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Full length article



Epigenetic footprints: Investigating placental DNA methylation in the context of prenatal exposure to phenols and phthalates

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ABSTRACT

Background: Endocrine disrupting compounds (EDCs) such as phthalates and phenols can affect placental functioning and fetal health, potentially via epigenetic modifications. We investigated the associations between pregnancy exposure to synthetic phenols and phthalates estimated from repeated urine sampling and genome wide placental DNA methylation.

Methods: The study is based on 387 women with placental DNA methylation assessed with Infinium MethylationEPIC arrays and with 7 phenols, 13 phthalates, and two non-phthalate plasticizer metabolites measured in pools of urine samples collected twice during pregnancy. We conducted an exploratory analysis on individual CpGs (EWAS) and differentially methylated regions (DMRs) as well as a candidate analysis focusing on 20 previously identified CpGs. Sex-stratified analyses were also performed.

Results: In the exploratory analysis, when both sexes were studied together no association was observed in the EWAS. In the sex-stratified analysis, 114 individual CpGs (68 in males, 46 in females) were differentially methylated, encompassing 74 genes (36 for males and 38 for females). We additionally identified 28 DMRs in the entire cohort, 40 for females and 42 for males. Associations were mostly positive (for DMRs: 93% positive associations in the entire cohort, 60% in the sex-stratified analysis), with the exception of several associations for bisphenols and DINCH metabolites that were negative. Biomarkers associated with most DMRs were parabens, DEHP, and DiNP metabolite concentrations. Some DMRs encompassed imprinted genes including *APC* (associated with parabens and DiNP metabolites), *GNAS* (bisphenols), *ZIM2*/*PEG3*/*MIMT1* (parabens, monoethyl phthalate), and *SGCE*/*PEG10* (parabens, DINCH metabolites). Terms related to adiposity, lipid and glucose

Abbreviations: BMI, body mass index; cx-MiNP, mono-4-methyl-7-carboxy-octyl phthalate; DEHP, di-2-ethylhexyl phthalate; DINCH, 1,2-cyclohexane dicarboxylic acid di-isononyl ester; DiNP, di-isononyl phthalate; DMR, differentially methylated region; EDC, endocrine disrupting compound; EDEN, Etude des Déterminants pré et postnataux du développement et de la santé de l'Enfant; eQTM, expression Quantitative Trait Methylation; EWAS, epigenome-wide association study; FDR, false discovery rate; IQR, interquartile range; LMP, last menstrual period; LOD, limit of detection; LOQ, limit of quantification; MBzP, monobenzyl phthalate; MCNP, monocarboxy-iso-nonyl phthalate; MCOP, monocarboxy-iso-octyl phthalate; MCPP, mono-3-carboxypropyl phthalate; MECPP, mono-2-ethyl-5-carboxypentyl phthalate; MEHHP, mono-2-ethyl-5-hydroxyhexyl phthalate; MEHP, mono-2-ethylhexyl phthalate; MEOHP, mono-2-ethyl-5-oxohexyl phthalate; MEP, monoethyl phthalate; MiBP, mono-iso-butyl phthalate; MMCHP, mono-2-carboxymethylhexyl phthalate; MnBP, mono-n-butyl phthalate; nRBC, nucleated red blood cell; OH-MINCH, 2-(((hydroxy-4-methyloctyl)oxy)carbonyl) cyclohexanecarboxylic acid; OH-MiNP, 2-(((4-methyl-7-oxooctyl)oxy)carbonyl) cyclohexanecarboxylic acid; OH-MPHP, mono-6-hydroxy-propylheptyl phthalate; oxo-MINCH, 2-(((4-methyl-7-oxooctyl)oxy)carbonyl)cyclohexanecarboxylic acid; oxo-MiNP, mono-4-methyl-7-oxooctyl phthalate; QC, quality control; SLK, Stouffer-Liptak-Kechrer correction; Σ DEHP, molar sum of DEHP metabolites; Σ DINCH, molar sum of DINCH metabolites; Σ DiNP, molar sum of DiNP metabolites.

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metabolism, and cardiovascular function were among the enriched phenotypes associated with differentially methylated CpGs. The candidate analysis identified one CpG mapping to imprinted *LGALS8* gene, negatively associated with ethylparaben.

Conclusions: By combining improved exposure assessment and extensive placental epigenome coverage, we identified several novel genes associated with the exposure, possibly in a sex-specific manner.

1. Introduction

The prenatal environment plays a crucial role in the Developmental Origins of Health and Disease (Gillman, 2005). Endocrine disrupting compounds (EDCs) are ubiquitous in our environment and have the ability to disturb hormonal homeostasis (reviewed by De Falco et al., 2015; Tang et al., 2020). In particular, phthalates and synthetic phenols are known or suspected EDCs. Phthalates are plasticizers used in solvents, paints, varnishes, and as common additives in polyvinyl chloride plastics or personal care products (Latini, 2005). Synthetic phenols are used as antimicrobial and preserving agents in the cosmetic, drug, and food (parabens) industries, as well as for the production of polycarbonates and epoxy resins (bisphenols), and cosmetic ultraviolet filters (benzophenone-3 also called oxybenzone).

Both phthalate and phenol exposure has been associated with various adverse health outcomes in pregnant women and offspring, such as impaired neurodevelopment and increased risk of childhood asthma (Eales et al., 2022; Kahn et al., 2020). Some of these compounds have been detected in placental tissue (Mose et al., 2007b, 2007a; Song et al., 2020; Valle-Sistac et al., 2016; Vela-Soria et al., 2017) and could interfere with fetal development by disrupting crucial placental functions including epigenetic regulation (Seymore et al., 2022; Strakovsky and Schantz, 2018).

Alterations of the placental methylome have been associated with adverse effects on placental health (Bianco-Miotto et al., 2016; Robinson et al., 2019), which in turn may have long-lasting consequences for the fetal and child development (Ravaei et al., 2023). Pregnancy complications such as miscarriage, preterm birth, preeclampsia, intrauterine growth restriction (Marsit, 2015; Robinson et al., 2019), or high blood pressure (Broséus et al., 2022) have also been associated with disrupted placental DNA methylation. However, although the placenta is a key organ for fetal programming and represents a molecular memory of the prenatal environment (Maccani and Marsit, 2009; Robinson et al., 2019), the impact of phenols and phthalates exposure on its epigenome has not been thoroughly studied.

A few studies (Grindler et al., 2018; Jedynak et al., 2021, 2022; LaRocca et al., 2014; Nahar et al., 2015; Song et al., 2021; Zhao et al., 2015, 2016) have begun characterizing the effects of these chemicals on the human placental methylome, but they have been limited by small sample sizes and a single isolated urine spot to assess exposure. Both phenols and phthalates have short biological half-lives (<24 h), are quickly metabolized, and show a substantial within- and between-day variation of urinary levels (reviewed by Casas et al., 2018; Vernet et al., 2018), thus a single spot urine sample is unlikely to represent exposure over a long period such as pregnancy. While sexual dimorphisms have been observed in many developmental conditions, including the health effects of phenols and phthalates, very few studies have considered effects modification by child's sex (Jedynak et al., 2023; LaRocca et al., 2014; Song et al., 2021). We aimed to address these gaps by investigating the relationships of pregnancy exposure to phthalates, 1,2-cyclohexane dicarboxylic acid di-isononyl ester (DiNCH), and synthetic phenols assessed by repeated urine sampling with the placental methylome, screening over 850,000 methylation sites of male and female offspring. We further aimed at validating associations previously identified in two of our studies using the 450 K array and a single urine spot (Jedynak et al., 2021, 2022).

2. Methods

2.1. Study design and population

484 mother–child couples were enrolled in the French SEPAGES study between 2014 and 2017 in the area of Grenoble (Lyon-Caen et al., 2019). Inclusion criteria were: being pregnant (singleton pregnancy) by less than 19 gestational weeks at inclusion, at least 18 years of age, ability to read and speak French fluently, to be affiliated to the French national social security system, and to plan to deliver in one of the four maternity clinics of the area. Prior to inclusion, both parents completed an informed consent and the ethical agreements were obtained from the Comité de Protection des Personnes Sud-Est V and the Commission Nationale de l'Informatique et des Libertés, the French data privacy institution. 478 of the 484 research subjects had at least one pooled urine sample available (Suppl. Fig. 1). Of those, 396 had placental samples collected and 395 had placental DNA extracted.

2.2. Maternal urine collection and assessment of exposure biomarker concentrations

Both at the second and third trimesters of pregnancy (median 18 and 34 gestational weeks, respectively), the women were instructed to gather three urine samples per day (morning, midday, and evening, with no specific instructions regarding the food intake) for seven straight days and to keep them at -20°C in their personal freezer. After the collection week, samples were moved to the biobank where they were kept at -20°C until they were thawed overnight at 4°C . Then, for each subject, equal volumes of all samples provided during each collection week were combined (21 urines per pool, on average, Philippat and Calafat, 2021; Vernet et al., 2019). Aliquots of these within-individual, within-period pools were stored at -80°C .

Concentrations of 13 phthalate metabolites, two DINCH metabolites, and 12 phenols were measured from the urine pools using high-performance liquid chromatography coupled with mass spectrometry (phthalates and DINCH metabolites, Sabaredzovic et al., 2015) and ultra-performance liquid chromatography coupled with mass spectrometry (phenols, Sakhi et al., 2018). In both methods, procedural blanks and quality assurance samples (in-house quality controls and standard reference material [SRM 3673] from National Institute of Standards and Technology) were analyzed along with the samples. The accuracy ranged from 70% to 126%, and the precision given as relative standard deviation was below 26% for all the compounds. Out of the 12 phenols measured, triclosan has been investigated in a prior publication (Jedynak et al., 2023) and four (bisphenols AF, B, F, and triclocarban) were detected in less than 2% of the samples leaving seven phenols included in the further analyses (Suppl. Table 1). For phenol biomarkers, free and conjugated forms were first measured independently in samples from 50 women. These preliminary measurements did not show external contamination (Rolland et al., 2020), thus for the remaining participants we relied on the analysis of the total form (free plus conjugated). Detailed procedure for biomarkers assessment is provided in the Supplement.

2.3. Placental tissue collection and DNA extraction

Samples of about five mm^3 were collected at delivery from the fetal side of the placenta, a few centimeters from the cord insertion, by a

clinical staff, using a standardized operating procedure. The placental sample was put at 4°C in a fridge in the maternity clinics until transfer to the biobank where it was frozen at -80°C. DNA was extracted using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany). We assessed DNA concentrations in duplicate using the Quant-IT kit (ThermoFisher, Asnières-sur-Seine, France). Samples that produced inconsistent results were verified in a second round of measurements. On a subset of samples, the DNA integrity number was calculated by migrating a small amount of DNA on an Agilent TapeStation 4200 (Les Ulis, France). The efficiency of amplification was evaluated by the simultaneous amplification of two microsatellite markers, and the sex of individuals was verified using a PCR-based method. 0.5–1 µg of genomic DNA was bisulfite treated with the EpiTect® Fast 96 DNA Bisulfite Kit (Qiagen, Courtaboeuf, France) following the manufacturer's instructions. After the conversion, samples' volumes were adjusted based on the starting amount of DNA to normalize the sample concentrations.

2.4. Placental DNA methylation assessment and quality control

DNA methylation was assessed for >850,000 CpGs using the Infinium Human MethylationEPIC Kit (Illumina, San Diego, USA), according to the manufacturer's protocol. Sample layout was designed with the R/Bioconductor OSAT package (Yan et al., 2012) and randomized by child's sex, maternal tobacco smoking during pregnancy, and maternity clinic. To assess batch effects, 14 samples were duplicated across the microarrays between two and five times. We extracted raw methylation data using the R/Bioconductor ChAMP package v. 2.14 (Morris et al., 2014; Tian et al., 2017). All samples met the quality threshold of below 10% of failed probes detection p-value (>0.01, global mean of 0.11% of failed probes). We next filtered the probes that met the following conditions: detection p-value >0.01 (n = 38,192 probes removed); beadcount <3 in at least 5% of samples (n = 2,081); non-CpG probes (n = 2,745); multi-hit probes as described by Nordlund et al. (Nordlund et al., 2013) (n = 8,419); CpG sites at a distance ≤2 bp from SNPs with minor allele frequency <0.05. Probes on chromosomes X and Y (n = 29,987) were removed using the R package DMRcate (Peters et al., 2015). Finally, cross-hybridizing probes identified in previous studies (n = 31,917) were also filtered out using the R package maxprobes (<https://github.com/markgene/maxprobes>, McCartney et al., 2016; Pidsley et al., 2016), leaving 752,577 methylation sites for further analyses.

Methylation levels (i.e., beta values) were normalized using Beta Mixture Quantile normalization (Jedynak et al., 2023; Teschendorff et al., 2013) and are expressed as the ratio of intensities between fully methylated and unmethylated alleles. We applied the Tukey's fences method to diminish the effect of outliers, i.e., we removed the methylation beta values above the third quartile plus three interquartile ranges (IQRs) or below the first quartile minus three IQRs (in total, 0.4 % of the methylation values).

2.5. Placental cell heterogeneity estimation

We estimated methylation profiles of six reference placental cell types (endothelial, Hofbauer, nucleated red blood cells [nRBCs], stromal, syncytiotrophoblasts, and trophoblasts) using the *planet* R/Bioconductor package (Yuan et al., 2021). First, we applied the Robust Partial Correlations from the R/Bioconductor package *EpiDISH* (Teschendorff et al., 2017) to the methylation data and obtained reference-based estimates of cell composition. The estimates were then used as adjustment factors in the regression models. We considered zero estimates obtained for a given cell type as below the limit of detection (LOD) and imputed their values by *impCoda* function from the R package *robCompositions* relying on an iterative regression-based procedure after KNN-initialization (Hron et al., 2010; Templ et al., 2011). As for each subject the six estimated cell proportions sum up to one, to avoid singularity in the regression models we used five cell types only (all but

nRBCs).

3. Statistical analyses

3.1. Handling of biomarker concentrations of exposure to pregnancy EDCs

For two phenols (bisphenol S and butylparaben), more than 30% of the samples were below the LOD so their concentrations were categorized as follows: below LOD for the second and third trimester (reference category) versus above LOD for the second or third trimester. For others chemicals, we singly imputed concentrations that were below the LOD and between the LOD and LOQ using the *NADA* (Lee, 2020) and *msm* (Jackson, 2011) R packages (Helsel, 1990; Lubin et al., 2004). The method consists of random drawing of the values below the LOD and between LOD and LOQ from the estimated distribution of the given compound. We calculated molar sums for di(2-ethylhexyl) phthalate (DEHP), 1,2-cyclohexane dicarboxylic acid di-isononyl ester (DINCH), and di-isononyl phthalate (DiNP) metabolites (Suppl. Table 1).

To account for between-subject variations related to differences in urine sampling conditions (duration of the sample transport from participant's home to the biobank, sample thawing time at 4°C during the pooling procedure) and analytical batch, when needed, we standardized exposure biomarker concentrations. Briefly, we estimated the associations between the conditions mentioned above and each exposure biomarker concentration using adjusted linear regression. If sampling conditions/analytical batch were associated with an exposure biomarker (p-value <0.2), we then used the measured concentrations and the estimated effects of the sampling conditions/analytical batch to predict standardized concentrations (i.e., concentrations that would have been observed if all samples had been collected under the same conditions and assayed in the same analytical batch, Guilbert et al., 2021; Mortamais et al., 2012; Nakiwala et al., 2022). We used these standardized concentrations in our statistical analyses.

To decrease the influence of outliers, continuous standardized biomarker concentrations were log₂-transformed. For each individual, we used the average of the standardized log₂-transformed concentrations assessed from the two weekly pools as a proxy of the pregnancy exposure. For women who had exposure biomarker concentration unavailable for one of the urine collection time points (n = 2 for time point one, n = 6 for time point two), the only available concentration was used instead of the average.

3.2. Adjustment factors

We chose the confounders *a priori*, given that they may affect both maternal exposure to phenols and/or phthalates and placental DNA methylation levels. These data were collected by questionnaires during pregnancy and included: parity (nulliparous; one or more children); maternal pre-pregnancy body mass index ([BMI], underweight; normal weight; overweight and obesity); maternal active cigarette smoking before and/or during pregnancy (did not smoke; smoked before and/or during pregnancy), maternal education level (below three years after high school; three-four years after high school; above four years after high school); maternal age (continuous); gestational age (continuous); season of conception (January-March; April-June; July-September; October-December); child's sex (except for sex-stratified analyses, female; male). We additionally adjusted the analyses for estimated placental cell proportions and for technical factors affecting DNA methylation measurements (batch, plate, and chip). Missing values for maternal smoking status (n = 28), education level (n = 2), and pre-pregnancy BMI (n = 4), were imputed by the mode.

3.3. Associations between prenatal exposure and placental DNA methylation

Associations of pregnancy exposure to EDCs and placental DNA methylation was estimated by an epigenome-wide association study (EWAS) followed by an analysis of differentially methylated regions (DMRs) (Fig. 1). For each CpG and each exposure biomarker we fitted robust linear regressions (from the MASS R package, Venables and Ripley, 2002), with DNA methylation levels as the dependent variable and the standardized log₂-transformed exposure averaged over two collection time points (or dichotomized value for bisphenol S and butylparaben) and confounders as independent variables. A false discovery rate (FDR, Benjamini and Hochberg, 1995) corrected p-value less than 0.05 was considered significant. Potential non-linearity of the associations between exposure and DNA methylation of individual probes was tested by modelling each exposure concentration as a restricted cubic spline with three knots followed by the FDR-adjusted Wald test to assess significance of the non-linear association. The EWAS in the entire cohort identified a very high number (18,151) of differentially methylated CpGs associated with maternal concentrations of some biomarkers of exposure (Suppl. Table 2). Most of these associations were detected for exposures modelled as restricted cubic splines and were non-linear. To test the robustness of these results, we performed an additional analysis removing 2% of the extreme (1% lowest and 1% highest) concentration values (n = 8 per continuous biomarker exposure). After this exclusion, none of over 18,000 associations identified in the entire cohort was retained in the only slightly smaller subsample (Suppl. Table 2). We therefore focused the subsequent analyses on the subsample excluding the highest and lowest exposure values that seemed to strongly influence the association results. The final sample size used in all of our analyses was therefore 387. Nominal p-values obtained in the EWAS without extreme exposure values were then used to identify DMRs.

DMR analysis was done with the *comb-p* Python module (Pedersen et al., 2012) allowing accounting for spatial correlations between p-values by the use of sliding windows and Stouffer-Liptak-Kechris correction (Kechris et al., 2010). This algorithm calculates p-values of the regions accounting for multiple testing by applying the Šidák correction (Šidák, 1967). To initiate a DMR, we set a threshold for the p-value of 0.001 and defined a maximum distance of 500 bp between the CpGs. To limit the number of spurious associations we focused on the

most reliable associations i.e., on DMRs containing five or more CpGs.

We further performed a candidate approach to analyze 20 CpGs previously identified in male placentas as associated with pregnancy phenol and phthalate concentrations measured in a single urine spot (Jedynak et al., 2021,2022). We considered a CpG as replicated if the nominal p-value for the association was below 0.05. It was not possible to account for the sign of the effect estimate since the previous studies mainly reported non-linear associations (Jedynak et al., 2021,2022). We also attempted to replicate the DMRs identified in our previous studies. For this analysis, we relied on the results of the EWAS performed for the 337,722 CpG sites overlapping with the CpGs present on the previously used BeadChip (Jedynak et al., 2021,2022). DMRs were identified as described above, except that we used a less restrictive p-value threshold of 0.05 to start a region (instead of 0.001). A region was considered replicated if it encompassed any of the 49 previously identified differentially methylated genes (Jedynak et al., 2021,2022).

To estimate the joint effect of all chemicals on placental DNA methylation in the whole and sex-stratified population, we used the quantile-based g-computation method from *qgcomp* R package (Keil et al., 2020) allowing for estimation of the effect on an outcome by simultaneously increasing all chemicals within the mixture by a quartile. DNA methylation beta values were transformed to M-values to approach normality prior the computations and all models were adjusted for confounders listed above. The effect of the mixture was first tested for the individual CpGs (a mixture effect was considered significant for the FDR-corrected p-values <0.05) and, subsequently, the nominal p-values yielded in that analysis were used to screen for DMRs with a threshold for the p-value of 0.001 and a maximum distance of 500 bp between the CpGs to initiate a region.

3.4. Biological interpretation

We derived gene annotations from the Illumina's hg19 reference genome database (*IlluminaHumanMethylationEPICanno.ilm10b4.hg19* R/Bioconductor package, Hansen, 2017) combining the information with annotations present in the University of California, Santa Cruz database (<https://genome.ucsc.edu>). Further information on genes of interest was retrieved from the GeneCards Human Gene Database (Stelzer et al., 2016).

We identified imprinted genes by screening the list compiled from the MetaImprint (Wei et al., 2014, <https://openebench.bsc>).

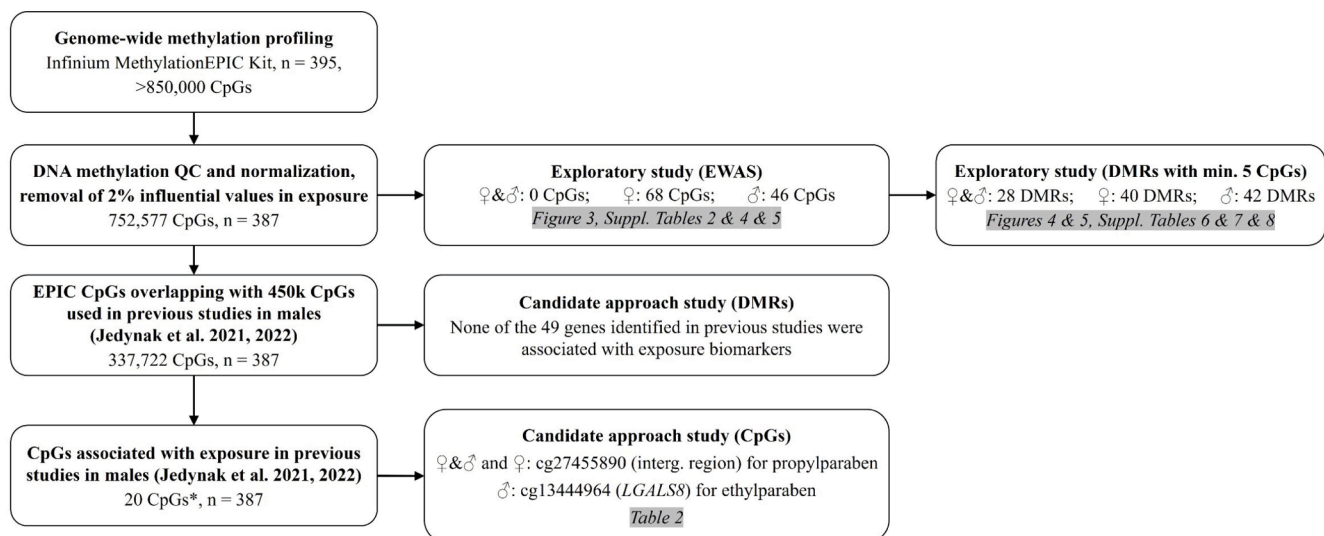


Fig. 1. Workflow of the study. Abbreviations: DMRs = differentially methylated regions. EWAS = epigenome-wide association study. QC = quality control. *Out of 24 CpGs previously identified as differentially methylated in association with phenol and phthalate biomarkers concentrations, four methylation sites were not present in the current study due to filtering at the methylation data pre-processing stage or because they were not covered by the current assay.

es/tool/metaimprint), GeneImprint (Jirtle, 1999, <https://www.geneimprint.com>), and igc.otago (Morison et al., 2005, <https://igc.otago.ac.nz>) databases, as well as among the imprinted loci defined by Yuen et al. (2010) and Hamada et al. (2016). From the initial list of 304 imprinted genes, 295 were covered by the Infinium MethylationEPIC Kit used in the current study.

To assess whether methylation levels of identified differentially methylated CpGs (individual and those included in the DMRs) were associated with the expression levels of the nearby genes, we looked them up in the placental expression Quantitative Trait Methylation (eQTM) lists available from Delahaye et al. (2018) and Deyssenroth et al. (2020). eQTM are transcript-CpG specific associations based on gene expression and DNA methylation of each CpG on a genome-wide level that improve further interpretations of epigenetic modifications considering their functional implications. Additionally, we investigated the potential biological functions of the differentially methylated CpGs by querying for enriched health outcomes and phenotypes within the database of Genotypes and Phenotypes (dbGaP, Tryka et al., 2014) among CpGs identified in the EWAS as differentially methylated (nominal p-values <0.001), using the R package *enrichR* (Xie et al., 2021). Only dbGaP terms that were linked to at least 20 genes and with FDR <0.01 were considered significantly enriched.

3.5. Research data and code

Data used in this study are confidential and can only be provided upon a reasonable request to the corresponding authors. The analyses were conducted using R v. 4.2.2 (R Core Team and R Foundation for Statistical Computing 2020), RStudio v. 2022.12.0 (RStudio Team, 2020) and Python v. 3.7.4. (van Rossum and Drake, 2009). The code is available in the public repository of the Team of Environmental Epidemiology applied to Development and Respiratory Health (<https://gricad-gitlab.univ-grenoble-alpes.fr/iab-env-epi>).

4. Results

4.1. Study population characteristics and exposure biomarker concentrations

The mothers were on average 32.2 years old at enrolment and median gestational duration was 40.0 weeks (Table 1). The mother-child pairs included in the study had a slightly longer gestational age at delivery compared to those not included (median = 40.0 and 39.7 weeks, respectively). The distribution of the other covariates did not differ between included and not included pairs. The median gestational age at urine sampling was 17.7 (25th centile: 16.4; 75th centile: 18.9) and 34.0 (32.1; 35.1) weeks for the first and the second urine collection week, respectively. Apart from two phenols (bisphenol S and butylparaben) whose concentrations were detected in less than 30% of samples, propylparaben had the lowest frequency of detection (78.4% and 77.4% for trimester two and three, respectively) (Suppl. Table 3). For the remaining chemicals, detection rates were close to 100%. The biomarker concentrations are presented in Fig. 2 and Suppl. Table 3.

4.2. Associations between individual pregnancy urinary biomarker concentrations and placental DNA methylation

4.2.1. Differentially methylated CpGs associated with biomarker concentrations in the subsample

When both sexes were studied together, no association was observed. For females, among the associations observed in the entire cohort, after exclusion of the 2% of extreme biomarker concentration values, associations were preserved mainly for two DEHP metabolites (mono-2-ethyl-5-carboxypentyl phthalate [MECPP]: n = 29 CpGs, mono-2-ethylhexyl phthalate [MEHP]: n = 20) (Suppl. Table 2). For males, 12 and 10 associations were preserved with ethyl- and methylparaben,

Table 1

Population characteristics for the mother-child pairs included (n = 395) and excluded (n = 84) from the study.

Characteristic	Mother-child pairs included in the study (n = 395)	Mother-child pairs excluded from the study (n = 84)
	n (%)	n (%)
Season of conception		
January-March	99 (25.1 %)	25 (29.8 %)
April-June	81 (20.5 %)	19 (22.6 %)
July-September	95 (24.1 %)	16 (19.0 %)
October-December	120 (30.4 %)	24 (28.6 %)
Maternal active tobacco smoking before and/or during pregnancy		
Did not smoke	320 (81.0 %)	63 (75.0 %)
Smoked before and/or during pregnancy	47 (11.9 %)	8 (9.5 %)
Missing	28 (7.1 %)	13 (15.5 %)
Parity		
Nulliparous	178 (45.1 %)	40 (47.6 %)
Multiparous	217 (54.9 %)	44 (52.4 %)
Maternal level of education (years after high school)		
<3	69 (17.5 %)	14 (16.7 %)
3-4	100 (25.3 %)	26 (31.0 %)
>4	224 (56.7 %)	44 (52.4 %)
Missing	2 (0.5 %)	0 (0 %)
Maternal pre-pregnancy BMI (kg/m²)^a		
Underweight (<18.5)	26 (6.6 %)	3 (3.6 %)
Normal weight (≥18.5 – <25)	296 (74.9 %)	65 (77.4 %)
Overweight and obesity (≥25)	69 (17.5 %)	16 (19.0 %)
Missing	4 (1.0 %)	0 (0 %)
Child's sex		
Male	210 (53.2 %)	47 (56.0 %)
Female	185 (46.8 %)	37 (44.0 %)
Maternal age (years)		
Median [25th; 75th centile]	32.2 [29.8; 35.1]	32.1 [30.0; 35.5]
Gestational age (weeks)^b		
Median [25th; 75th centile]	40.0 [39.1; 40.7]	39.7 [37.8; 40.6]*

Before the analyses, missing values for maternal education level, pre-pregnancy BMI, and smoking status were replaced by the mode.

^aCategorized according to the World Health Organization definitions.

^bBased on the date of the LMP or gestational duration assessed by the obstetrician if it differed from the LMP-based estimate by more than 2 weeks.

*Wilcoxon signed-rank test p-value <0.05. Other characteristics did not significantly differ between included and excluded mother-child pairs (chi squared or Wilcoxon signed-rank test p-value >0.05).

Abbreviations: BMI = body mass index. LMP = last menstrual period.

respectively. In total, 114 CpGs were differentially methylated in association with urinary biomarker concentrations in males (68 CpGs) and females (46 CpGs), encompassing 74 genes (38 for females and 36 for males, Suppl. Tables 2 and 4). None of the identified CpGs overlapped between the two sexes. Among the 114 CpGs, 27 were non-linearly associated probes, detected mostly for phthalates (FDR-corrected p-value <0.05, Suppl. Table 4). For 93% of the linear associations, DNA methylation levels increased with higher biomarker concentration.

In the enrichment analysis on the whole population, we observed the effect of exposure to bisphenols A and S, parabens (methyl- and ethylparaben), and, to a lesser extent, benzophenone-3 and DEHP metabolites

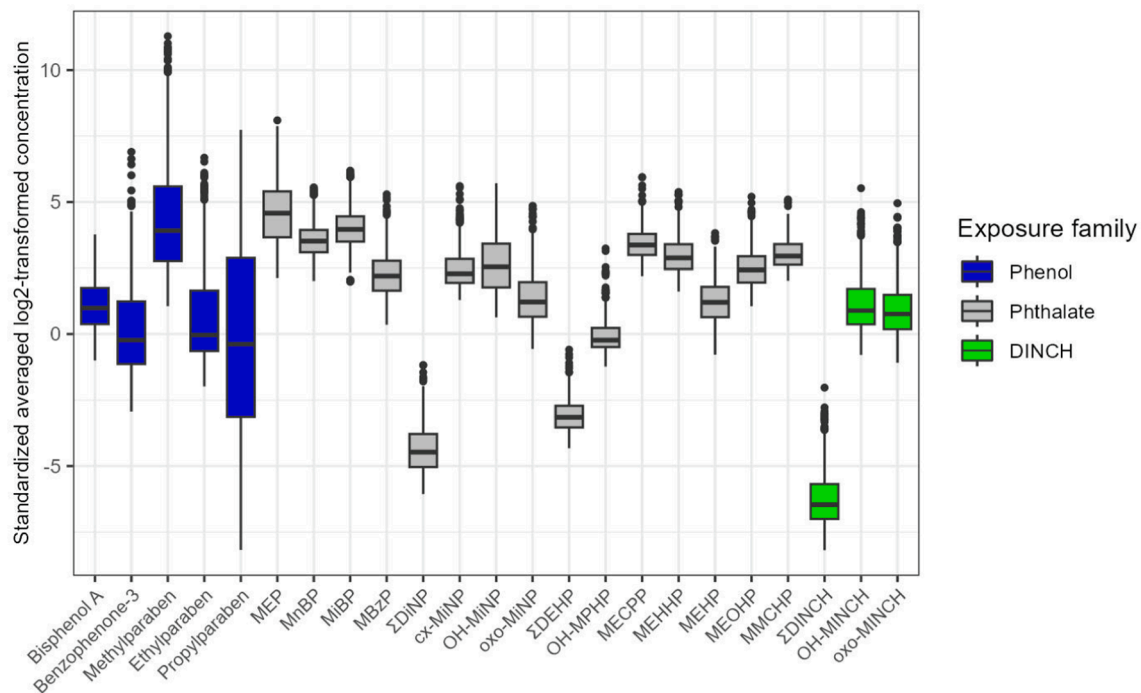


Fig. 2. Maternal urinary concentrations of synthetic phenols, phthalates, and DINCH metabolites (concentrations standardized for analytical batch and sampling conditions and log₂-transformed), restricted to the reduced population ($n = 387$ pregnant women), where 2% of the extreme continuous exposure biomarker values ($n = 8$ for each biomarker) were removed. Due to detection rate $<30\%$, bisphenol S and butylparaben biomarker concentrations were categorized and therefore their concentrations are not displayed. Exposure biomarker concentrations were, when needed, standardized to account for the effects of urine processing and assay (sample transport time from participant's home to the biobank, individual samples thawing time at 4°C during the pooling procedure, and analytical batches). Concentrations of exposure biomarkers are expressed as $\mu\text{g/L}$ while concentrations of molar sum of DEHP, DINCH, and DiNP metabolites as $\mu\text{mol/L}$. *Abbreviations:* cx-MiNP = mono-4-methyl-7-carboxyoctyl phthalate. DEHP = di(2-ethylhexyl) phthalate. DINCH = 1,2-cyclohexane dicarboxylic acid di-isononyl ester. DiNP = di-isononyl phthalate. MBzP = monobenzyl phthalate. MECPP = mono(2-ethyl-5-carboxypentyl) phthalate. MEHHP = mono(2-ethyl-5-hydroxyhexyl) phthalate. MEHP = mono(2-ethylhexyl) phthalate. MEOHP = mono(2-ethyl-5-oxohexyl) phthalate. MEP = monoethyl phthalate. MiBP = mono-*iso*-butyl phthalate. MMCHP = mono-2-carboxymethylhexyl phthalate. MnBP = mono-*n*-butyl phthalate. OH-MINCH = 2-(((hydroxy-4-methyloctyl)oxy)carbonyl) cyclohexanecarboxylic acid. OH-MiNP = mono-4-methyl-7-hydroxyoctyl phthalate. OH-MPHP = mono-6-hydroxy-propylheptyl phthalate. oxo-MINCH = 2-(((4-methyl-7-oxooctyl)oxy)carbonyl) cyclohexanecarboxylic acid. oxo-MiNP = mono-4-methyl-7-oxooctyl phthalate. ΣDEHP = molar sum of DEHP metabolites (MECPP, MEHHP, MEHP, MEOHP, MMCHP). ΣDINCH = molar sum of DINCH metabolites (OH-MINCH, oxo-MINCH). ΣDiNP = molar sum of DiNP metabolites (cx-MiNP, OH-MiNP, oxo-MiNP).

(MEHP and mono-2-carboxymethylhexyl phthalate [MMCHP]) mainly on adiposity, lipid and glucose metabolism, and cardiovascular function as well as blood cell traits (bisphenol S, benzophenone-3, methyl- and ethylparaben, MEHP) (Fig. 3, Suppl. Table 5). In a sex-stratified analysis, the enrichment was more pronounced in females compared to males and the most involved phenotypes were adiposity, lipid and glucose metabolism, and cardiovascular function (Fig. 3, Suppl. Table 5) in association with the exposure to phthalates (monoethyl phthalate [MEP], mono-*iso*-butyl phthalate [MiBP], mono-6-hydroxy-propylheptyl phthalate [OH-MPHP], DEHP metabolites), DiNP metabolites, and, to a lower extent, to phenols (butylparaben). For males, the enrichment on the phenotypes was mostly observed for benzophenone-3, methylparaben, mono-4-methyl-7-carboxyoctyl phthalate [cx-MiNP], and MECPP and, to a lower extent, for bisphenol A, MiBP, and OH-MPHP.

None of the CpGs identified in the EWAS appeared on the eQTM lists associating methylation levels with the expression of the nearby genes.

4.2.2. DMRs associated with biomarkers concentrations in the subsample

When both sexes were analyzed together, the regional analysis identified 28 DMRs with at least five probes as associated with pregnancy exposure biomarker concentrations (Šidák-corrected p -value <0.05 , Fig. 4, Suppl. Table 6), including 188 CpGs and encompassing 23 genes (Fig. 4, Suppl. Table 7). Most DMRs were associated with DEHP (individual metabolites and molar sum) concentrations ($n = 14$ DMRs), DiNP metabolites, and parabens ($n = 5$ DMRs for each sub-family of exposure) (Figs. 4 and 5, Suppl. Tables 6 and 7). For most DMRs ($n = 26$), DNA methylation levels increased with higher exposure, except for

bisphenol A and propylparaben (Fig. 5).

For the sex-stratified analysis, we observed 82 significant DMRs (40 for females and 42 for males, containing 603 CpGs and encompassing 28 and 32 genes, respectively) (Figs. 4 and 5, Suppl. Tables 6 and 7). Similarly to the results obtained in the entire cohort, for most DMRs (60 % of the DMRs [$n = 49$]) DNA methylation levels increased with higher exposure, except for some parabens and DiNP metabolites in males and females, bisphenol A in males, and bisphenol S and DINCH metabolites in females, which were negatively associated with DNA methylation in most DMRs.

Interestingly, 12 CpGs constituting the identified DMRs are reported placental eQTMs that downregulate expression of four genes: *BIN2* and *C11orf9* (associated with MEHP metabolite concentrations in females), *MKRN3* (propylparaben in males), DINCH metabolites and their sum in females), and *TSTD1* (benzophenone-3 in both sexes) (Suppl. Table 8).

Overall, for the phenols, while the observed associations were mostly negative for the bisphenols, there was no clear pattern in the direction of the associations for the parabens. Of interest, the same genomic locus (*C1orf86*; *LOC100128003*) was identified as positively associated with two different parabens (ethyl- and propylparaben). We also detected one imprinted locus (*APC*) as negatively associated with propylparaben concentrations and we found two positive associations between ethyl- and methylparaben concentrations and methylation of the imprinted loci *PEG10* and *ZIM2;PEG3;MIMT1*, respectively. The latter genomic location was also identified as positively associated with MEP concentrations, but only in male placenta. For bisphenol S, a positive association with a DMR encompassing an imprinted gene *GNAS* and *GNASAS*

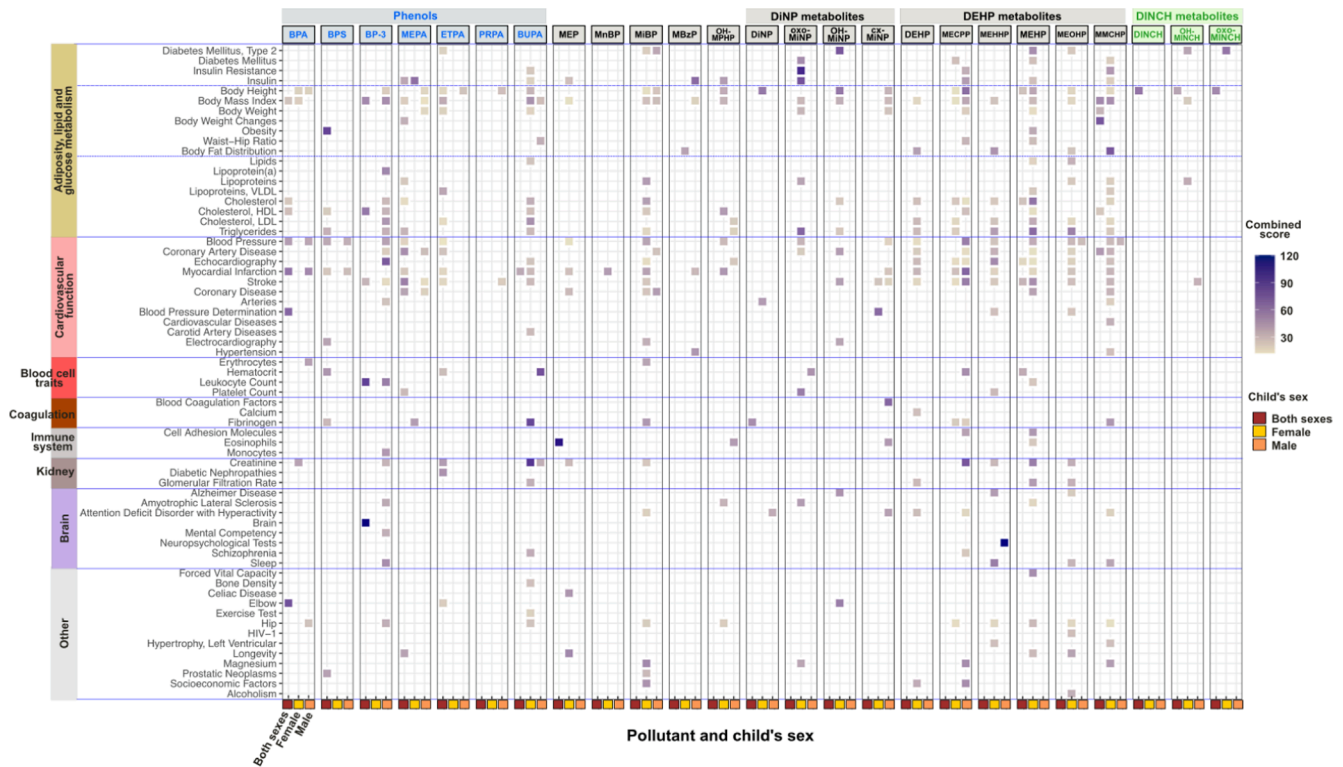


Fig. 3. Heat map summarizing the enrichment analysis on differentially methylated CpGs associated with pregnancy exposure in the exploratory study (p-value <0.001), when both sexes were studied together and separately (n = 387, 752,577 CpGs). Regression models were performed in the reduced cohort after removal of 2% extreme concentration values for each continuous exposure biomarker (thus except for bisphenol S and butylparaben) and adjusted for maternal active smoking before and/or during pregnancy, maternal age, gestational age at delivery, parity, maternal education level, maternal pre-pregnancy BMI, season of conception, batch, plate, chip, and estimated placental cell-type proportions. Squares represent identified phenotype terms and colors represent the combined score of enrichment. Phenotype terms were retrieved from the database of Genotypes and Phenotypes (<https://www.ncbi.nlm.nih.gov/gap>). Abbreviations: BMI = body mass index.

(or *GNAS-AS1*) was identified for males.

Overall, for the phthalates, the observed associations were mostly positive for the metabolites of DEHP and DiNP, while negative associations were detected for DINCH and only in females. A few isolated associations were identified for the other phthalate metabolites. Of interest, the same genomic loci (*ARSB*, *NIPAL4*, *SCAND3*, and *TCL1A*) were identified as associated with at least two different DEHP metabolites. Two loci (*NOX4* and the imprinted *SGCE;PEG10* locus) were demethylated in association with pregnancy concentrations of all three DINCH metabolites. While the vast majority of the associations detected for the DiNP sub-family were positive, three negatively methylated DMRs were associated with the imprinted *APC* gene. Four genomic locations (*APC*, *CYP1A1*, *H2AFY2*, and *UCP1*) were differentially methylated in association with concentrations of more than one DiNP metabolite.

Six DMRs were common to different biomarker families: *APC* (propylparaben and DiNP metabolites), *FOXP2* (bisphenol A, MiBP), *RNF39* (bisphenol A, DiNP metabolites), *SGCE;PEG10* (ethylparaben and DINCH metabolites), *UBE2U* (methylparaben and DiNP metabolites), *ZIM2;PEG3;MIMT1* (ethylparaben and MEP).

4.2.3. Differentially methylated CpGs and DMRs from the candidate approach relying on hits from Jedynak et al., 2021, 2022

When both sexes were studied together and for females only, we identified a propylparaben-associated increase in DNA methylation of one CpG mapping to an intergenic region (Table 2). For males, we identified one CpG negatively associated with ethylparaben concentrations and mapping to the *LGALS8* imprinted gene. The CpG was non-linearly associated with ethylparaben in the previous study used to select our candidate CpGs. In the candidate DMR study carried on a

restricted subset of 337,722 candidate CpGs we did not identify any regions, either when females and males were studied together or separately. None of the identified CpGs appeared on the eQTM lists associating methylation levels with the expression of the nearby genes.

4.3. Associations between the mixture of phenol, phthalate, and DINCH metabolite concentrations and placental DNA methylation

We did not identify individual CpGs or DMRs associated with the mixture of chemicals (FDR-corrected p-values for the overall mixture effect >0.05), independently if both sexes were considered simultaneously or in sex-stratified analyses.

5. Discussion

In the presented study, we estimated maternal concentrations of short half-life chemicals using multiple urine sampling, which provides higher precision and less measurement error compared to studies with similar sample size using a single urine spot samples. Additionally, screened over 850,000 methylation sites in the placenta, providing the largest methylome coverage up to date. The large majority of detected associations were linear and positive, which is consistent with what we observed in the French EDEN cohort (Jedynak et al., 2021,2022). The few exceptions relate to parabens and DiNP metabolites concentrations in both males and females, bisphenol A in males, and bisphenols S and DINCH metabolites in females, which we found mostly negatively associated with DNA methylation. None of the identified CpGs or DMRs overlapped between males and females, suggesting effect modification by child's sex. Given our limited sample size, these results should be interpreted cautiously. Most DMRs were related to DEHP, DiNP and

Gene	Phenols						DINP metabolites				DEHP metabolites				DINCH metabolites									
	BPA	BPS	BP-3	MEPA	ETPA	PRPA	BUFA	MEP	MiBP	OH-MPHP	ΣDINP	cx-MiNP	OH-MiNP	oxo-MiNP	ΣDEHP	MECPP	MEHHP	MEHP	MEOHP	MMCHP	ΣDINCH	OH-MINCH	oxo-MINCH	
ACSS3																								
ADAM3																								
ADD3																								
AKAP2, PALM2, AKAP2																								
ALOX12																								
ANKRD90B																								
APC																								
AR5B																								
AR5B																								
BIN2																								
C11orf9, DKFZP444K03																								
C2, CFB																								
CDC144N1																								
CLEC9A																								
CO45Y1																								
CYP11A1																								
FAM134B																								
FGF12																								
FLOT1																								
FOXJ1, TTC9																								
FOXP2																								
G452																								
GNAS45, GN45																								
GNGL1																								
GPR51																								
HJAFY2																								
HAND2, NBLA00301																								
HC92																								
HRS																								
KCNIP2																								
KCNJ13																								
KCNJ8																								
KSRI																								
LOC101927560, LOC1019																								
27587, MIR5-484F																								
MARVELD3																								
MKRN3																								
NCAM2																								
NG2																								
NIPAL4																								
NKAP1																								
NOX3																								
NPR3																								
OVRI																								
PCK3																								
PENK																								
PPAP2C																								
RG53																								
RIMS1																								
RNF39																								
ROBO4																								
SCAND3																								
SEPT4																								
SGCE, PEG10																								
SLIT3																								
SP9																								
SYNPR																								
TAP1, PSMB8																								
TBC1D10																								
TCF4																								
TCL1A																								
TRIL																								
TS1D1																								
UBE2U																								
UBXN10, UBXN10-AS1																								
UCP1																								
VWASB2, MIR1234																								
ZFP90																								
ZIM2, PEG3, MIMT1																								

Fig. 4. DMRs (≥ 5 probes) associated with pregnancy exposure (Šidák-corrected p-value < 0.05 , $n = 387, 752, 577$ CpGs) in females (yellow), males (orange), and when both sexes were studied together (red). Regression models were performed on the reduced sample set after removal of 2% extreme concentration values for each continuous exposure biomarker (thus except for bisphenol S and butylparaben) and adjusted for maternal active smoking before and/or during pregnancy, maternal age, gestational age at delivery, parity, maternal education level, maternal pre-pregnancy BMI, season of conception, batch, plate, chip, and estimated placental cell-type proportions. Imprinted genes are in bold. *Abbreviations:* BMI = body mass index. cx-MiNP = mono-4-methyl-7-carboxyoctyl phthalate. DEHP = di(2-ethylhexyl) phthalate. DINCH = 1,2-cyclohexane dicarboxylic acid di-isononyl ester. DiNP = di-isononyl phthalate. DMR = differentially methylated region. MECPP = mono(2-ethyl-5-carboxypentyl) phthalate. MEHHP = mono(2-ethyl-5-hydroxyhexyl) phthalate. MEHP = mono(2-ethylhexyl) phthalate. MEOHP = mono(2-ethyl-5-oxohexyl) phthalate. MEP = monoethyl phthalate. MiBP = mono-*iso*-butyl phthalate. MMCHP = mono-2-carboxymethylhexyl phthalate. OH-MINCH = 2-(((hydroxy-4-methyloctyl)oxy)carbonyl) cyclohexanecarboxylic acid. OH-MiNP = mono-4-methyl-7-hydroxyoctyl phthalate. OH-MPHP = mono-6-hydroxypropylheptyl phthalate. oxo-MINCH = 2-(((4-methyl-7-oxooctyl)oxy)carbonyl) cyclohexanecarboxylic acid. oxo-MiNP = mono-4-methyl-7-oxooctyl phthalate. ΣDEHP = molar sum of DEHP metabolites (MECPP, MEHHP, MEHP, MEOHP). ΣDINCH = molar sum of DINCH metabolites (OH-MINCH, oxo-MINCH). ΣDiNP = molar sum of DiNP metabolites (cx-MiNP, OH-MiNP, oxo-MiNP). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

DINCH metabolites, and parabens, and very few DMRs were common to the different biomarker families. We identified a number of exposure-associated differentially methylated CpGs and DMRs encompassing imprinted genes (*APC*, *GNAS45;GNAS*, *MKRN3*, *PPAP2C*, *SGCE;PEG10*, *ZIM2;PEG3;MIMT1*) whose epigenetic modifications could be of biological importance due to the major effects of these genes on fetal development and placental biology. Interestingly, some differentially methylated CpGs are reported placental eQTM that regulate expression of four genes, including the imprinted *MKRN3*. Moreover, one gene potentially involved in phthalate metabolism (*CYP11A1*) was also identified. In the enrichment analysis, we identified associations between several phenols, phthalate and DiNP metabolite concentrations and phenotypes related to adiposity, lipid and glucose metabolism, cardiovascular function, and blood cell traits. Finally, we did not observe associations between the chemical mixture and placental DNA methylation.

5.1. Parabens

Out of a number of differentially methylated loci identified as associated with maternal paraben concentration in our study, of particular interest in the context of placental functioning are the imprinted loci. Among them, we observed a decreased *APC* methylation in the entire cohort associated with propylparaben, an increased *SGCE;PEG10* methylation in males associated with methylparaben, and increased *ZIM2;PEG3;MIMT1* methylation in males and the entire cohort associated with increased ethylparaben. Moreover, one DMRs positively associated with propylparaben concentrations in males contains eQTM CpGs that downregulate the expression levels of *MKRN3* imprinted gene.

APC is a tumor suppressor protein acting as an antagonist of the Wnt signaling pathway which influences embryonic development, stem cell maintenance, and tissue differentiation during placentation (reviewed by Sonderegger et al., 2010). *APC* also plays a role in cell migration and adhesion, transcriptional activation, or apoptosis (O’Leary et al., 2016). *In vitro* studies have demonstrated that increased methylation of *APC*

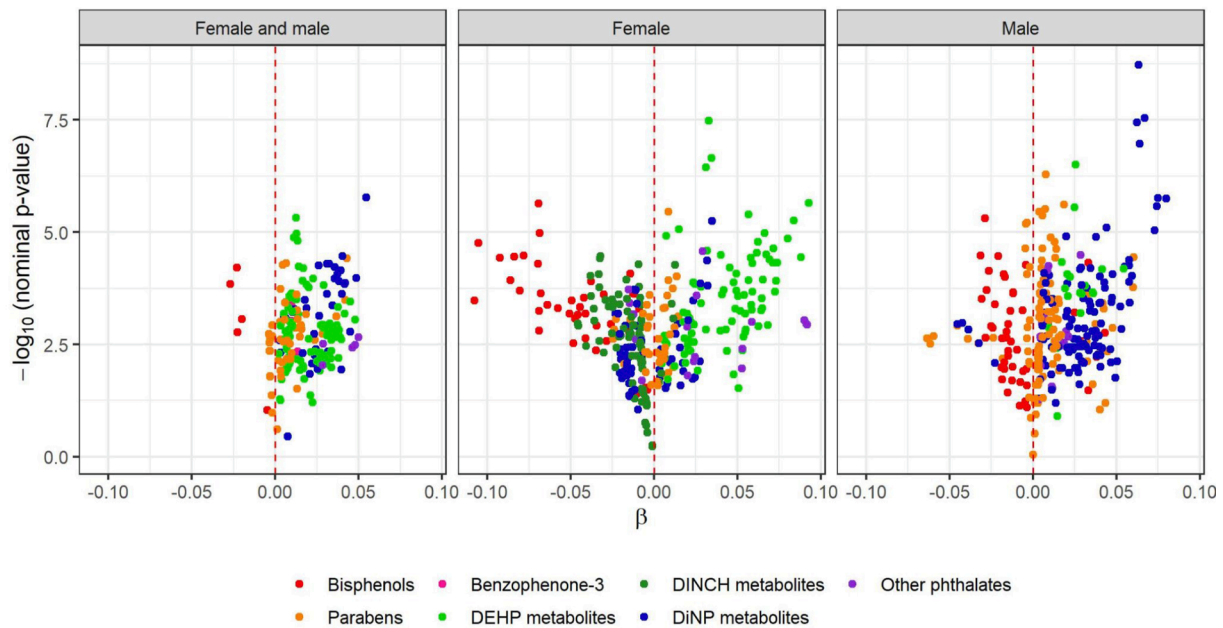


Fig. 5. Volcano plot for CpGs within DMRs (≥ 5 probes) associated with pregnancy exposure in the exploratory study ($\text{Šidák-corrected } p\text{-value} < 0.05$, $n = 387$, 752,577 CpGs), when both sexes were studied together and separately. Regression models were performed in the reduced cohort after removal of 2% extreme concentration values for each continuous exposure biomarker (thus except for bisphenol S and butylparaben) and adjusted for maternal active smoking before and/or during pregnancy, maternal age, gestational age at delivery, parity, maternal education level, maternal pre-pregnancy BMI, season of conception, batch, plate, chip, and estimated placental cell-type proportions. *Abbreviations:* BMI = body mass index. DEHP = di(2-ethylhexyl) phthalate. DINCH = 1,2-cyclohexane dicarboxylic acid di-isononyl ester. DiNP = di-isononyl phthalate. DMR = differentially methylated region.

may be related to trophoblast cancer cell progression (Novakovic et al., 2008; Wong et al., 2008). A previous study, relying on spot urine sample and a smaller sample size ($n = 202$), did not identify association between maternal urinary concentrations of parabens and DNA methylation of placental *APC* (Jedynak et al., 2021).

Methylparaben was associated with increased levels of DNA methylation of *SGCE; PEG10* in males and the DMR was among the most populated identified in the study ($n = 19$ CpGs). *SGCE; PEG10* play a role in growth-promoting activities and placental formation during the first trimester of pregnancy (Chen et al., 2015; Ono et al., 2006). Previous studies on the EDEN cohort showed increased DNA methylation of placental *SGCE; PEG10* in males as associated with elevated maternal concentrations of triclosan but not parabens ($n = 202$, Jedynak et al., 2021) and as associated with smoking during pregnancy in the entire cohort ($n = 668$, Rousseaux et al., 2020), suggesting that DNA methylation at this genomic locus may be sensitive to environmental exposure. Studies on cord blood showed contrasting results with increased *SGCE; PEG10* methylation related to low birth weight ($n = 90$, Lim et al., 2012) and impaired anthropometric scores at 1 ($n = 288$) and 3 years ($n = 294$) in males (Gonzalez-Nahm et al., 2018) and higher birth weight in other studies ($n = 508$ infants of both sexes, Liu et al., 2012; $n = 496$ infants of both sexes, Hoyo et al., 2014).

In males and the entire cohort, we observed increased methylation in *PEG3* imprinted domain (*ZIM2; PEG3; MIMT1*) associated with elevated maternal ethylparaben concentrations. This locus was not associated with any phenol considered in our previous study. *PEG3* may play a role in cell proliferation and p53-mediated apoptosis (Stelzer et al., 2016) and its increased DNA methylation in cord blood has been linked to higher placental weight in humans (Haggarty et al., 2013).

Only one study investigated the associations between pregnancy concentrations of parabens (butyl-, methyl-, and propylparaben) and placental DNA methylation levels. This study investigated only two imprinted genes (*H19* and *IGF2*, $n = 179$, LaRocca et al., 2014) and reported a decrease in DNA methylation of *IGF2* DMR2 in males in association with elevated levels of maternal butyl- and methylparaben concentrations. In our analysis, we did not find any studied paraben to

be associated with this gene, neither at individual CpGs nor at the DMR level.

5.2. Candidate CpG study

In our candidate approach, among the 20 candidate CpGs (Jedynak et al., 2021,2022) we identified only one (mapping to imprinted *LGALS8*) negatively associated with ethylparaben exposure in males. The low replication rate could be due to lower concentrations of exposure biomarkers in the SEPAGES cohort compared to the EDEN cohort (Philippat et al., 2021; Rolland et al., 2020) and the use of different omic platforms (HumanMethylation450k vs. MethylationEPIC array) which could affect the replication rate. *LGALS8* is a maternally imprinted gene (Metsalu et al., 2014) expressed, apart from tumor tissues, in villous and extravillous trophoblasts (Kolundžić et al., 2011). The encoded protein is a cytosolic lectin playing a role in detecting bacterial invasion (Stelzer et al., 2016) thus important in controlling infections and inflammation while modulating the maternal immune system during pregnancy, enabling fetal survival and development. Nevertheless, there is no direct evidence for ethylparaben to be involved in the immune system disruption (reviewed by Popescu et al., 2021).

5.3. Bisphenols

Three studies have investigated the role of bisphenols (especially bisphenol A) in DNA methylation modifications in placenta (Jedynak et al., 2021; LaRocca et al., 2014; Song et al., 2021), with either candidate or genome-wide approaches, among which two did not find any significant associations (Jedynak et al., 2021; LaRocca et al., 2014), while the third showed prenatal BPA exposure to be associated with a higher methylation level of *HLA-DRB6* pseudogene in both genome-wide ($n = 6$) and candidate gene ($n = 146$) analyses (Song et al., 2021).

A few studies also investigated methylation in cord blood and five reported association for bisphenol A measure either in urine (Huang et al., 2021; Junge et al., 2018; Kundakovic et al., 2015; McCabe et al., 2020) or cord blood (Miura et al., 2018), while one did not report any

Table 2
Candidate CpGs selected from the study on males from the French EDEN cohort (2003–2006) (Jedynak et al., 2021,2022) and differentially methylated in the current study (nominal p-value <0.05) in association with pregnancy exposure biomarker concentrations (n = 387).

Exposure	Child's sex	CpG	Chr	Position	Gene ^a	Location of CpG	Location in gene	Current study			Jedynak et al., 2021,2022		
								Mean meth.	SD meth.	β (CI)	Nominal p-value	β (CI)	Nominal p-value
Propylparaben	Female and male	cg27455890	10	118976677	Intergenic region	Island	No group	0.046	0.030	0.0009 (0.0003;0.0016)	0.004	Non-linear	2.0E-07
Propylparaben	Female	cg27455890	10	118976677	Intergenic region	Island	No group	0.046	0.030	0.0018 (0.0006;0.0031)	0.003	Non-linear	3.3E-08
Ethylparaben	Male	cg13444964	1	236687053	IGALS8;LGALS8;	North Shore	5'UTR;	0.050	0.021	-0.0017 (-0.0033;-0.0001)	0.034	Non-linear	3.3E-08
					IGALS8;LGALS8		TSS200;5'UTR;5'UTR						

Regression models were performed on the reduced population after removal of 2% extreme concentration values for each continuous biomarker of exposure (thus except for bisphenol S and butylparaben) and adjusted for maternal active smoking before and/or during pregnancy, maternal age, gestational age at delivery, parity, maternal education level, maternal pre-pregnancy BMI, season of conception, batch, plate, chip, and estimated placental cell-type proportions.

^a According to the University of California, Santa Cruz Genome Browser (<https://genome.ucsc.edu>).

Abbreviations: BMI = body mass index. Chr = chromosome. CI = confidence interval. TSS = transcription start site. UTR = untranslated region.

association (Montrose et al., 2018).

In our study, we did not detect differential DNA methylation of any of the genes previously identified in cord blood or placenta (Song et al., 2021) as associated with bisphenol A exposure. Nevertheless, we found five DMRs associated with bisphenol A and four with bisphenol S, a newer bisphenol not investigated in previous studies. One DMR encompassed imprinted *GNAS*; *GNAS* locus being positively associated with pregnancy bisphenol S concentrations in males. One of the several products of *GNAS* is the stimulatory alpha subunit of the G protein, a signaling protein whose function involves regulating production of hormones by endocrine glands (Steer, 1975). As for the epigenetic regulation of placental *GNAS*, genome-wide studies have shown an increase in its methylation levels associated with being large for gestational age (n = 12, Shen et al., 2020) as well as associated with pregnancy exposure to another synthetic phenol triclosan (n = 202, Jedynak et al., 2021), and with high total phthalate exposure (sum of concentrations of 23 phthalate metabolites) in first trimester placentas of women undergoing elective pregnancy terminations (n = 16, Grindler et al., 2018).

5.4. DEHP metabolites

Three studies investigated the link between pregnancy DEHP metabolite concentrations (individually or summed) and DNA methylation in placenta. Two candidate studies showed negative associations between mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP), mono-2-ethyl-5-oxohexyl phthalate (MEOHP), and ΣDEHP and methylation of *IGF2* (n = 179, LaRocca et al., 2014; n = 181 fetal growth restricted infants, Zhao et al., 2016), while in a genome-wide study in the EDEN cohort we have showed that MECPP, MEHP, and MEOHP concentrations were almost exclusively positively associated with DNA methylation of several placental genes, including transcription and nucleotide exchange factors (n = 202, Jedynak et al., 2022). As for cord blood, several studies (either targeted or genome-wide, Chen et al., 2018; Miura et al., 2021; Montrose et al., 2018; Petroff et al., 2022; Solomon et al., 2017; Tindula et al., 2018) reported associations of DEHP metabolite concentrations (individual or summed) with imprinted genes, or enrichment in genes implied in various pathways including metabolism, growth, endocrine regulation, including one study reporting sex-specific effects (Petroff et al., 2022). None of these genes or pathways was identified in our study. We identified several other genes differentially methylated in association with individual DEHP metabolites or their molar sum, including *TCL1A* positively associated with MEHP and MECPP biomarker levels (two DMRs). The exact function of this gene is unknown, but it has been previously linked with DNA methylation in cord blood as associated with lung function (increased methylation) and asthma (decreased methylation) in late childhood (den Dekker et al., 2019). Finally, two DMRs positively associated with the MEHP metabolite concentrations contain eQTM CpGs that downregulate the expression levels of *BIN2* and *C11orf9* (or *MYRF*) (Delahaye et al., 2018). *BIN2* encodes a protein enabling phospholipid binding activity and *C11orf9* encodes a transcription factor that is required for central nervous system myelination and may regulate oligodendrocyte differentiation, but none of these genes has been previously described in the context of MEHP exposure and placental DNA methylation.

5.5. DINCH metabolites

To our knowledge, this study is the first one investigating associations between DINCH, a DEHP substitute, and placental DNA methylation. An epigenome-wide study investigated cord blood DNA methylation (Petroff et al., 2022) and reported interaction of ΣDINCH with child's sex regarding methylation of the long non-coding RNA gene *LOC101926942* and *HES2*. In our study, we found five genes with differential methylation levels, only in females, associated with DINCH metabolite concentrations, including one demethylated imprinted locus

that was associated with 2-(((hydroxy-4-methyloctyl)oxy)carbonyl)cyclohexanecarboxylic acid (OH-MINCH), 2-(((4-methyl-7-oxooctyl)oxy)carbonyl)cyclohexanecarboxylic acid (oxo-MINCH), and Σ DINCH – the previously described *SGCE*/*PEG10*. Within placenta, *SGCE*/*PEG10* was also found being differentially methylated in association with methylparaben (this study) and triclosan concentrations (Jedynak et al., 2021), but not with phthalate concentrations (Jedynak et al., 2022).

5.6. DiNP metabolites

For DiNP metabolites, most of the associations for DMRs were observed in male placentas. *CYP1A1* methylation increased in association with mono-4-methyl-7-oxooctyl phthalate (oxo-MiNP) and Σ DiNP concentrations in males. *CYP1A1* encodes a member of the cytochrome P450 superfamily of enzymes, which may be involved in phthalate metabolism. *APC* methylation decreased in female placentas in association with maternal levels of 2-(((4-methyl-7-oxooctyl)oxy)carbonyl)cyclohexanecarboxylic acid (OH-MiNP) and oxo-MiNP. We also identified increased DNA methylation of *RNF39* in association with cx-MiNP in females. *RNF39* encodes a protein playing an important role in an early phase of synaptic plasticity (Stelzer et al., 2016). While no study has previously reported associations for DiNP, methylation changes on *RNF39* in placenta have been reported as negatively associated with the concentration of a phthalate mixture that included 20 phthalate metabolites but not DiNP (Grindler et al., 2018). In cord blood, *RNF39* methylation was negatively associated with MEHP concentrations (Solomon et al., 2017). While the sign of the associations was not always consistent across studies, these results may point to the increased sensitivity of the *RNF39* locus to pregnancy exposure to phthalates. Finally, we identified one more hypermethylated gene, *FOXA1*, whose cord blood DNA methylation has been previously related to pregnancy MEHP, but not DiNP metabolite concentrations (Miura et al., 2021). *FOXA1* is a transcription factor crucial for embryonic development as it plays a role in proliferation and invasion of trophoblast cells (Wang et al., 2014). Moreover, *FOXA1* has been found to be significantly enriched in placental villi from patients with recurrent pregnancy loss in the hypomethylated region near *PRDM1*, a gene that undergoes dysregulation in this pathological condition, potentially leading to an increased migration and apoptosis in trophoblast cells (n = 6, Du et al., 2020).

5.7. Enriched health outcomes and phenotypes

We identified phenotypes enrichment for several phenols (all but propylparaben), phthalate (MiBP, OH-MPHP), DiNP (cx-MiNP) and DEHP (MEHP, MMCHP) metabolite concentrations. They were predominantly related to adiposity, lipid and glucose metabolism, blood pressure, and more generally cardiovascular function. The enrichment was more pronounced in women carrying female fetuses, especially for phthalate and DiNP metabolites. Both phthalates and phenols are suspected to promote metabolic changes and influence the risk of offspring obesity and cardiovascular diseases later in life (Egusquiza and Blumberg, 2020; Haverinen et al., 2021). This may be due to their adverse effect on activation of peroxisome proliferator-activated receptor γ coupled with the generation of reactive oxygen species (e.g., Aboul Ezz et al., 2015; Hao, 2013; Hu et al., 2013; Saura et al., 2014; Shin et al., 2020).

6. Sexually dimorphic placental response associated with prenatal exposure

The lack of overlap between differentially methylated CpGs between sexes could be explained by a lack of statistical power to detect an effect, if any. It could also result from a sex-specific placental response to the exposure. The previous studies investigating sex-specific effects of phenols and phthalates on placental DNA methylation reported interactions

with sex for methylation of candidate genes for several phenols and phthalate metabolites (n = 179, LaRocca et al., 2014; n = 146, Song et al., 2021). Sexually dimorphic placental responses to a variety of maternal factors have been also observed (e.g., Alexander et al., 2018; Loke et al., 2018). While the underlying mechanisms of such sex-specific effects are so far not well understood, they might be linked to the fact that phenols and phthalates may interact with the endocrine system, which is sex-specific itself. Moreover, during its development, the placenta undergoes sex-specific changes and its DNA methylation patterns depend on the infant's sex (e.g., Andrews et al., 2022; Bozack et al., 2022). Altogether, placental/fetal sex is an important factor that should be considered when studying the associations between exposure to environmental chemicals and epigenetic placental outcomes (Strakovsky and Schantz, 2018).

7. Strengths and limitations

The presented study is one of the very few studies relying on a genome-wide analysis investigating the association between placental DNA methylation and short half-life chemicals measured in pooled repeated urine samples. These multiple samples (two pools of 21 samples for each woman, on average), when compared to the most commonly used spot sampling, should improve the precision of exposure assessment, especially for compounds with high and moderate intra-individual variability such as bisphenols, parabens, or DEHP metabolites. Moreover, our study is one of the very few existing epigenome-wide studies on placenta (Grindler et al., 2018; Jedynak et al., 2021, 2022; Song et al., 2021) that additionally relies on the broad epigenome coverage by Infinium MethylationEPIC BeadChip that covers more than 850,000 CpGs. This might partly explain the low rate of overlap between exposure-associated differentially methylated loci and regions identified by us and by other studies. Besides, many previous studies relied on a smaller sample size, with lower statistical power to detect associations, or focused on a few candidate loci and therefore were unable to investigate differentially methylated regions.

This study has nonetheless several limitations. Although larger than all previous studies, our sample size is still modest regarding the number of compounds and DNA methylation sites studied, especially for the sex-stratified analyses. Taken together with the relatively low exposure levels we observed compared to previous studies (Jedynak et al., 2021, 2022), these could result in a low power, especially for the compounds with low detection rate that were dichotomized (bisphenol S, butylparaben). It should be noted though that our study is the first one to report on bisphenol S and placental DNA methylation.

As we averaged the concentrations of exposure biomarkers assessed over the two collection time points, we decreased the measurement error if the window of sensitivity is the entire pregnancy (Perrier et al., 2016). This allowed reducing the number of comparisons performed, but prevented us from exploring specific periods of epigenetic sensitivity to environmental exposure. For false discovery, we only accounted for the number of CpGs tested for each metabolite, but we did not correct for the number of tested compounds, which might have led to spurious associations.

However, we focused our interpretation on regional changes in DNA methylation, which are more robust and more biologically meaningful than isolated differentially methylated probes (Svendsen et al., 2016). An additional factor complicating replication of the results is the tissue specificity of methylation patterns, which is especially important for complex multilayer organs like placenta. Residual confounding by factors that we did not account for (maternal behaviors such as diet) cannot be ruled out.

8. Conclusion

In conclusion, based on a robust analysis framework, our results suggest that short half-life phenols and phthalates could alter placental

methylation marks, including some imprinted genes, possibly in a sex-specific manner. Further research is needed to replicate these findings and investigate whether these DNA methylation changes persist into childhood and what is their role in child development.

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10. Role of the funder/sponsor

The funding sources had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review or approval of the manuscript; decision to submit the manuscript for publication.

CRedit authorship contribution statement

Paulina Jedynak: Conceptualization, Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. **Valérie Siroux:** Funding acquisition, Investigation, Supervision, Writing – review & editing. **Lucile Broséus:** Formal analysis, Methodology, Software, Writing – review & editing. **Jörg Tost:** Data curation, Formal analysis, Resources, Writing – review & editing. **Florencia Busato:** Data curation, Formal analysis, Writing – review & editing. **Stephan Gabet:** Methodology, Writing – review & editing. **Cathrine Thomsen:** Resources, Writing – review & editing. **Amrit K. Sakhi:** Resources, Writing – review & editing. **Azemira Sabaredzovic:** Resources, Writing – review & editing. **Sarah Lyon-Caen:** Project administration, Writing – review & editing. **Sam Bayat:** Resources, Writing – review & editing. **Rémy Slama:** Resources, Funding acquisition, Methodology, Writing – review & editing. **Claire Philippat:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Writing – review & editing. **Johanna Lepeule:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Software,

Supervision, Writing – review & editing.

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 material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envint.2024.108763>.

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