the participants is provided in Table 1.

3.2. Exposure to phthalates and DINCH

Eleven phthalate and 2 DINCH metabolites with levels > LOD in more than 90 % of the samples were included in the analyses. Median and interquartile range (IQR) levels for each compound and period are presented in Table 2 (see TS2A-C for more details). MEP had the highest levels measured (median 19 weeks: 47.51 ng/mL, 35 weeks: 40.04 ng/mL, whole pregnancy: 45.16 ng/mL). The weighted sums of metabolites of DEHP (4 metabolites), DiNP (2 metabolites), and DINCH (2 metabolites) showed median levels of 67.71, 40.51 and 10.19 nmol/L at 19 weeks, 45.23, 25.56, 6.13 nmol/L at 35 weeks, and 68.57, 40.13, 10.12 nmol/L for whole pregnancy, respectively (Table 2, TS2A-C).

The metabolites from different parent compounds presented a moderate to weak correlation, with the highest correlation between oh-MPHP and the oxo-MiNP (Spearman's correlation coefficient of 0.59 at 19 weeks, 0.61 at 35 weeks, and 0.64 for whole pregnancy) (Fig. 1A–C, Fig. S2A–C). The correlation within metabolites from the same parent compound was higher, ranging from 0.73 to 0.95 at 19 weeks, 0.70 to 0.95 at 35 weeks, and 0.72 to 0.97 for whole pregnancy (Figs. 1A, 1B, Fig. S2C).

The intra-class correlation of 19 weeks and 35 weeks ranged between 0.29 and 0.58 (Fig. S3A), indicating moderate correlation within the

same women between trimesters. The differences in phthalate levels between trimesters for each individual and each metabolite are shown in Fig. S3B. Finally, the metabolite levels at 19 weeks and 35 weeks were moderately correlated with creatinine (Spearman's correlation coefficient at 19 weeks: 0.28–0.43, 35 weeks: 0.30–0.46) (Figs. S4A, S4B).

3.3. Epigenome-wide association study by exposure period

In the main analysis of phthalates and DINCH metabolites and placental DNA methylation, a total of 38 associations were identified after Bonferroni correction (p-value < 1E-07), and 1065 at suggestive statistical significance (p-value < 1E-05) (Fig. 2, Table S3). The genomic inflation factors (λ) ranged from 0.95 to 1.22 at 19 weeks, from 0.84 to 1.27 at 35 weeks, and from 0.89 to 1.32 for whole pregnancy analyses (Table S3).

In particular, at 19 weeks, 24 Bonferroni-adjusted significant associations were found, which represented 10 unique metabolites and 21 unique CpGs, distributed in 10 loci (defined as a region < 1 Mb) and annotated to 11 unique genes (Table S4). Twelve CpGs from the same locus at chromosome 7 (cg12969170 as the lead CpG) were inversely associated with MEP levels. The rest of CpGs represented different loci and were associated with one or a few metabolites of the same parental compound (Fig. S5A). At 35 weeks, we identified 8 Bonferroni-adjusted significant associations, representing 7 unique metabolites and 6 unique CpGs (6 loci, 5 unique genes) (Table S4). Finally, phthalate levels over the whole pregnancy period were associated with 6 CpGs, representing 6 loci (4 genes) (Table S4C). Statistically suggestive associations (p-value < 1E-05) for each exposure window (N = 493 at 19 weeks, N = 295 at 35 weeks, and N = 277 for whole pregnancy) are shown in Table S5A-C.

Table 1
Descriptives of the study population.

	Whole population (N = 469)		Population at 19w (N = 349)		Population at 35w (N = 404)	
	N	%	N	%	N	%
Hospital of delivery						
Hospital Sant Pau	217	46	158	45	195	48
Hospital Sant Joan de Déu	235	50	182	52	194	48
Other	17	4	9	3	15	4
Covid-19 confinement						
Pregnancy before confinement	210	45	130	37	190	47
Pregnancy during confinement	109	23	89	26	82	20
Pregnancy after confinement	150	32	130	37	132	33
Maternal education						
Primary or secondary school	143	30	105	30	119	29
University	326	70	244	70	285	71
Maternal smoking during pregnancy						
Non-smoker	434	93	323	93	376	93
Smoker	35	7	26	7	28	7
Parity						
Multiparous	207	44	161	46	179	44
Nulliparous	262	56	188	54	225	56
Child ethnicity						
European	329	70	243	70	292	72
Other	140	30	106	30	112	28
Child sex						
Female	238	51	181	52	212	52
Male	231	49	168	48	192	48
	Mean	SD	Mean	SD	Mean	SD
Maternal age (years)	34.55	4.55	34.63	4.5	34.7	4.37
Maternal body mass index (BMI) at 12 weeks of pregnancy (kg/m ²)	24.53	4.44	24.51	4.39	24.33	4.31
Gestational age at birth (weeks)	39.75	1.4	39.68	1.42	39.85	1.22
Birth weight (g)	3287.42	465.65	3281.88	492.92	3309.06	429.68
Cell type proportions						
Trophoblasts	0.08	0.07	0.07	0.06	0.07	0.07
Stromal	0.01	0.01	0.01	0.01	0.01	0.01
Hofbauer	<0.01	0.01	<0.01	0.01	<0.01	0.01
Endothelial	0.05	0.03	0.05	0.03	0.05	0.03
Nucleated red blood cells (nRBC)	0.02	0.02	0.01	0.02	0.02	0.02
Syncytiotrophoblast	0.84	0.1	0.84	0.1	0.84	0.1

N: sample size; SD: standard deviation

Table 2
Descriptives of the phthalate and DINCH metabolite levels in maternal urine samples collected at 19 weeks (N = 349) and 35 weeks (N = 404).

Variable	Name	19w	35w	Whole pregnancy
		Median (IQR)	Median (IQR)	Median (IQR)
MEP	Monoethyl phthalate	47.51 (22.16–89.09)	40.04 (21.03–92.27)	45.16 (24.16–86.18)
MiBP	Mono-iso-butyl phthalate	11.58 (7.50–17.95)	11.38 (7.14–18.15)	11.81 (7.45–17.55)
MnBP	Mono-n-butyl phthalate	10.51 (6.73–15.50)	9.81 (6.59–16.91)	10.52 (6.90–16.66)
MBzP	Mono benzyl phthalate	1.24 (0.75–2.15)	1.16 (0.59–2.13)	1.19 (0.68–2.05)
MEHP	Mono-2-ethylhexyl phthalate	1.82 (1.05–3.11)	1.38 (0.68–2.71)	1.58 (0.81–2.83)
MEHHP	Mono-2-ethyl-5-hydroxyhexyl phthalate	5.68 (3.85–8.30)	5.06 (3.54–8.68)	5.63 (3.79–8.30)
MEOHP	Mono-2-ethyl-5-oxohexyl phthalate	4.71 (3.13–7.00)	4.61 (3.07–7.25)	4.75 (3.17–6.99)
MECPP	Mono-2-ethyl 5-carboxypentyl phthalate	8.52 (6.31–12.11)	8.29 (5.79–12.64)	8.38 (6.21–12.59)
oxo-MiNP	Mono-4-methyl-7-oxooctyl phthalate	4.31 (2.81–7.32)	4.32 (2.627.333)	4.48 (2.90–7.03)
cx-MiNP	Mono-4-methyl-7-carboxyoctyl phthalate	7.97 (5.51–11.76)	7.02 (5.08–11.02)	7.54 (5.50–11.06)
oh-MPHP	6-Hydroxy Monopropylheptylphthalate	1.81 (1.17–2.99)	1.87 (1.13–3.29)	1.90 (1.18–3.02)
oh-MINCH	2-(((Hydroxy-4-methyloctyl)oxy)carbonyl)cyclohexanecarboxylic acid	1.94 (1.33–3.37)	1.71 (1.10–3.13)	1.87 (1.18–3.08)
oxo-MINCH	2-(((4-Methyl-7-oxooctyl)oxy)carbonyl)cyclohexanecarboxylic acid	1.21 (0.73–2.08)	1.19 (0.71–2.19)	1.17 (0.71–2.10)
sum DEHP	molar sum of MEHP, MEHHP, MEOHP and MECPP (nmol/L)	67.71 (50.00–99.75)	63.75 (45.23–102.30)	68.57 (48.42–100.35)
sum DiNP	molar sum of oxo-MiNP and cx-MiNP (nmol/L)	40.51 (28.22–63.59)	37.59 (25.55–60.12)	40.13 (28.36–59.42)
sum DINCH	molar sum of oh-MINCH and oxo-MINCH (nmol/L)	10.19 (6.61–17.78)	9.66 (6.13–17.81)	10.12 (6.55–17.59)

Only the 13 phthalate and DINCH metabolites (and the sums) included in this study are reported.

N: sample size
Units: ng/mL urine
IQR: interquartile range

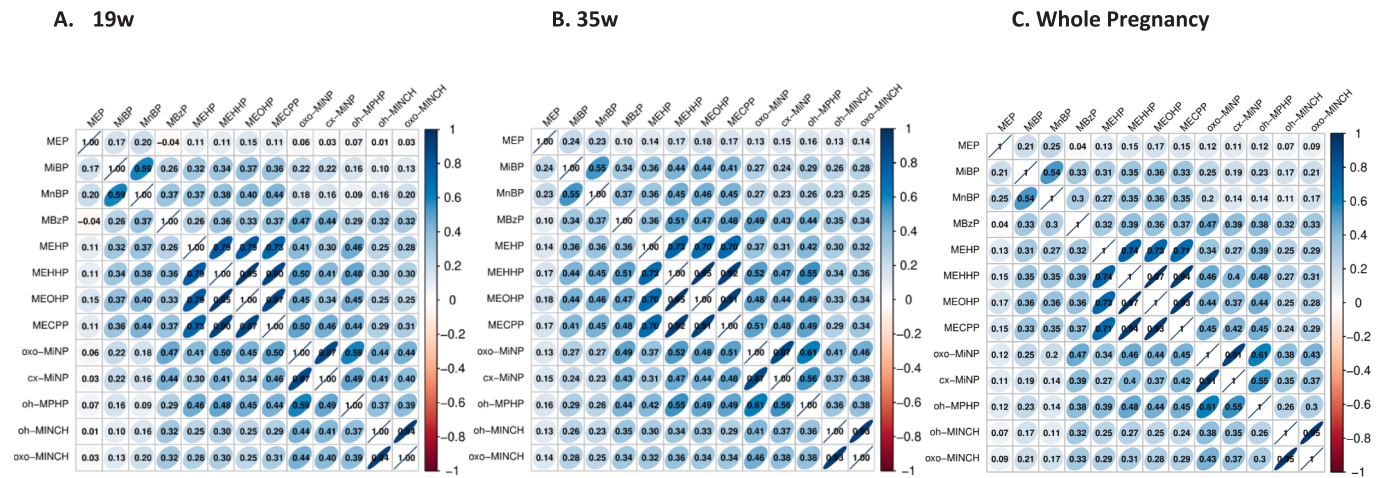


Fig. 1. Correlation of phthalate and DINCH metabolite levels. The heatmap shows the Pearson's correlation of the 13 phthalate and DINCH metabolite levels measured at 19w (A), at 35w (B), and in whole pregnancy (C). Positive correlations are indicated in blue and inverse correlations in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The effect sizes of the significant CpGs ranged from -0.047 to 0.019 for a doubling in the exposure levels, with most of them (71 %) showing decreased methylation levels with increasing exposure levels (Table S4). In general, at the same time point, DNA methylation levels at significant CpGs presented consistent directions of effects across compounds with a few exceptions (Fig. S5A–C). For instance, at 19 weeks, the inverse associations between MEP and CpGs at chromosome 7 were in the same direction in most of the compounds, but MiBP, MnBP or MBzP, which showed increased levels of methylation at these CpGs. The overlap of Bonferroni significant CpGs across compounds and the correlation of effect sizes across compounds are shown in Fig. S5A–C.

The overlap of Bonferroni significant CpGs across time periods was limited, with only two phthalate-CpG association pairs shared between 35 weeks and whole pregnancy analyses. Fig. 3 and Fig. S6 show the correlation of the effect size of the Bonferroni significant CpGs across the periods. Effect sizes between 35 weeks and whole pregnancy were quite correlated, reflecting the high correlation of exposure levels across these periods. In contrast, some of significant associations in one exposure period (19 weeks or 35 weeks) showed weak effect sizes in the other,

suggesting period-specific effects (Fig. 3). For instance, statistically significant CpGs that were associated with exposure to MEP metabolite at 19 weeks showed a weak effect at 35 weeks, while cg09012471 associated with MEHP metabolite at 35 weeks showed no effect at 19 weeks.

3.4. Sum of metabolites and mixture analyses

The results for the sum of metabolites were consistent with the associations observed for individual compounds at all exposure windows (Tables S6–S8).

We also conducted mixture analyses for the statistically suggestive CpGs (p -value $< 1E-05$), identifying six Bonferroni-significant associations at 19 weeks, seven at 35 weeks, and also seven associations in the whole pregnancy period (Tables S9A–C). There was no overlap among the CpGs identified in the mixture analyses across time points. Only two CpGs identified surviving the multiple-testing correction in the mixture analyses were also detected in the single-metabolite EWAS at Bonferroni significance: cg05811594 (gene *ZIC4*) at 19 weeks and cg07964716

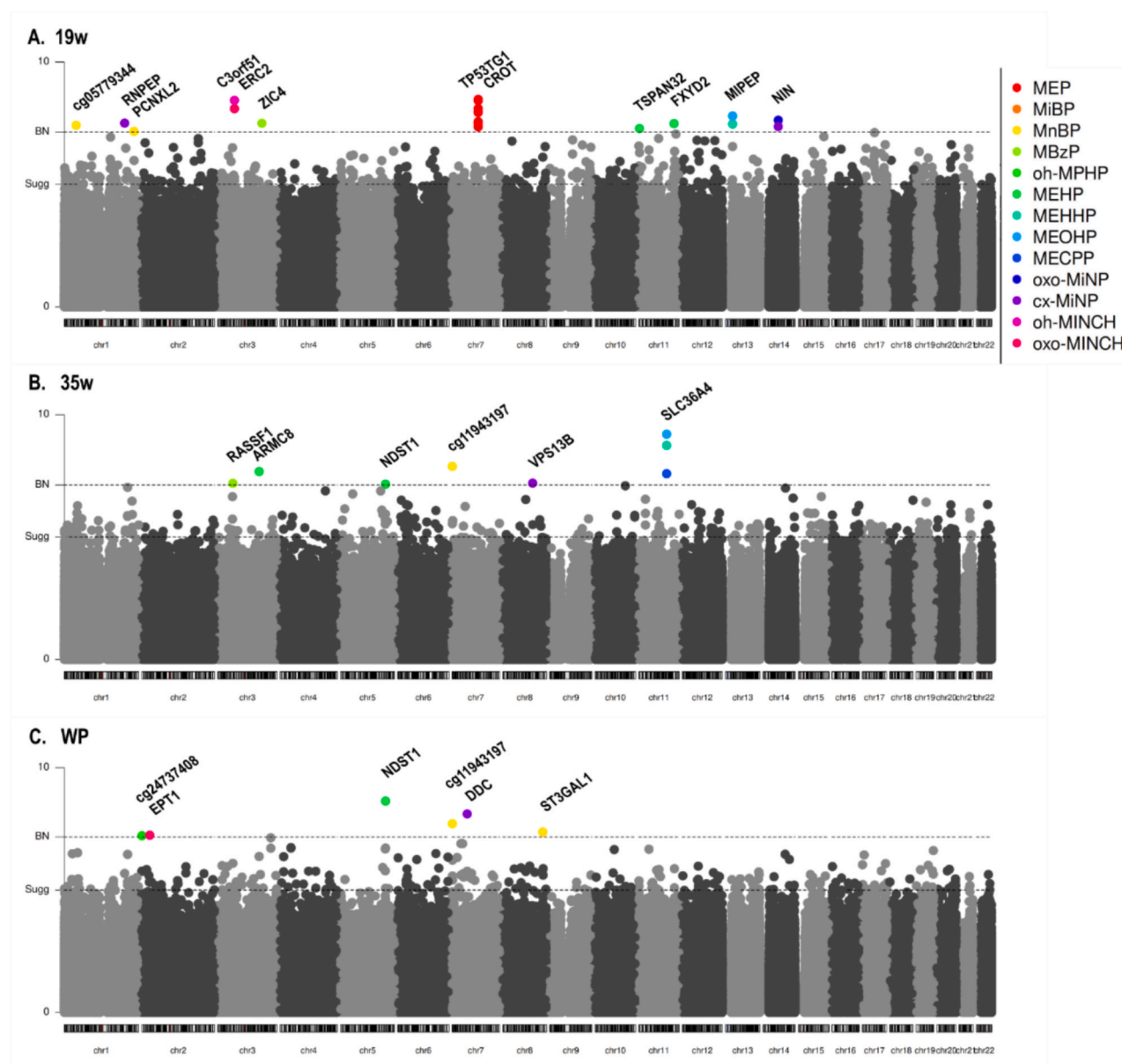


Fig. 2. Manhattan plots of the association between genome-wide DNA methylation and phthalate and DINCH metabolite levels, by exposure period. Each dot is the association of the DNA methylation levels at one CpG with the levels of one of the phthalate or DINCH metabolites measured at 19w (A), at 35w (B), and in whole pregnancy (C). The x-axis represents the position of the CpGs and the y-axis the $-\log_{10}$ p value of the association. The horizontal dashed lines are the suggestive ($1E-05$ p value $< 1E-05$) and Bonferroni (p value $< 1E-7$) multiple-testing thresholds. Associations surviving Bonferroni correction are coloured according to the legend. For CpGs annotated to a gene, the gene is shown, for the rest, the CpG ID is shown.

(gene *SLC36A4*) at 35 weeks. The overall effect sizes for the mixture associations for these two CpGs were -0.429 and 0.025 , respectively, in the same direction as the effects observed for the corresponding individual metabolites. The partial contributions of the metabolites to the differentially methylated CpGs in the mixture analyses are shown in Fig. S7.

3.5. Epigenome-wide association analysis stratified by sex

Results of the sex-stratified analyses by exposure period are shown in Table S10A and B. After applying the Cochran's Q heterogeneity test, 43 associations showed evidence of differences in effect sizes between females and males at 19 weeks, 31 associations at 35 weeks, and 17 associations for whole pregnancy (Tables S11, S12, S13). The associations at 19 weeks included 23 unique CpGs (20 loci, 19 unique genes) and 10 unique metabolites in females, and 17 unique CpGs (17 loci, 17 unique

genes) and 10 unique metabolites in males. The associations at 35 weeks included 14 CpGs (14 loci, 9 unique genes) and 8 metabolites in females, and 16 unique CpGs (13 loci, 10 unique genes) and 7 unique metabolites in males. Finally, the associations for whole pregnancy included 10 CpGs (8 loci, 6 unique genes) and 5 metabolites in females, and 7 unique CpGs (5 loci, 6 unique genes) and 6 unique metabolites in males.

The effect sizes of the sex-specific CpGs across sexes are shown in Fig. 4. No overlap in significant CpG-metabolite pairs was observed between 19 and 35 weeks, or between 19 weeks and the whole pregnancy period, in either females or males. However, in males, two CpG-metabolite pairs were shared between 35 weeks and the whole pregnancy period, as expected given the higher correlation in metabolite levels between these two periods.

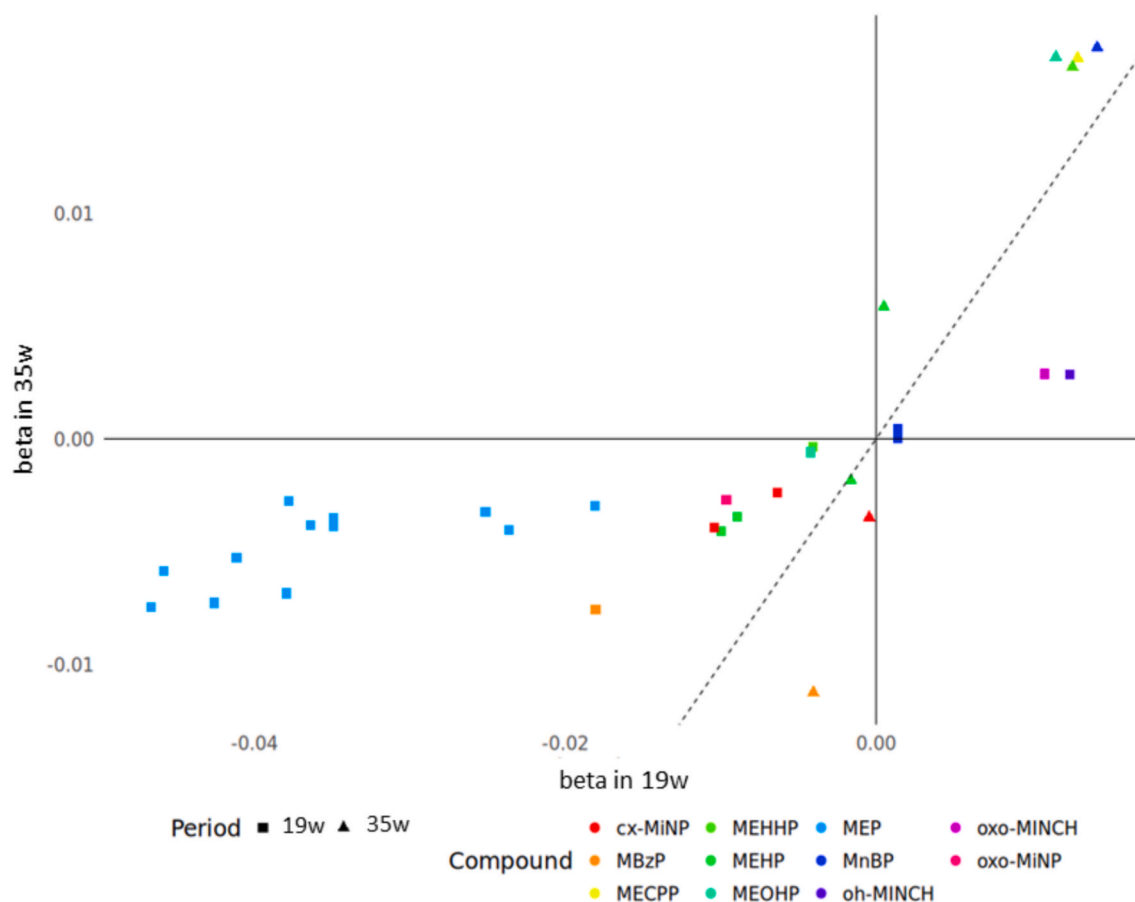


Fig. 3. Correlation of the effect sizes of the Bonferroni significant CpGs between exposure periods (19w and 35w). Each symbol is the association of the DNA methylation levels at one CpG with the levels of one of the phthalate or DINCH metabolites. The x-axis represents the effect size of the association with the exposure levels measured at 19w, and the y-axis at 35w. The shape of the symbols represents the period when the association is significant, and the color the phthalate or DINCH metabolite (see legend). Symbols at the diagonal indicate similar effect sizes, while at the axes indicate period-specific effects.

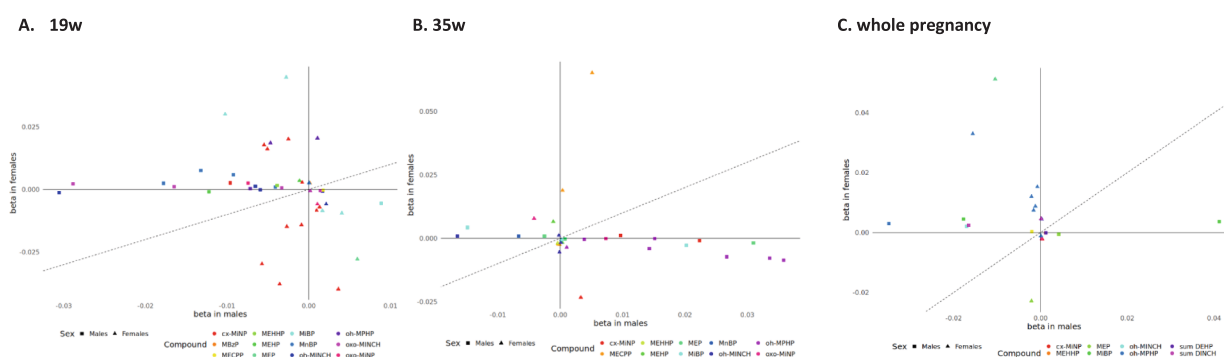


Fig. 4. Correlation of the effect sizes of the Bonferroni significant CpGs between males and females, by exposure periods. Each symbol is the association of the DNA methylation levels at one CpG with the levels of one of the phthalate or DINCH metabolites measured at 19w (A), at 35w (B), and in whole pregnancy (C). The x-axis represents the effect in males, and the y-axis the effect in females. The shape of the symbols represents the sex for which the CpG is significant, and the color the phthalate or DINCH metabolite (see legend). Symbols at the diagonal indicate similar effect sizes, while at the axes indicate sex-specific effects.

3.6. Sensitivity analyses

To confirm robustness of the findings from the main model, we conducted several sensitivity analyses, which are summarized in Table S14A-D. Although the Bonferroni significant CpGs varied across the sensitivity models, the effects sizes remained very similar to those of the main model with minor fluctuations (Figs. S8-S12). The model adjusted for gestational age showed more similar effect sizes to the main model than the other sensitivity analyses (Fig. S8). On the other hand,

the analysis restricting to the participants with European origin presented the largest differences in effect sizes, however, their direction remained the same (Fig. S9). Suggestive CpGs (p -value < $1E-05$) of the sensitivity analyses are presented in Tables S15-S18.

3.7. Downstream analyses

The 31 Bonferroni-significant CpGs of the main model as well as the 87 sex-specific CpGs were annotated using different tools (Tables S4 for

main model, S7 for sum of metabolites, S11 for sex-specific results). In the main model, we found that 28 out of the 31 CpGs were annotated to genes, 11 to CpG islands, and one (cg04915058 annotated to *C3orf51* and *ERC2* genes) had mQTLs. None of the CpGs was annotated to placental PMDs or gDMRs. In females and males, 39 and 36 of the CpGs were annotated to genes, ten and five to CpG islands, 20 and eight to placental PMDs, and 14 and four had mQTLs. In males, one CpG (cg01224063 at gene *IGF1R*) was also annotated to a gDMR.

We, then, took suggestive CpGs from the main model (p -value $< 1E-05$) and run pathway enrichment analyses for each phthalate and DINCH metabolite and period (going from 10 to 71 CpGs per analysis). The identified pathways included immune and vascular regulation (MiBP), and signaling through growth factors (oh-MPHP) at 19 weeks, steroid biosynthesis (MECPP) (Table S19B), and obesity for whole pregnancy levels (MECPP, MEHHP) (Table S19C). Moreover, enrichment for several transcription factors was identified, including regulation by androgen receptor (AR) (MEHHP) and estrogen receptor 1 (ESR1) (oxo-MINCH) at 19 weeks.

3.8. Comparison with previous studies

We compared our findings with those previously published in the EDEN and SEPAGES cohorts. In EDEN cohort, phthalates were measured in urine samples collected between 22w and 29w of pregnancy (Jedynak et al., 2022). SEPAGES conducted both whole and sex-stratified analyses with whole pregnancy phthalate and DINCH exposures, defined as the median of levels measured at 18w and 34w of gestation (Jedynak et al., 2024). None of our CpGs were among the list of FDR significant signals in any of the cohorts. FDR significant CpGs in EDEN and SEPAGES were not among suggestive CpGs in our results, regardless of the time period, the metabolites, or the main or sex-stratified analysis. A comparison of the FDR significant CpGs observed in EDEN and SEPAGES cohorts with our results is shown in Table S20. Most CpGs did not replicate, but some showed nominal associations in the same direction.

4. Discussion

Our study identified a number of associations between in utero exposure to phthalate and DINCH metabolites measured at 19 weeks, 35 weeks and across the entire pregnancy, and placental DNA methylation at birth. Most of the associations showed decreased DNA methylation with higher exposure levels. A substantial number of sex-specific associations were observed, which also differed across the exposure periods. Furthermore, mixture analyses of statistically suggestive CpGs revealed additional associations. The effect sizes were, in general small, and most of the associations showed decreased DNA methylation with higher exposure levels. Enrichment analysis revealed pathways important for placental function and fetal development.

4.1. Exposure window-specific effects

4.1.1. Week 19 of gestation

At 19 weeks, we identified 21 unique CpGs annotated to 11 genes, six of which (*TP53TG1*, *ERC2*, *MIPEP*, *RNPEP*, *CROT*, *FXD2*) have been previously reported as differentially expressed following phthalate exposure in animal models according to CTD (Alhasnani et al., 2022; Johnson et al., 2011; Kalo et al., 2019; Labaronne et al., 2017; Mathieu-Denoncourt et al., 2016). Among them, four of these loci are particularly relevant to placental metabolism. For instance, the gene *CROT*, which regulates fatty acid β -oxidation and supports placental energy supply (Calabuig-Navarro et al., 2017) showed reduced methylation at 12 promoter CpGs with higher MEP levels. These CpGs are also annotated to *TP53TG1*, a p53-inducible non-coding RNA that may protect the hypoxic early placenta through stress-response pathways (Zhang et al., 2022; Zhang et al., 2021). Moreover, *MIPEP* exhibited reduced gene body methylation with MEHP and MEOHP exposure. This gene

participates in oxidative phosphorylation, a critical energy-generating process in the placenta (Carter, 2000; Chew et al., 1997). In addition, *FXD2* showed lower 3'UTR methylation with MEHP; it encodes a sodium-potassium ATPase subunit, and such ion transporters are known to be essential for placental nutrient exchange (Lee et al., 2019). Lastly, *RNPEP* showed decreased gene body methylation with cx-MiNP. This gene encodes an arginyl aminopeptidase, and reduced *RNPEP* expression has been reported in placentas from cases of growth restriction, suggesting a potential link with impaired fetal growth and altered arginine availability (Chen et al., 2015).

Enrichment analyses identified immune-related pathways associated with MiBP exposure at 19 weeks, involving genes such as *AKT2*, *PPP3R1*, and *GNG12*. MiBP has previously been reported to be associated with inflammation during pregnancy (Ferguson et al., 2015; Han et al., 2025). In addition, genes associated with oh-MPHP (*SPRY1*, *HRAS*, and *ARHGAP26*) were enriched for FGF, EGF receptor, and PDGF pathways. These pathways are especially critical for trophoblast proliferation, survival, and migration, ensuring proper villi formation, invasion, and vascular remodeling during early and mid-gestation (Dawid et al., 2021; Holmgren et al., 1991; Liu et al., 2024; Forbes and Westwood, 2010). Several epidemiological and animal studies earlier reported vascular alterations in placenta following exposure to phthalates such as DEHP, DHP or DCHP (Labaronne et al., 2017; Mathieu-Denoncourt et al., 2016), although none have directly linked these effects to oh-MPHP.

We also investigated whether annotated genes were enriched for the regulation by certain transcription factors. CpGs associated with MEHHP were enriched in genes regulated by the androgen receptor (AR). Previous *in vitro* and *in silico* studies found no androgenic activity for DEHP, the parental compound of MEHHP, but reported conflicting results regarding its anti-androgenic potential (Beg and Sheikh, 2020; Engel et al., 2017; Kambia et al., 2019; Kim et al., 2019; Sarath Josh et al., 2016). Moreover, CpGs related to oxo-MINCH were enriched for genes regulated by the estrogen receptor 1 (ESR1). This finding is consistent with *in vitro* study, that reported ESR1 activation induced by DINCH metabolites, including oxo-MINCH (Engel et al., 2017). In contrast, another *in vitro* study found no ESR1 activity in response to DINCH metabolites; however, the use of lower metabolite concentrations in that study may explain the discrepancy (Wenzel et al., 2021).

4.1.2. Week 35 of gestation

At 35 weeks of gestation, six CpGs significantly associated with phthalate levels were annotated to five genes. Four of these genes (*NDST1*, *SLC36A4*, *ARMC8* and *RASSF1*) have previously been reported as differentially expressed in phthalate-exposed animal or cell models (Johnson et al., 2011; Kalo et al., 2019; Albert et al., 2018; Gaido et al., 2007). In our study, CpG within the gene body of *SLC36A4*, an amino acid transporter, presented higher methylation with higher MECPP, MEHHP, and MEOHP levels. In the placenta, both the functional capacity and protein abundance of these transporters are related to fetal growth in human and experimental studies (Kalo et al., 2019; Albert et al., 2018). In late pregnancy, proper transporter function becomes critical, as fetal growth increasingly depends on the placenta's capacity to supply amino acids and other nutrients (Bell and Ehrhardt, 2002). We also observed reduced methylation at the *RASSF1* promoter with higher MBzP levels. *RASSF1* regulates cell cycle and apoptosis and has been linked to intrauterine growth restriction and preeclampsia (Iglesias-Platas et al., 2015; Salvianti et al., 2015). In addition, CpGs in *ARMC8* (promoter) and *NDST1* (gene body) were differentially methylated in relation to MEHP. *ARMC8* belongs to the armadillo superfamily involved in cellular communication, while *NDST1* plays a role in heparan sulfate biosynthesis and inflammation (Gul et al., 2019; Vallet et al., 2023; van der Weyden and Adams, 2007). However, their roles in placental development have not been established.

The enrichment analyses at 35 weeks highlighted steroid biosynthesis (MECPP), among other pathways. Several studies have shown the impact of exposure to DEHP or MEHP on steroidogenesis in rat and

mouse placentas (Saadeldin et al., 2018; Xu et al., 2021; Zhang et al., 2020). The genes identified in the pathway were *CYP24A1*, that inactivates vitamin D levels and indirectly could affect other steroid hormones, and *FDFT1*, that participates in cholesterol biosynthesis, the precursor of steroid hormones (Dinour et al., 2013; Sidhu and Mishra, 2024). Furthermore, regulation by seven transcription factors was found, including YY1, which is known to be important for placentation and several placental functions (Wang et al., 2025).

4.1.3. Whole pregnancy

Six CpGs, annotated to 4 genes (*NDST1* also detected at 35 weeks), were associated with whole pregnancy phthalate exposure levels. Two of the other genes, *DDC* and *ST3GAL1*, have been reported in response to phthalates in animal or cell models (Johnson et al., 2011; Kalo et al., 2019; Albert et al., 2018; Gaido et al., 2007). Notably, we found an association between cx-MiNP and lower methylation at the *DDC* promoter. *DDC* encodes Dopa-decarboxylase, an enzyme expressed in placenta that catalyzes the conversion of L-DOPA to dopamine, a neurotransmitter crucial for fetal brain development (Bertoldi, 2014; Gratz et al., 2018; Rosenfeld, 2021). In utero phthalate exposure has been previously related to neurodevelopmental outcomes and behavior (Sprowles et al., 2022). We also identified lower DNA methylation at the *ST3GAL1* promoter with higher MnBP exposure. *ST3GAL1* participates in protein glycosylation, a process that, when deregulated, can lead to several pregnancy complications (Yung et al., iScience 2023; Zhong et al., 2024).

Enrichment analyses revealed genes associated with obesity, including *PPARGC1B*, *FTO*, and *AIP*, for both MECPP and MEHHP. Previous epidemiological studies have linked these chemicals with childhood and adult obesity (Wu et al., 2023). Additionally, enrichment for transcription factors with roles in placental progenitor maintenance, differentiation and function was found: *POU5F1* (MEHP), *TCF7L2* (oh-MINCH), and *TBX3* (oxo-MiNP). Experimental studies have shown both increased and decreased *POU5F1* during early embryogenesis in response to MEHP metabolite (Chu et al., 2013; Grossman et al., 2012).

4.2. Sex-specific effects

We identified a substantial number of differentially methylated CpGs associated with phthalate and DINCH metabolites in a sex-specific manner at 19 weeks, 35 weeks, and whole pregnancy. This goes in line with the expected sex-specific endocrine disruptor activity of the phthalate and DINCH metabolites (Ouidir et al., 2024), and with the sex-specific epigenetic pattern of the placenta (Martin et al., 2017; Tekola-Ayele et al., 2019; Tekola-Ayele et al., 2025).

Due to the small number of Bonferroni significant CpGs per chemical metabolite and sex, we did not conduct functional enrichment analyses; however, we have extensively annotated these sex-specific CpGs. Among the 49 female-specific CpGs, we highlight two loci related with placental vascularization: cg00752628 annotated to the *RSPO3* gene (associated with MiBP levels), and cg22505312, cg22505312 and cg23710890 annotated to the *CTSB* gene (associated with cx-MiNP), both at 19 weeks. *RSPO3* encodes an evolutionarily conserved angiogenic factor which participates in the formation of the labyrinthine layer of the placenta (Aoki et al., 2007; Kazanskaya et al., 2008), and *CTSB* encodes the cathepsin B enzyme, which participates in the degradation of the extracellular matrix during trophoblast invasion facilitating placental vascularization (Varanou et al., 2006). Altered levels of both genes have been found in preeclamptic placentas (Ueland et al., 2020; Tanrıverdi Kılıç et al., 2024). Moreover, overexpressed levels of *CTSB* have been shown in frog embryos exposed to DCHP, DMP or MMP (Mathieu-Denoncourt et al., 2016).

Among the 42 male-specific CpGs, we highlight two loci. The first locus involves four CpGs associated with oh-MPHP levels at 35 weeks and located at the *IGF1R* gene body. One of the CpGs (cg01224063) was located in a maternal gDMR described in the placenta (Hamada et al.,

2016). The insulin-like growth factors are involved in placental and fetal growth, carrying out various functions including trophoblast proliferation, migration, and invasion (Sferruzzi-Perri et al., 2017). Another interesting male-specific CpG was cg23939844 associated with oxo-MINCH at 19 weeks and annotated to *LRTM2* and *CACNA2D4* genes. *CACNA2D4* has been previously reported to be underexpressed in human embryonic stem cells in relation to exposure to DEHP (Fang et al., 2019).

4.3. Mixture analyses

The mixture analysis identified 20 Bonferroni significant associations among the overall suggestive CpGs investigated. Only two of these CpGs were also identified in single-metabolite analyses at the Bonferroni significance, therefore the mixture analyses provide additional insights into the effects of phthalate and DINCH metabolites. Eight of these additional genes (19 weeks: *JAG1* and *GABPB1*; 35 weeks: *HOXC9*, *RPH3AL*, *INTS3*, and *ITPKB*; whole pregnancy: *BBS4* and *PHF21B*) have previously been related to phthalate exposure in animal or cell studies (Tando et al., 2021; Lahousse et al., 2006; Jahreis et al., 2018; Moral et al., 2011; Alhasnani et al., 2022; Fang et al., 2019; Johnson et al., 2011; Gaido et al., 2007; Zhang et al., 2015; Chen et al., 2022). These genes are involved in diverse and important biological processes, including Notch signaling (*JAG1*), which regulates cell fate determination, differentiation and development (Hunkapiller et al., 2011; Zhao and Lin, 2012); mitochondrial metabolism (*GABPB1*) (Liu et al., 2019); pattern formation and cell differentiation during embryonic development (*HOXC9*) (Morgan, 2006); vesicle trafficking and exocytosis (*RPH3AL*) (Yuan et al., 2017); RNA processing and DNA repair (*INTS3*) (Skaar et al., 2009); calcium signaling (*ITPKB*) (Apicco et al., 2021); ciliary transport (*BBS4*) (Wingfield et al., 2018); and chromatin regulation (*PHF21B*) (Chin et al., 2022).

For the two overlapping CpGs between the mixture and the single-metabolite EWAS, the overall effect of the mixture was in the same direction as in the individual analysis for all significant CpG-metabolite pairs, but some of the partial effects within the mixture were in the opposite direction. For instance, at 35 weeks, MEHHP, MEOHP, MECPP were individually significantly associated with higher DNA methylation of cg07964716. However, in the mixture analysis MEHHP showed a negative effect on this CpG. This can be explained by the high correlation among these metabolites, which could lead to reversed partial effects in the mixture model.

Although mixture analyses better reflect the real-life exposure, they also have limitations, including limited generalizability (effects depend on the specific joint distribution of exposures in a given population), or potential issues with exposure scaling, as the method relies on quartiles, rather than actual concentrations. For all these reasons, mixture analyses in this study were considered secondary.

4.4. Differences across gestation

The differences observed between exposure windows, both in the main model and in the sex-stratified model, may have a number of explanations. One possibility is that exposure to phthalate and DINCH metabolites affects distinct genes and pathways at each stage of pregnancy, leaving permanent and specific DNA methylation marks that can be detected at birth. Under this hypothesis, early-mid exposure (19 weeks) appears to impact immune-related pathways and placental vascularization, whereas later exposure may primarily influence steroid hormone production (35 weeks) and fetal growth (whole pregnancy). Consistently, the transcription factors enriched at each window differed: 19 weeks exposures were associated with AR and ESR2, while other periods highlighted factors critical for placental development and function, albeit without a clear temporal pattern (ie. *POU5F1* is known to be important in early gestation and we identified it for the whole pregnancy exposure). Alternatively, the observed differences could

reflect incomplete overlap in mother–child pairs or variations in metabolite levels across time points, leading to differing lists of significant CpGs and pathways. In addition, the relatively low number of suggestive CpG sites may have limited the ability to detect broader biological pathways. Further research is required to clarify these temporal and exposure-dependent dynamics.

4.5. Comparison with previous studies

The lack of replication of the CpGs observed in EDEN and SEPAGES cohorts could have been resulted due various reasons. First, the exposure assessment was not equivalent in the three cohorts in term of exposure window (22–29 weeks in EDEN, whole pregnancy in SEPAGES and 19 and 35 weeks in BiSC), number of urine voids tested (1 in EDEN, and > 10 in the other two cohorts) and the laboratory performing the analyses (EDEN: National Center for Environmental Health; BiSC, SEPAGES: Norwegian Institute of Public Health). Second, different DNA methylation arrays were used: EDEN used the Infinium HumanMethylation450 BeadChip, BiSC and SEPAGES Infinium Methylation EPIC BeadChip. Additionally, differences in population characteristics (ie. ethnicity, socio-economic status, etc.), exposure levels, and statistical modelling may also have contributed to the discrepancies. In future studies, conducting meta-analyses across cohorts will be essential to increase statistical power and validate the observed associations.

Only a few studies have performed mixture analyses relating combined exposure to phthalates with cord blood DNA methylation (Khodasevich et al., 2024; Sol et al., 2022), but none have identified CpGs overlapping with those found in our study.

4.6. Strengths and limitations

Our study has several strengths. First, exposure assessment was based on the first and last voids of the day over six consecutive days (10–12 samples per urine pool) in two different gestational periods, providing a more robust and accurate measure compared to studies relying on a single urine void. Second, with a sample size of 469, this study modestly exceeds the size of previous research. Third, the robustness of our findings was supported by multiple sensitivity analyses, and potential causality was further explored through integration with evidence from experimental studies. Lastly, we conducted mixture analyses of suggestive CpGs offering deeper and more realistic insights into the impact of combined exposures on placental DNA methylation, although the limitations described above.

This study also faced some limitations. First, while we investigated different exposure windows, we could not fully disentangle time-specific effects due to incomplete overlap of participants across windows and differences in overall exposure levels. Second, although we used the EPIC array, which contains > 0.8 M CpG sites, we still did not cover all possible CpG sites across the genome, limiting our ability to detect all potential DNA methylation changes associated with the exposures and outcomes of interest. Third, analyses were based on bulk tissue, preventing assessment of cell type-specific effects and, potentially overlooking distinct roles of different placental cell types. Finally, despite adjusting for a wide range of relevant covariates, residual confounding cannot be completely ruled out, and causal inference requires complementary studies such as interventional studies or *in vitro* or *in vivo* models.

5. Conclusion

Our study suggests that in utero exposure to phthalate and DINCH metabolites is associated with differential placental DNA methylation. These CpGs are annotated to genes implicated in endocrine disruption, metabolism, and vascular and immune regulation. Moreover, sex-specific effects were observed, consistent with the known endocrine-disrupting properties of phthalate and DINCH metabolites. Further

studies are needed to confirm these findings, to investigate the differences across exposure windows, and to determine whether the placental epigenetic signatures linked to in utero exposure have implications for short- and long-term health effects.

6. Ethics

The BiSC study, from pregnancy up to the 18 month-visit, was approved by the Clinical Research Ethics Committee of the Parc de Salut Mar project (2018/8050/I), Medical Research Committee of the Fundació de Gestió Sanitària del Hospital de la Santa Creu i Sant Pau de Barcelona (EC/18/206/5272), and Ethics Committee of the Fundació Sant Joan de Déu (PIC-27-18). Before joining the cohort during their regular first trimester hospital visit, participants were informed by a BiSC midwife or nurse about the study's details, duration, and their option to withdraw without penalty. If they agreed to take part, they signed consent forms permitting the collection of biological samples and genetic studies, receiving a copy for themselves.

CRedit authorship contribution statement

Hana Vespalcova: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Formal analysis, Data curation, Conceptualization. **Bethany Knox:** Writing – review & editing, Data curation. **Amrit Kaur Sakhi:** Writing – review & editing, Resources, Methodology. **Cathrine Thomsen:** Writing – review & editing, Resources, Methodology. **Sofía Aguilar-Lacasaña:** Writing – review & editing, Data curation. **Marta Cosin-Tomas:** Writing – review & editing, Data curation. **Laura Gómez-Herrera:** Writing – review & editing, Data curation. **Olga Sánchez García:** Writing – review & editing, Resources. **Elisa Llurba:** Writing – review & editing, Resources. **María Dolores Gómez-Roig:** Writing – review & editing, Resources. **Jordi Sunyer:** Writing – review & editing, Resources, Funding acquisition. **Payam Dadvand:** Writing – review & editing, Resources, Funding acquisition. **Mariona Bustamante:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Martine Vrijheid:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

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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.envint.2025.109946>.

Data availability

Genome-wide DNA methylation summarized results are uploaded to Zenodo repository (DOI: 10.5281/zenodo.15746984). Individual level data may be available by contacting BiSC (<https://projectebisc.org/en/contact/>). Code can be shared upon request with the authors.

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