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## Prenatal exposure to triclosan assessed in multiple urine samples and placental DNA methylation<sup>☆</sup>

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### ABSTRACT

A previous study reported positive associations of maternal urinary concentrations of triclosan, a synthetic phenol with widespread exposure in the general population, with placental DNA methylation of male fetuses. Given the high number of comparisons performed in -omic research, further studies were needed to validate and extend on these findings. Using a cohort of male and female fetuses with repeated maternal urine samples to assess exposure, we studied the associations between triclosan and placental DNA methylation. We assessed triclosan concentrations in two pools of 21 urine samples collected among 395 women from the SEPAGES cohort. We used Infinium Methylation EPIC arrays to measure DNA methylation in placental biopsies collected at delivery. We performed a candidate study restricted to a set of candidate CpGs ( $n = 500$ ) identified in a previous work as well as an exploratory epigenome-wide association study to investigate the associations between triclosan and differentially methylated probes and regions. Analyses were conducted on the whole population and stratified by child's sex. Mediation analysis was performed to test whether heterogeneity of placental tissue may mediate the observed associations. In the candidate approach, we confirmed 18 triclosan-associated genes when both sexes were considered. After stratification for child's sex, triclosan was associated with 72 genes in females and three in males. Most of the associations were positive and several CpGs mapped to imprinted genes: *FBRSL1*, *KCNQ1*, *RHOBTB3*, and *SMO1*. A mediation effect by placental tissue heterogeneity was identified for most of the observed associations. In the exploratory analysis, we identified a few isolated associations in the sex-stratified analysis. In line with a previous study on male placentas, our approach revealed several positive associations between triclosan exposure and placental DNA methylation. Several identified loci mapped to imprinted genes.

### 1. Introduction

Triclosan is a synthetic phenol showing biocidal features that is used in healthcare and many consumer products including personal care products, household cleaning products, plastics, toys, certain textiles, and paints. Exposure to triclosan is widespread in the general population (Haug et al., 2018; Rolland et al., 2020), which is of concern due to its

endocrine disrupting properties (Weatherly and Gosse, 2017). Triclosan can cross the placental barrier (Bai et al., 2020) and has been detected in mammalian placental tissue (Feng et al., 2016). It has been associated with adverse effects on placenta, a key organ for fetal development. *In vitro*, triclosan decreased cell viability and affected hormone secretion in placental cells derived from choriocarcinoma (Honkisz et al., 2012) as well as induced apoptosis in human syncytiotrophoblasts (Zhang et al.,

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2015). It also altered trophoblast viability, migration, and angiogenesis as well as led to increased expression of *H19* and secretion of IGF2 (Li et al., 2022; Ma et al., 2021). In mice, triclosan exposure (8 mg/kg) decreased placental weight and volume, impeded activity and expression of placental glucose and amino-acid transporters (Cao et al., 2017), and increased placental thrombosis and fetal death (Wang et al., 2015). Moreover, pregnancy exposure to triclosan in rats (100 and 300 mg/kg) caused upregulation of expression levels of placental steroid metabolism enzymes (transferases and reductases) as well as of genes encoding progesterone, estrogen, and androgen receptors (Feng et al., 2016). Despite this evidence from *in vitro* and rodent studies, few epidemiological studies have investigated effects of triclosan on the human placenta. Two of them, focusing on placental weight at birth as a marker of its function and health, reported a negative association with triclosan concentrations during pregnancy. One reported this effect in males ( $n = 473$  males, Philippat et al., 2019) while the other identified effects in females only with no effect observed in males ( $n = 42$ , Ferguson et al., 2018). Two other studies ( $n = 202$  males, Jedynak et al., 2021;  $n = 179$ , LaRocca et al., 2014) investigated the associations with placental DNA methylation, a potential mechanism through which *in utero* exposure to environmental chemicals can affect health of the fetus and child later in life (Alvarado-Cruz et al., 2018; Vlahos et al., 2019). A candidate study focusing on two imprinted genes *H19* and the insulin-like growth factor 2 (*IGF2*), selected due to their major role in fetal and placental growth, did not report any associations with triclosan exposure (LaRocca et al., 2014). The other one analyzing about 340,000 CpGs in male placentas showed significant associations (mostly an increase in placental DNA methylation), both at the level of individual CpGs and differentially methylated regions (DMRs) (Jedynak et al., 2021). Limitations of these studies included the low number of considered CpGs (LaRocca et al., 2014), the limited sample size, and the reliance on a spot urine sampling which, for short half-life chemicals such as triclosan, likely lead to exposure misclassification, attenuation bias as well as, for a given sample size, reduced statistical power.

The mechanisms by which triclosan could influence placental DNA methylation are uncertain, with one of the hypotheses suggesting it may be through endocrine disruption. Triclosan, like other phenolic compounds such as parabens (Nowak et al., 2018; Pollock et al., 2017) and bisphenols (Matthews et al., 2001; Routledge et al., 2000), has estrogenic properties enabling it to interact with estrogen receptors (Lee et al., 2014; Yoon and Kwack, 2021). Disruption of estrogen signaling may in turn lead to change in CpG methylation (Romagnolo et al., 2014). Existing studies on associations between maternal phenols other than triclosan and DNA methylation in term placenta showed parabens-associated decrease in methylation of the *IGF2* DMR2 in males (LaRocca et al., 2014) or changes of the DNA methylation pattern of several genes associated with bisphenol A ( $n = 6$  and 146, Song et al., 2021) as well as with 2,4-dichlorophenol, benzophenone-3, methyl-, and propylparaben (Jedynak et al., 2021).

The aim of the present study was to explore the associations between triclosan exposure during pregnancy and placental DNA methylation in a population with repeated urine samples to assess pregnancy exposure and with higher sample size and larger epigenome coverage (>750,000 CpGs) compared to previous studies.

## 2. Methods

### 2.1. Study design and population

The present study included mother-child pairs recruited between 2014 and 2017 in the Grenoble area to participate in the French cohort SEPAGES (Lyon-Caen et al., 2019). All parents of the expected child signed an informed consent form for themselves and their child, prior to inclusion. Ethical approvals were obtained from the *Comité de Protection des Personnes Sud-Est V* (13-CHUG-44, ID RCB: 2013-A01491-44) and the *Commission Nationale de l'Informatique et des Libertés* (914, 138).

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. Out of the 484 SEPAGES study participants, 478 had at least one pooled urine sample available for exposure assessment. Of those, 396 had placental samples collected and placental DNA was extracted for 395 subjects (Supplementary Fig. 1).

### 2.2. Maternal urine collection and assessment of triclosan concentrations

At two time points during pregnancy (median 18 and 34 gestational weeks, respectively) the women were asked to collect three urine samples per day (morning, midday, evening) for seven consecutive days (Supplementary Fig. 2) and store them at  $-20^{\circ}\text{C}$  in their personal freezer. At the end of the follow-up week, samples were transferred to a certified biobank and stored at  $-20^{\circ}\text{C}$  until they were thawed at  $4^{\circ}\text{C}$  and equal volumes of all samples collected during each collection week for each participant were pooled (21 urine samples per pool, on average (Philippat and Calafat, 2021; Vernet et al., 2019)). Aliquots of the pools were stored at  $-80^{\circ}\text{C}$  before being sent on dry ice to the Norwegian Institute of Public Health for triclosan measurement.

For 50 samples, we assessed conjugated and total (free plus conjugated) urinary triclosan concentrations to check for external contamination. The results did not reveal contamination (Rolland et al., 2020) and therefore we assessed the total form for the entire cohort using ultra-performance liquid chromatography coupled to mass spectrometry (UPLC-MS/MS) (Sakhi et al., 2018). Briefly, 200  $\mu\text{L}$  of urine sample was mixed with internal standards and enzyme solution (beta-glucuronidase/sulfatase in ammonium acetate buffer, pH 5.0) and incubated for 4 h at  $37^{\circ}\text{C}$ . Next, the enzymatic reaction was stopped by addition of 40% formic acid. The samples were centrifuged and 80  $\mu\text{L}$  of the supernatant was transferred into the UPLC-MS/MS system. For quality assurance and control purposes, in-house pooled urine samples were analyzed along with the standard reference material provided by the National Institute of Standards and Technology (NIST, USA). The limits of detection (LOD) and limits of quantification (LOQ) for triclosan were 0.04 and 0.10 ng/mL, respectively. The mean accuracy, computed for each batch by comparing the NIST true value to the NIST concentration measured in that batch was 101% (range from 78% to 118%) and the precision given as relative standard deviation was below 15%.

### 2.3. Placental tissue collection and DNA extraction

At birth, a midwife collected placental tissue samples using a standardized procedure. Samples of approximately five  $\text{mm}^3$  were obtained from the fetal side, a few centimeters from the insertion of the cord, and immediately frozen at  $-80^{\circ}\text{C}$ . DNA was extracted using the DNeasy Blood & Tissue Kit. DNA concentrations were determined in duplicate using the Quant-IT Kit (ThermoFisher, Asnières-sur-Seine, France). Samples with discordant results were verified in a second series of measurements. DNA quality and correspondence with sample characteristics were evaluated on a subset of the samples by 1) migrating a small amount of DNA on a TapeStation 4200 (Agilent, Les Ulis, France) to calculate the DNA Integrity Number, 2) assessing amplification efficiency through the simultaneous amplification of two microsatellites markers, and 3) as a routine quality measure confirming the sex of individuals using PCR-based verification to detect potential sample swaps.

0.5–1  $\mu\text{g}$  of genomic DNA was bisulfite treated with the EpiTect® Fast 96 DNA Bisulfite Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. After the conversion, samples' volumes were adjusted based on the starting amount of DNA to normalize the sample concentration.

## 2.4. Placental DNA methylation assessment and quality control

DNA methylation was analyzed for >850,000 CpGs with the Infinium Human Methylation EPIC Kit (Illumina, San Diego, USA), according to the manufacturer's instructions. Sample plate layout was designed with the R/Bioconductor *OSAT* package (Yan, 2023; Yan et al., 2012) and randomized based on child's sex, maternal tobacco smoking during pregnancy, and delivery place. Fourteen samples were duplicated between two to five times across the microarrays to assess any technical batch effects.

Raw methylation data were extracted using the R/Bioconductor *ChAMP* package (Morris et al., 2014; Tian et al., 2017, 2023). All 432 samples (395 samples and replicates) were below the quality threshold of less than 10% of failed probes detection p-value ( $>0.01$ , global mean of 0.11% of failed probes) and therefore no sample was removed. Probes were further filtered as follows: detection p-value  $>0.01$  (38,192 probes removed); beadcount  $<3$  in at least 5% of samples (2,081 probes); non-CpG probes (2,745 probes); multi-hit probes as described by Nordlund et al., (2013) (8,419 probes). CpG sites at a distance  $\leq 2$  bp from SNPs with minor allele frequency  $<0.05$  and chromosomes X and Y (29,987 probes) were removed using the R package *DMRcate* (Peters, 2023; Peters et al., 2015). Cross-hybridized probes (31,917) identified in previous studies were then filtered out using the R package *maxprobes* (Benton et al., 2015; Chen, 2023; Chen et al., 2013). 752,577 methylation sites remained after quality control, normalization, and filtering of outliers. Beta values, i.e., methylation levels expressed by the ratio of intensities between methylated and unmethylated alleles (between 0 and 1, with 0 being unmethylated and 1 fully methylated) were normalized using Beta Mixture Quantile (BMIQ) normalization (Teschendorff et al., 2013) (Supplementary Fig. 3). We applied the Tukey's fences method to reduce the influence of outliers, i.e., methylation beta values above the third quartile plus three interquartile ranges (IQRs) or below the first quartile minus three IQRs were removed (in total, 0.4% of methylation values).

## 3. Statistical analyses

### 3.1. Adjustment factors

We adjusted our analyses for *a priori* selected factors potentially affecting both triclosan concentrations and placental DNA methylation levels or those affecting DNA methylation only. Those included maternal active tobacco cigarette smoking before and/or during pregnancy (did not smoke; smoked before and/or during pregnancy), maternal age (continuous), maternal education level (below three years after high school; three-four years after high school; above four years after high school), parity (nulliparous; one or more children), season of conception (January–March; April–June; July–September; October–December), maternal pre-pregnancy body mass index [(BMI), underweight; normal weight; overweight and obesity], gestational age (continuous), and child's sex (except for sex-stratified analyses, female; male). The data on these factors were collected by questionnaires during pregnancy. We additionally adjusted our analyses for technical factors related to the methylation measurements (batch, plate, and chip)

### 3.2. Imputation of the missing data

Triclosan concentration values below the limit of detection (LOD) and between the LOD and limit of quantification (LOQ) were singly imputed using the *NADA* (Lee, 2020) and *msm* (Jackson, 2011, 2022) R packages to implement a method (Helsel, 1990; Lubin et al., 2004) consisting of randomly drawing values below the LOD and between LOD and LOQ from the estimated distribution of the compound. Missing values for maternal education level ( $n = 2$ ), pre-pregnancy BMI ( $n = 4$ ), and smoking status ( $n = 28$ ) were replaced by the mode.

## 3.3. Associations between triclosan and placental DNA methylation

To assess the associations between triclosan concentrations and methylation levels of each CpG site we used robust linear regression (using the *MASS* R package, Ripley et al., 2023; Venables and Ripley, 2002) adjusted for potential confounders. To reduce the impact of outliers on regression fits, triclosan concentrations were  $\log_2$ -transformed. We averaged the  $\log_2$ -transformed triclosan concentrations measured in the two weekly pools for each participant and used this average in our main statistical analyses. Eight women had no triclosan concentration available for either of two assessment time points, so for those cases the concentration that was available was used instead of the average.

We performed two complementary approaches: a candidate approach focused on genomic locations previously shown to be affected by triclosan exposure and an exploratory approach investigating all genomic locations of the BeadChip. These two approaches are explained in detail below. Since among the two previous published studies one relied on male fetus placentas only (Jedynak et al., 2021) and the other suggested interaction with the child's sex for some associations (LaRocca et al., 2014), we also performed a sex-stratified analysis in both candidate and exploratory study (males  $n = 210$ ; females  $n = 185$ ).

### 3.4. Candidate approach

We first focused on CpGs whose methylation has been associated with triclosan concentrations assessed in spot urine samples collected between 22 and 29 gestational weeks in the only previous epigenome-wide study reporting associations for this chemical (Jedynak et al., 2021). Out of 543 candidates, 43 were not present in the current study due to filtering at the stage of methylation data pre-processing or because they were not covered by the EPIC BeadChip (Fig. 1). As this was a candidate approach, we considered a CpG as significantly associated with triclosan if the nominal p-value for the association was below 0.05. Finally, we compared  $\beta$  coefficient estimates with those previously published (Jedynak et al., 2021) by calculating Pearson correlation coefficients.

We next focused on the 162 DMRs (containing at least two CpGs) that were previously associated with triclosan concentrations (Jedynak et al., 2021). We relied on the 337,722 CpG sites of the EPIC BeadChip overlapping with the CpGs of the Infinium HumanMethylation450 BeadChip used in the previous study (Fig. 1). We used the *comb-p* Python module (Pedersen et al., 2012) that accounts for spatial correlations of nominal p-values obtained for each CpG by the use of sliding windows and Stouffer-Liptak-Kechris correction (Kechris et al., 2010). This algorithm returns regional p-values with Šidák correction applied to account for multiple testing (Šidák, 1967). As this was a candidate approach, we used a p-value threshold of 0.05 to start a region and a maximum distance of 500 bp to initiate a DMR. Finally, we considered DMRs including at least two probes and with Šidák-corrected p-values below 0.05 as regions of interest. A region was considered replicated if it encompassed genes previously identified as associated with triclosan.

### 3.5. Exploratory study

For the exploratory approach, we utilized the 752,577 CpGs that passed quality control (Fig. 1). We considered a CpG as significantly associated with triclosan if the false discovery rate (FDR) (Benjamini and Hochberg, 1995) corrected p-value obtained for the association was below 0.05. As for the regional analysis, DMRs were identified as described above except that a region could be detected for all 752,577 genomic loci and that we used a more restrictive threshold to start a region (p-value  $<0.001$  instead of 0.05).

### 3.6. Genomic control

We drew Q-Q plots and calculated genomic inflation factor ( $\lambda$ ) using

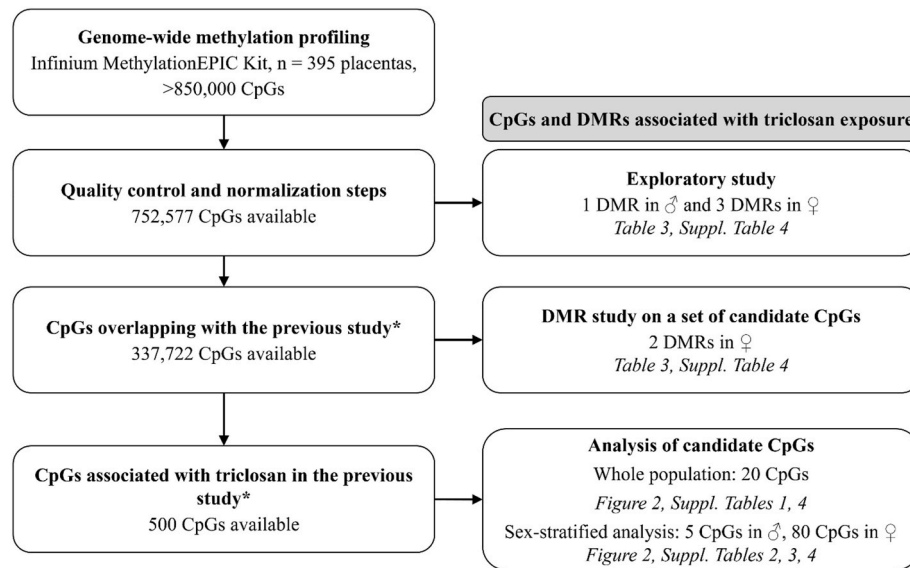


Fig. 1. Workflow of the study.

\* Jedynak et al. 2021

Abbreviations: DMR = differentially methylated region.

the QCEWAS R package (Van der Most et al., 2017, 2023) and the Bayesian inflation factor (BIF) was obtained using *bacon* R/Bioconductor package (Iterson et al., 2017; Iterson and Zwet, 2023).

### 3.7. Gene annotations

We retrieved gene annotations from the Illumina annotation hg19 reference genome available in *IlluminaHumanMethylationEPICanno.ilm10b4.hg19* R/Bioconductor package (Hansen, 2017) and from the database of the University of California, Santa Cruz (UCSC, <https://genome.ucsc.edu>). We obtained basic information on genes identified as differentially methylated in association with triclosan concentrations from the GeneCards Human Gene Database (Stelzer et al., 2016).

### 3.8. Identification of imprinted genes

To determine if any identified triclosan-associated genes are imprinted, we screened the list of imprinted genes in the following databases: MetaImprint (Wei et al., 2014) (<https://openebench.bsc.es/tool/metaimprint>), GeneImprint (Jirtle, 1999) (<http://www.geneimprint.com>), igc.otago (Morison et al., 2005) (<http://igc.otago.ac.nz>) 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 Methylation EPIC Kit used in the current study.

### 3.9. Analysis of mediation via the placental cell composition

We did not adjust our main analyses for placental cell heterogeneity because it may act as a mediator between pregnancy exposure to triclosan and DNA methylation (Jedynak et al., 2021). Nevertheless, to explore whether cell composition could mediate the associations between triclosan concentrations and DNA methylation levels, for CpGs significantly associated with triclosan exposure in the candidate approach, we ran additional analysis adjusted for cell proportions. We obtained estimates of methylation profiles of six placental cell types [endothelial, Hofbauer, nucleated red blood cells (nRBC), stromal, syncytiotrophoblasts, and trophoblasts] using a reference-based method from the *planet* R/Bioconductor package (Yuan et al., 2021; Yuan and Robinson, 2023) (Supplementary Fig. 4). First, we used the Robust

Partial Correlations algorithm implemented in the R/Bioconductor package *EpiDISH* (Teschendorff et al., 2017; Teschendorff and Zheng, 2023) on the methylation data to calculate the reference-based estimates of placental cell composition. We then adjusted our models with those estimates by adding them to the regression equations as explanatory variables. We considered zero estimates, if obtained for a cell type, as below the LOD and we imputed those values with *impCoda* function from the R package *robCompositions* that was designed to handle compositional data and that relies on an iterative regression-based procedure after KNN-initialization (Hron et al., 2010; Templ et al., 2011, 2021). As the six estimated cell proportions sum up to one for each individual, in the regression models we used five cell types (all but nRBC) to avoid singularity.

We estimated the percentage difference between the effect estimates obtained for the models unadjusted and adjusted for the placental tissue heterogeneity using the following formula:  $[(\beta_{\text{adjusted}} - \beta_{\text{unadjusted}}) / \beta_{\text{unadjusted}}] \times 100\%$ . Then, for the CpGs showing  $\geq 20\%$  absolute difference between adjusted and unadjusted effect estimates, additional mediation analysis was run using the *mediation* R package (Tingley et al., 2014, 2019). The six estimated placental cell types were reduced to the first principal component (PC1) explaining above 50% of the variance of the cell heterogeneity, which was then used in the mediation analysis. The cell types most strongly contributing to the PC1 were those most abundant in the cell mixture (syncytiotrophoblast, trophoblast, and stromal cells). P-values for average causal mediation effect were reported as the measure of mediation and those below 0.2 were treated as significant.

### 3.10. Sensitivity analysis – exploring specific periods of sensitivity to triclosan exposure

To explore potential periods of sensitivity to triclosan exposure during pregnancy, we used concentrations assessed in each urine pool separately (median 18 and 34 gestational weeks, respectively) to study associations with DNA methylation of 500 pre-selected CpGs (candidate approach) and all 752,577 available CpGs (exploratory study).

### 3.11. Research data and code

Data used in this study are confidential and can only be provided

upon a reasonable request to the SEPAGES steering committee. The analyses were conducted using R v. 4.1.2 (R Core Team and R Foundation for Statistical Computing, 2020), RStudio v. 2021.09.1–372 (RStudio Team, 2020), and Python v. 3.7.4 (van Rossum and Drake, 2009). The code is available under the link: [https://gricad-gitlab.univ-grenoble-alpes.fr/iab-env-epi/jedynak\\_prenatal\\_2023](https://gricad-gitlab.univ-grenoble-alpes.fr/iab-env-epi/jedynak_prenatal_2023).

## 4. Results

### 4.1. Study population characteristics and triclosan concentrations

The mothers were 32.2 years old on average and median gestational duration was 40.0 weeks (Table 1). The median gestational age at two urine samples collection times was 17.7 weeks (25th centile: 16.4; 75th centile: 18.9) and 34.0 (32.1; 35.1), respectively. The median number of urine samples provided by the participants was 21 (25th centile: 20; 75th centile: 21) for each pool (see Supplementary Table 1 for detailed numbers of samples per participant). The frequency of triclosan detection in the first collected sample was 98.4% for women carrying female fetuses, 98.6% for male fetuses, and 98.5% when both sexes were considered. For the second urine collection, it was 97.2%, 98.6%, and 97.9%, respectively. The median triclosan concentration averaged over

**Table 1**  
Population characteristics for the 395 mother-child pairs included in the study and recruited between 2014 and 2017.

Characteristics	Distribution	
	n (%)	Median [25th; 75th centile]
<b>Season of conception</b>		
January–March	99 (25.1)	
April–June	81 (20.5)	
July–September	95 (24.1)	
October–December	120 (30.4)	
<b>Maternal active smoking before and/or during pregnancy</b>		
Did not smoke	320 (81.0)	
Smoked before and/or during pregnancy	47 (11.9)	
Missing	28 (7.1)	
<b>Parity</b>		
Nulliparous	178 (45.1)	
≥ 1 child	217 (54.9)	
<b>Maternal level of education after high school (years)</b>		
<3	69 (17.5)	
3–4	100 (25.3)	
>4	224 (56.7)	
Missing	2 (0.5)	
<b>Maternal pre-pregnancy BMI<sup>a</sup></b>		
Underweight (<18.5 kg/m <sup>2</sup> )	26 (6.6)	
Normal weight (≥18 - <25 kg/m <sup>2</sup> )	296 (74.9)	
Overweight and obesity (≥25 kg/m <sup>2</sup> )	69 (17.5)	
Missing	4 (1.0)	
<b>Child's sex</b>		
Female	185 (46.8)	
Male	210 (53.2)	
<b>Maternal age (years)</b>		32.2 [29.8; 35.1]
<b>Gestational age at delivery (weeks)<sup>b</sup></b>		40.0 [39.1; 40.7]

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

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

<sup>a</sup> Categorized according to the World Health Organization definitions.

<sup>b</sup> Based 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.

the two collection time points was 1.1 µg/L (5th centile ranging from 0.2 for female fetuses to 0.3 for male fetuses and both sexes; 95th centile ranging from 181.0 for male to 199.4 for female fetuses) (Table 2).

### 4.2. Associations between triclosan concentrations and placental DNA methylation

P-value distributions obtained for associations between triclosan concentrations and DNA methylation were close to the theoretical distributions as indicated by the genomic inflation factor values [ranging from 0.81 to 0.84 for the candidate approach (337,722 CpGs) and exploratory approach (752,577 CpGs), respectively] and BIF values (0.84–0.87, Supplementary Fig. 5).

### 4.3. Candidate approach

When boys and girls were studied together, 20 out of the 500 candidate CpGs were significantly associated with triclosan (nominal p-value <0.05, Fig. 2A, Supplementary Table 2). Of interest, the sign of those associations was identical as in the previous study (Jedynak et al., 2021) (positive associations for 18 CpGs, negative associations for two). Identified CpGs mapped to two intergenic regions and 18 genes, all of which are protein coding. When we compared  $\beta$  coefficient estimates for all 500 candidate CpGs, the correlation with those obtained in the previous study was high [Pearson correlation coefficient ( $\rho$ ) = 0.6], but the overall effect size was smaller (Fig. 2A, Supplementary Table 2).

In the sex-stratified analysis when only males were studied, we identified five triclosan-associated CpGs located within three genes (*uromodulin like 1* overlapping with the whole population analysis) and two intergenic regions, with two CpGs whose methylation increased (nominal p-value <0.05, Fig. 2B, Supplementary Table 4). The effect estimates for the 500 candidate CpGs were not correlated with those observed in the previous study ( $\rho$  = 0.06, Fig. 2B).

When analyses were restricted to female placentas, 80 CpGs mapping to 72 genes and 22 intergenic regions were significantly associated with triclosan, with 12 genes in common with the 20 loci identified in the whole population analysis (nominal p-value <0.05, Supplementary Table 6). Most of the associations (95%) identified in female placenta were positive, and their signs were identical as those in the previous work conducted on boys (positive associations for 76 CpGs, negative associations for four). Four triclosan-associated genes are confirmed or predicted imprinted loci: *fibrosin like 1 (FBRSL1)*, *potassium voltage-gated channel subfamily Q member 1 (KCNQ1)*, *rho related BTB domain containing 3 (RHOBTB3)*, and *SPARC related modular calcium binding 1 (SMOC1)* (Supplementary Table 8). Surprisingly, we observed a high correlation between the effect estimate values for females and those obtained in the previous study on males ( $\rho$  = 0.7, Supplementary Table 6).

Among the DMRs detected in the previous study conducted in male placentas (Jedynak et al., 2021), we found two genes [*Septin 9 (SEPT9 or SEPTIN9)* and *synaptic Ras GTPase activating protein 1 (SYNGAP1)*, Table 3, Supplementary Table 8] encompassed by two DMRs whose association with triclosan exposure was replicated, but these associations were seen in female placentas only.

### 4.4. Mediation by placental cell composition

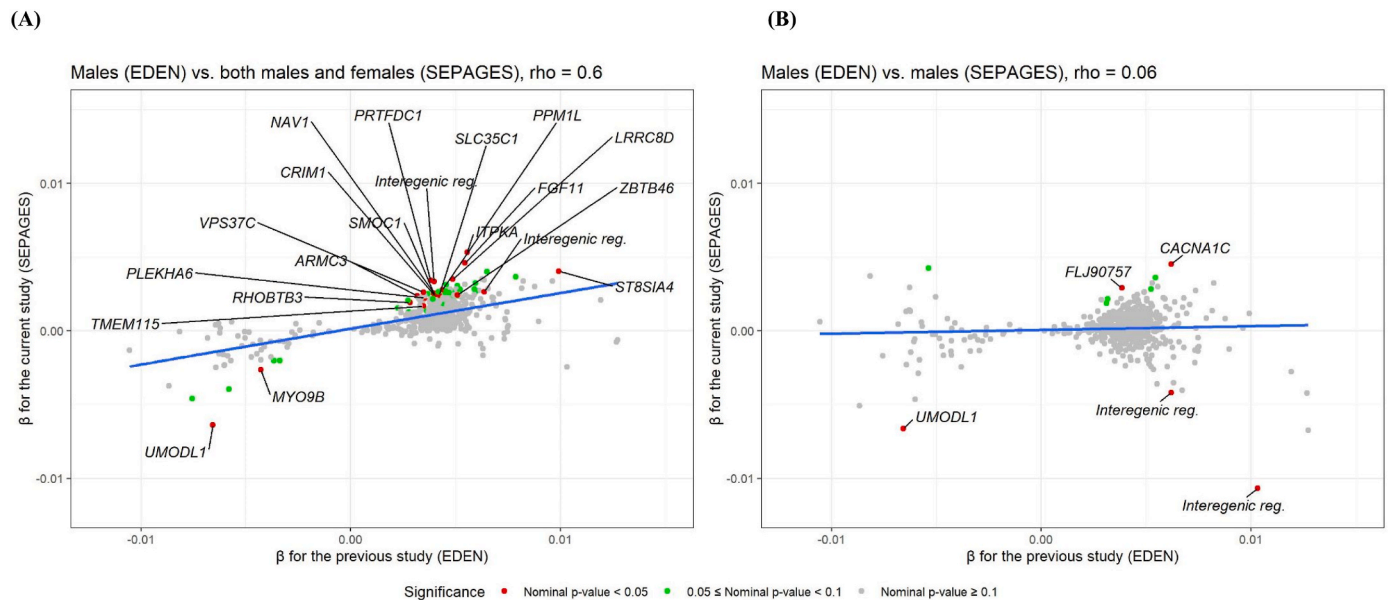
Mediation analysis revealed that part of the observed associations in the candidate approach were mediated by the placental cell heterogeneity. When males and females were studied together, additional adjustment for placental cell proportions overall caused a shrinkage of regression coefficients and reduced the number of differentially methylated CpGs from 20 to seven (Supplementary Table 2). Eighteen out of 20 triclosan-associated CpGs showed ≥20% absolute difference between adjusted and unadjusted effect estimates and the formal mediation analysis indicated mediation effect for eleven out of these 18 CpGs

**Table 2**

Average maternal urinary triclosan concentrations assessed in weekly pools collected at two time points during pregnancy (n = 395 pregnant women).

	LOD (µg/L)	LOQ (µg/L)	>LOD urine pool 1 (%)	>LOD urine pool 2 (%)	Measured pregnancy triclosan concentrations								
					Percentiles (µg/L)								
					Urine pool 1			Urine pool 2			Averaged over urine pools 1&2		
					5th	50th	95th	5th	50th	95th	5th	50th	95th
Female fetuses	0.04	0.1	98.4	97.2	0.2	0.9	176.4	0.2	1.0	178.3	0.2	1.1	199.4
Male fetuses			98.6	98.6	0.2	1.0	115.5	0.2	0.9	171.9	0.3	1.1	181.0
Both sexes			98.5	97.9	0.2	0.9	148.7	0.2	0.9	177.5	0.2	1.1	195.7

Abbreviations: LOD = limit of detection. LOQ = limit of quantification.



**Fig. 2.**  $\beta$  regression coefficient estimates obtained for the whole population (A) and in analysis in males only (B) in function of the regression coefficient estimates obtained for the 500 CpGs associated with triclosan in the previous study on males (Jedynek et al., 2021). The dots represent regression coefficient estimates with nominal p-value <0.05 (red),  $0.05 \leq$  nominal p-value <0.1 (green), and nominal p-value  $\geq 0.1$  (gray). The blue line represents the best linear fit for the association.  $\beta$  estimates correspond to a change in the DNA methylation level for doubling of the urinary triclosan concentration. Regression models in the current study were 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, child's sex (for analyses not stratified by child's sex), as well as the technical parameters batch, plate, and chip. Abbreviations: BMI = body mass index. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

**Table 3**

DMRs associated with concentrations of triclosan during pregnancy (Šidák-corrected p-value <0.05; males n = 210, females n = 185).

Study based on a subset of candidate CpGs (p-value to start a region <0.05, 337,722 CpGs)						
Child's sex	Gene <sup>a</sup>	DMR (chromosome:start-end)	No. of CpGs	SLK p-value	Šidák p-value	Direction of association
Female	SEPT9	chr17:75315486–75315838	6	3.83E-07	3.68E-04	+
Female	SYNGAP1	chr6:33396160–33396296	8	1.98E-06	4.90E-03	+
Exploratory study (p-value to start a region <0.001, 752,577 CpGs)						
Child's sex	Gene <sup>a</sup>	DMR (chromosome:start-end)	No. of CpGs	SLK p-value	Šidák p-value	Direction of association
Female	CLASP2	chr3:33701101–33701224	5	1.50E-12	9.17E-09	+
Female		chr7:27192056–27192188	3	8.13E-07	4.62E-03	+
Female		chr15:37403184–37403243	3	1.70E-06	2.14E-02	+
Male	LURAP1; POMGNT1	chr1:46668781–46668871	5	7.70E-09	6.44E-05	+

Epigenome-wide regression models were based on were 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, as well as the technical parameters batch, plate, and chip. No associations were detected when both sexes were studied together.

Abbreviations: chr = chromosome. DMR = differentially methylated region. SLK = Stouffer-Liptak-Kechrchis correction.

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

(mediation effect p-value <0.2, [Supplementary Table 2](#)).

Similar results were observed in the sex-stratified analysis. For males, additional adjustment for placental cell heterogeneity caused a shrinkage of the regression coefficient by more than 20% for one of five triclosan-associated CpGs, which was not associated with triclosan anymore, but this effect did not prove to be mediated by the cell composition (mediation effect p-value >0.3, [Supplementary Table 4](#)). For females, additional adjustment caused a notable shift of regression coefficient values towards zero [all but one triclosan-associated CpG (n = 79) showed  $\geq 20\%$  absolute difference between adjusted and unadjusted effect estimates] and reduced the number of differentially methylated CpGs (from 80 to 14, [Supplementary Table 6](#)). Formal mediation analysis indicated a mediation effect for most (n = 77) of the 79 tested CpGs (mediation effect p-value <0.2, [Supplementary Table 6](#)).

#### 4.5. Potential periods of sensitivity to triclosan exposure

In the analysis stratified by the time of urine collection, we observed more CpGs significantly associated with exposure (nominal p-value <0.05) when triclosan concentrations were measured earlier in pregnancy compared to the later time point, both in the whole population (19 vs. 9 CpGs for urine pools 1 and 2, respectively, [Supplementary Fig. 6A](#), [Supplementary Table 3](#)) and in the sex-stratified analysis (10 vs. 4 in male placenta, [Supplementary Fig. 6B](#), [Supplementary Table 5](#); 82 vs. 23 CpGs in female placenta, [Supplementary Table 7](#)). We also observed slightly higher correlation with the estimates reported in the previous study on triclosan assessed in spot urine samples collected between 22 and 29 weeks of gestation ([Jedynak et al., 2021](#)) for the earlier pregnancy time point, both in the whole population ( $\rho = 0.6$  and  $0.5$  for the urine pools 1 and 2, respectively) and when only females placenta were studied ( $\rho = 0.7$  and  $0.6$ , respectively). For males, we did not detect such trend ( $\rho = 0.06$  and  $0.07$ , respectively). Four intergenic regions (one for males, three for females) and seven genes (*ARMC3*, *FGF11*, *UMOLD1* for the whole population and *FARP1*, *GRK5*, *LRRC8D*, *PLEKHA6* for females) were significantly associated with triclosan and overlapped between the two time points of exposure assessment. The seven genes had been also identified as associated with exposure averaged over pregnancy in the whole population and females, respectively.

#### 4.6. Exploratory study

In the exploratory approach performed on all 752,577 CpGs, we did not identify any associations between triclosan concentrations during pregnancy and placental DNA methylation (either for the concentrations averaged over pregnancy or for time point-specific exposure), either in the entire population or in a sex-stratified analysis (FDR-corrected p-values >0.05). As for the DMR study, only when males and females were studied separately, we observed four regions positively associated with pregnancy average triclosan concentrations. All identified DMRs encompassed protein-coding genes. For males, we found one DMR encompassing *leucine rich adaptor protein 1* (*LURAP1*) and *protein O-linked mannose N-acetylglucosaminyl-transferase 1 (beta 1,2-)* (*POMGNT1*) genes ([Table 3](#), [Supplementary Table 8](#)). For females, we identified three DMRs encompassing two intergenic regions and *cytoplasmic linker associated protein 2* (*CLASP2*) gene ([Table 3](#), [Supplementary Table 8](#)).

## 5. Discussion

Relying on a recent cohort including multiple urine samples to assess triclosan exposure, a large epigenome-wide coverage to measure placental DNA methylation, and both male and female placentas, we identified significant associations between prenatal exposure to triclosan and placental DNA methylation among a list of candidate CpGs previously identified in male placentas ([Jedynak et al., 2021](#)). In particular, we identified 18 differentially methylated genes (including two imprinted) in the whole population, 72 (including four imprinted

genes) in females, and three in males. Overall, most detected associations were positive, which is in line with the previous results ([Jedynak et al., 2021](#)). Our results confirm that part of the identified associations could be mediated by cell composition heterogeneity. Besides, we identified more associations for the earlier time point (median gestational age: 18 weeks), which may suggest different sensitivity of placental DNA methylation to triclosan exposure during pregnancy. Finally, in our exploratory approach investigating all CpGs of the EPIC BeadChip, we identified a few new DMRs that are candidates for future replication.

### 5.1. Candidate approach

Among the 500 candidate CpGs selected from a previous study on male placentas, 20 CpGs were associated with triclosan when males and females were studied together in the present study. In the sex-stratified analysis, only 6% of the identified associations were observed in male fetuses' placenta (5 CpGs in males vs. 80 CpGs in females), which was unexpected as the previous work that only analyzed male placentas reported over 500 CpGs associated with triclosan ([Jedynak et al., 2021](#)). The low number of differentially methylated CpGs observed in males compared to females could be explained by lack of statistical power or could result from a sex-specific response of placenta to triclosan exposure. Interaction with the child's sex has been previously reported for the triclosan-related methylation of *IGF2* DMR2 ([LaRocca et al., 2014](#)). While the underlying mechanisms of such sex-specific effects are so far not well understood, they might result from the fact that triclosan interacts with the endocrine system, which is sex-specific itself. On the other hand, the placenta undergoes sex-specific development and infant's sex is associated with placental DNA methylation patterns (e.g., [Andrews et al., 2022](#); [Bozack et al., 2022](#)), which might explain the observed sexually dimorphic placental responses to a variety of maternal factors (e.g., [Alexander et al., 2018](#); [Loke et al., 2018](#)). Taking together, this suggests that placental/fetal sex is an important factor to consider when looking at the associations between environmental chemicals and epigenetic placental outcomes ([Strakovsky and Schantz, 2018](#)).

### 5.2. Imprinted genes associated with pregnancy triclosan exposure

DNA methylation sites identified as positively associated with triclosan in the candidate CpG approach, either in both sexes or in females only, mapped to four confirmed or predicted imprinted loci: *FBRSL1*, *KCNQ1*, *RHOBTB3*, and *SMOC1*. Two of these genes (*KCNQ1* and *RHOBTB3*) show high expression in placenta, suggesting their important role during its development. *KCNQ1* is a maternally expressed growth-regulating gene ([Stelzer et al., 2016](#)) whose upregulation has been reported in human intrauterine growth restriction placentas (n = 25, [Cordeiro et al., 2014](#)). The *RHOBTB3* protein is involved in small GTPase-mediated signal transduction and the organization of the actin filament system ([Stelzer et al., 2016](#)). *RHOBTB3* is present within the mouse middle layer of blood vessels ([Lutz et al., 2014](#)) and was hypothesized to play a role in mammalian placental vasculature and to be predictive of transcriptional signaling for optimal placental development and formation ([Rempel et al., 2019](#)). As for the two remaining genes, *FBRSL1* encodes a protein involved in RNA binding ([Stelzer et al., 2016](#)) while *SMOC1* is a maternally-expressed gene ([Santoni et al., 2017](#)) that encodes a multi-domain secreted protein that may have a critical role in ocular and limb development ([Stelzer et al., 2016](#)).

### 5.3. Non-imprinted genes associated with pregnancy triclosan exposure

We discuss below genes that may play a role in placental development and function or whose methylation level or expression in placenta or cord blood has been linked with child health. These include *AHNAK*, *AQP1*, *CD93*, *COL4A1*, *EMILIN1*, *HDAC4*, *TBX3*, and *TFAP2E* identified in female placenta and *CRIM1*, *FGF11*, and *NAV1* identified in both the



whole population and in the analysis restricted to female placentas. All associations identified for these genes were positive.

Aquaporin 1 (AQP1) is a member of the water channel proteins family and plays an important role in the balance of maternal-fetal fluids during animal (Luo et al., 2018; Mann et al., 2005; Zheng et al., 2014) and human pregnancy (reviewed by Zhang et al., 2012). Increased AQP1 expression associated with decreased placental DNA methylation has been linked with a greater frequency of oligohydramnios in pre-eclamptic placentas (n = 863, Ding et al., 2022).

*Collagen type IV alpha 1 chain (COL4A1)* is one of six genes encoding human collagen type IV (col-IV) proteins (Shi et al., 2020) that are integral components of basement membranes and extracellular matrix structural constituents (Stelzer et al., 2016). Among several other functions, they facilitate trophoblast cell invasion by taking part in remodeling maternal spiral arteries to facilitate sufficient blood flow to the developing fetus, therefore playing a crucial role in early placentation (Oefner et al., 2015). Recent proteomic analysis of fetal membranes identified COL4A1 as an upregulated gene in preterm but not full-term births (n = 20, Pan et al., 2020), while in maternal blood COL4A1 has also been identified as differentially methylated in preeclamptic cases compared to controls (n = 13, Ariff et al., 2019), suggesting epigenetic modifications of COL4A1 to be involved in pathologic pregnancies.

Differential methylation of two placental genes identified in this study as associated with triclosan exposure [*transcription factor AP-2 epsilon (TFAP2E)* and *histone deacetylase 4 (HDAC4)*] has been previously linked to fat accretion later in life. TFAP2E encodes a protein activating genes involved in embryogenesis, growth, and organ development. Decreased placental DNA methylation of TFAP2E along with decreased mRNA levels was associated with higher early-childhood adiposity (n = 262, Gagné-Ouellet et al., 2020). As for HDAC4, it belongs to proteins responsible for histone acetylation/deacetylation altering chromosome structure and affecting transcription factor access to DNA (Stelzer et al., 2016). Methylation of HDAC4 locus in the placenta has been associated with changes in the overall BMI in early and late childhood (n = 426, Clark et al., 2019).

*AHNAK nucleoprotein (AHNAK)* may play a role in diverse processes such as blood-brain barrier formation, cell structure and migration, cardiac calcium channel regulation, and tumor metastasis (Stelzer et al., 2016). An *in vitro* study showed that AHNAK was upregulated in preeclamptic placental tissues and that its overexpression decreased proliferative and migratory potentials and induced apoptosis in human trophoblast cells (n = 48, Dong et al., 2020).

As for the *CD93 molecule (CD93)*, which is a cell-surface glycoprotein and type I membrane protein (Stelzer et al., 2016), it is potentially important for guidance of the extravillous cytotrophoblast migration in uterine spiral arteries during placentation in early pregnancy (n = 36, Fantone et al., 2022). Decreased CD93 expression in late pregnancy and in preeclamptic placentas was hypothesized to be associated with the impaired migration of the extravillous cytotrophoblast cells.

*Elastin microfibril interfacier 1 (EMILIN1)* gene encodes an extracellular matrix glycoprotein that may play a role in the development of elastic tissues (Stelzer et al., 2016) as well as in the placentation process by facilitating trophoblast invasion of the uterine wall (Spessotto et al., 2006). Finally, *T-box transcription factor 3 (TBX3)* was shown *in vitro* to be a key regulator of human trophoblast differentiation i.e., transition from the cytotrophoblast into syncytiotrophoblast during peri-implantation period (Lv et al., 2019).

*Cysteine rich transmembrane BMP regulator 1 (CRIM1)* encodes a transmembrane protein that may play a role in tissue development through interactions with members of the transforming growth factor beta family (Stelzer et al., 2016) and was shown to be necessary for trophoblast differentiation (Pennisi et al., 2012). An *in vitro* study on preeclamptic rats showed decreased expression of *Crim1* while enhancement of its expression promoted cell proliferation and reduced apoptosis of trophoblast cells (Jiang et al., 2021).

*FGF11* encodes a protein of the fibroblast growth factor (FGF) family

(Stelzer et al., 2016). FGF11 transcripts were detected in mouse trophoblast stem cells, early gestation human placenta, and human trophoblast cells (Zhong et al., 2006). Placental epigenetic modifications of CRIM1 or FGF11 have so far not been described.

*Neuron navigator 1 (NAV1)* is thought to play a role in neuronal development and regeneration, however its functions, especially in the placenta, are not known (Stelzer et al., 2016). Nevertheless, a study on *Nav1* knock-out mice showed increased embryonic lethality, decreased birth weight, and infertility in female offspring (Kunert, 2014), which suggests its importance for healthy fetal development.

#### 5.4. Mediation by placental cell heterogeneity

Additional adjustment for the estimated placental cell types' proportions notably shrunk almost all the regression estimates of identified triclosan-associated CpGs and reduced the overall number of significant associations. This was in line with results of a previous study (Jedynak et al., 2021) and suggested that part of the associations observed between pregnancy triclosan and placental DNA methylation could be mediated by the placental cell heterogeneity. Mediating effect could be linked to the fact that pregnancy triclosan exposure may affect placental size and weight (Cao et al., 2017; Feng et al., 2016; Philippat et al., 2019) and trigger tissue necrosis and thrombosis of placenta (Wang et al., 2015) which could affect placental cell composition and thus DNA methylation patterns.

#### 5.5. Exploratory study

In the exploratory epigenome-wide association study, we did not observe any associations between pregnancy triclosan and methylation of individual CpGs, either in the entire population or sex-stratified analysis. As for the DMR, we identified a few associations in the sex-stratified analysis only. We found one DMR encompassing *LURAP1/POMGNT1* genes for males and three DMRs encompassing two intergenic regions and *CLASP2* gene for females. LURAP1 is involved in upregulation of inflammatory reactions and immunological response (Stelzer et al., 2016) and POMGNT1 is a transmembrane protein that resides in the Golgi apparatus and participates in O-mannosyl glycosylation (Brown et al., 2015). Finally, CLASP2 enables binding activity of the cytoskeletal protein, dystroglycan, and protein tyrosine kinase (Brown et al., 2015) and is involved in several processes including microtubule cytoskeleton organization and regulation of extracellular matrix organization. To our knowledge, none of the genes identified in our exploratory study has been previously associated with exposure to triclosan or other synthetic phenols such as bisphenol A, benzophenone-3, dichlorophenols, or parabens (Jedynak et al., 2021; LaRocca et al., 2014; Song et al., 2021).

#### 5.6. Strengths and limitations

In the present study, we relied on repeated collection and pooling of several maternal biospecimens (two pools of 21 urine samples each, on average). Such an approach should, compared to studies relying on spot sampling, improve precision of the exposure assessment and decrease the resulting bias in effect estimates (Perrier et al., 2016).

Our sample size was increased compared to those of previous studies (Jedynak et al., 2021; LaRocca et al., 2014). The use of the Methylation EPIC Kit allowed to cover more than 750,000 CpGs in the exploratory analysis while previous studies included approx. 340,000 (Jedynak et al., 2021) or 21 (LaRocca et al., 2014) CpG sites. Although overall per-sample correlations between 450k and EPIC chips are very high, some individual CpG sites, especially those with low variance of methylation, showed lower correlations that might affect results comparisons across studies using different chips (Fernandez-Jimenez et al., 2019; Solomon et al., 2018).

Although we adjusted our analysis for a broad range of potential

confounders, we cannot rule out residual confounding by factors that we did not account for. Among other factors, our observations could have been also affected by co-exposure. However, since in our cohort we observed low correlations between concentrations of triclosan and other common environmental pollutants (Guilbert et al., 2021; Rolland et al., 2020), it is rather unlikely.

As for the exploratory study, despite the broad coverage of the epigenome (>750,000 tested CpGs), we identified only a few isolated associations with triclosan exposure. This could be explained by the relatively low triclosan concentrations observed in SEPAGES compared to the previous studies (Jedynak et al., 2021; LaRocca et al., 2014) and moderate sample size, all of which could lead to a limited power and could have prevented us from observing associations, if any. As for the genes identified as differentially methylated in association with pregnancy triclosan concentrations, it should be noted that interpretation of the differential DNA methylation in context of gene expression is not straightforward (Lim et al., 2017).

## 6. Conclusions

Relying on a candidate approach, we identified associations between pregnancy triclosan concentrations assessed in repeated pooled biospecimens and placental DNA methylation. Similarly to our previous work, most of the observed associations were positive. In the sex-stratified analysis, the associations were mainly observed in females, which could suggest sex-specific differences in the sensitivity of placental DNA methylation to environmental exposure. We further showed that part of the identified associations could be mediated by cell composition heterogeneity. An additional exploratory approach investigating a broad range of CpGs identified a few new DMRs that are candidates for future replication.

## Credit author statement

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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.

## 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.

## Data availability

The data that has been used is confidential.

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## Appendix A. Supplementary data

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