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Prenatal Exposure to Environmental Phenols and Phthalates and Altered Patterns of DNA Methylation in Childhood

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Abstract

Introduction: Epigenetic marks are key biomarkers linking the prenatal environment to health and development. However, DNA methylation associations and persistence of marks for prenatal exposure to multiple Endocrine Disrupting Chemicals (EDCs) in human populations have not been examined in great detail.

Methods: We measured Bisphenol-A (BPA), triclosan, benzophenone-3 (BP3), methyl-paraben, propyl-paraben, and butyl-paraben, as well as 11 phthalate metabolites, in two pregnancy urine samples, at approximately 13 and 26 weeks of gestation in participants of the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) study (N = 309). DNA methylation of cord blood at birth and child peripheral blood at ages 9 and 14 years was measured with 450K and EPIC arrays. Robust linear regression was used to identify differentially methylated probes (DMPs), and *comb-p* was used to identify differentially methylated regions (DMRs) in association with pregnancy-averaged EDC concentrations. Quantile g-computation was used to assess associations of the whole phenol/phthalate mixture with DMPs and DMRs.

Results: Prenatal BPA exposure was associated with 1 CpG among males and Parabens were associated with 10 CpGs among females at Bonferroni-level significance in cord blood. Other suggestive DMPs (unadjusted p-value $< 1\times10^{-6}$) and several DMRs associated with the individual phenols and whole mixture were also identified. A total of 10 CpG sites at least suggestively associated with BPA, Triclosan, BP3, Parabens, and the whole mixture in cord blood were found to persist into adolescence in peripheral blood.

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Conclusions: We found sex-specific associations between prenatal phenol exposure and DNA methylation, particularly with BPA in males and Parabens in females. Additionally, we found several DMPs that maintained significant associations with prenatal EDC exposures at age 9 and age 14 years.

Keywords

DNA methylation; Phenols; Prenatal; Mixture; Phthalates

Introduction

Endocrine-disrupting chemicals (EDCs) are a class of chemicals capable of interacting with components of the endogenous endocrine system. Several well-studied EDCs have been associated with a wide range of outcomes including reproductive anomalies, certain cancers, obesity, neurodevelopment, and birth weight.[1,2] Common EDCs include a range of environmental phenols and phthalates used in several applications. Bisphenol-A (BPA) is a chemical involved in polycarbonate production forming plastics which are commonly used in food and beverage containers, as well as in can linings, dental sealants, and paper receipts.[3] Triclosan is an antimicrobial agent used in a wide variety of settings including as a general material preservative and in personal care products like mouthwashes, and was formerly a prominent ingredient in antibacterial hand soaps before being phased out in recent years.[4] Parabens are a group of antimicrobial agents used as preservatives in a wide range of cosmetics and pharmaceutical products.[5] Benzophenone-3 (BP3) is a UV light absorber widely used in sunscreens and other personal care products.[6] Phthalates are a large group of industrial chemicals, including diethyl phthalate (DEP) and di 2-ethylhexyl phthalate (DEHP), that are used in an array of products including plastics, personal care products, and food packaging.[7] Parent phthalate compounds undergo hydrolysis into their monoester forms, which are often measured to quantify exposure. These EDCs vary in sources and functions, but they are connected through their suggested estrogen- and androgen-linked mechanisms of action. Although EDCs are capable of affecting health and development throughout the lifecourse, the Developmental Origins of Health and Disease (DOHaD) hypothesis emphasizes the importance of early life exposures in establishing adult trajectories of health and disease, highlighting the importance of studying EDC exposure during fetal development.[8,9]

Some of the earliest influences of prenatal EDC exposure can be seen with alterations of birth outcomes and developmental trajectories in childhood. Prenatal EDC exposure has been linked to key elements of development and health across multiple epidemiologic studies, including several within the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) cohort. One large meta-analysis involving 3004 participants found that prenatal exposure to BPA is associated with higher birthweight.[10] Another pooled analysis of 6045 participants found that certain phthalate metabolites, as well as the overall phthalate mixture, was associated with higher odds of preterm birth.[11] The influence of prenatal EDC exposure on puberty and obesity in childhood has also received particular attention. Within the CHAMACOS cohort, prenatal exposure to monoethyl phthalate (MEP) and triclosan were associated with earlier attainment of

pubertal milestones in girls including timing of pubic hair development and menarche.[12] Similarly, DEHP has been found to be associated with later menarche, while BP3 and mono-methyl phthalate exposure were associated with earlier menarche in a population of 200 Chilean girls.[13] Additionally, DEHP metabolites, monobenzyl phthalate (MBzP), and BPA were associated with later attainment of pubertal milestones including thelarche, pubarche, and menarche in girls, and earlier attainment of gonadarche and pubarche in boys.[14] Furthermore, MEP, monocarboxy-isononly phthalate (MCNP), propylparaben, and a phthalate/phenol mixture were positively associated with higher measures of obesity by age 5,[15] and prenatal exposure to MEP, mono-n-butyl phthalate (MBP), and DEHP metabolites were positively associated with BMI z-scores and odds of overweight status by age 12.[16] A larger meta-analysis consisting of data from a diverse collection of 13 cohorts around the world has also highlighted positive associations between BPA exposure and childhood obesity measures.[17]

Epigenetics is a key biomarker that might explain relationships between prenatal EDC exposure, health, and developmental outcomes later in life. Studies in agouti mouse models, a common model organism for studying the impacts of environmental exposures on the epigenome, have revealed that prenatal exposure to physiologically relevant doses of BPA resulted in shifts towards a yellow obese coat color phenotypes and decreased DNA methylation in the *Agouti* gene locus in genetically identical offspring.[18] Several epidemiologic studies with sample sizes ranging from 69 to 408 have reported associations between prenatal exposure to BPA and DNA methylation at birth in cord blood utilizing both candidate gene approaches[19] and epigenome-wide approaches,[20–22] often highlighting sex-specific associations and impact on genes associated with obesity and endocrine signaling. Similarly, several studies with sample sizes ranging from 64 to 262 have examined associations between prenatal phthalate exposure and DNA methylation in cord blood,[23,24,25] including a previous analysis of 336 mother-child pairs in the CHAMACOS cohort that found altered methylation in cord blood across various CpG sites and regions involved in biological processes including inflammatory response, male fertility, and endocrine function.[26] However, epigenetic associations of related EDCs including Parabens, BP3, and Triclosan have not been systematically studied. One study of 202 mother-son pairs found several differentially methylated regions in placenta associated with prenatal triclosan, BP3, and parabens.[27] Similarly, the potential influence of EDC mixtures on DNA methylation remains a largely unexplored topic, with only one recent study of 306 mother-child pairs examining associations between prenatal exposure to a phthalate/BPA mixture and DNA methylation in cord blood, finding only suggestive associations and citing the need to explore the topic in additional populations.[28] Critically, most prior studies on the relationship between prenatal EDC exposure and DNA methylation have relied on single-timepoint analysis, limiting their ability to identify persistent changes in DNA methylation that could potentially better link these prenatal exposure with health and developmental outcomes occurring later in life.

To better understand the influences of prenatal EDC exposure on DNA methylation, we sought out to characterize associations between multiple environmental phenols measured during pregnancy and DNA methylation at birth, along with persistence of those relationships into childhood. We further aimed to characterize associations between

an environmental phenol/phthalate mixture and DNA methylation. We harnessed prenatal phenol and phthalate exposure data, as well as cord blood DNA methylation data, from the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) study. Utilizing follow-up DNA methylation measurements taken at 9 and 14 years of age, we additionally examined the persistence of associations observed in cord blood later in childhood and into adolescence.

Methods

Study Population.

Between October 1999 and October 2000, the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) study recruited 601 pregnant women from farmworker communities of the Salinas Valley in California. At enrollment, women were ≤20 weeks of gestation, English- or Spanish-speaking, Medicare eligible, planning to deliver at the county hospital, and attending prenatal care visits at one of six local community clinics or hospitals. Of 601 initial enrollees of the cohort, 526 were followed to delivery of live, singleton newborns in 2000–2001. The initial CHAMACOS enrollee population represents a relatively low income and educational attainment population, and detailed descriptions of the study population have been previously reported.[29] Maternal covariate information and spot urine samples were obtained during two pregnancy interviews timed at around 13 (mean=13.8, SD=5.0) and 26 (mean=26.6, SD=2.6) weeks of gestation. Maternal covariates included pre-pregnancy BMI, maternal education (≤ 6th grade, 7^{th}-12^{th} grade, >=High School Graduate), and pregnancy smoking (yes, no). Child sex was abstracted from medical records. Infant gestational age was obtained by asking mothers about the date of their last menstrual period (LMP) and, if unknown (n = 46), was based on ultrasound methods abstracted from medical records. Medical records were systematically biased downward due to being rounded to the closest completed week. To account for this systematic downward bias, half a week was added to all gestational age estimates obtained from medical records.

Maternal pregnancy phenol concentrations.

Three environmental phenols (Bisphenol A (BPA), Triclosan (TCS), Benzophenone-3 (BP3)) and three parabens (methyl paraben (MP), propyl paraben (PP), and butyl paraben (BP)) were measured in urine samples collected from mothers at the two pregnancy timepoints. Samples were collected in polypropylene urine cups, aliquoted into glass vials, and stored at −80°C until shipment on dry ice to the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia, for analysis. Total urinary phenol concentrations were obtained using online solid phase extraction coupled to high performance liquid chromatography-isotope dilution tandem mass spectrometry.[30] A summary paraben measure was generated by calculating the molar sum of the three individual paraben measures.

Maternal pregnancy phthalate concentrations.

11 phthalate metabolites were measured in urine samples collected from mothers during the two pregnancy timepoints. These included metabolites of three low molecular weight phthalates [monoethyl phthalate (MEP), mono-n-butyl phthalate (MBP), and mono-

isobutyl phthalate (MiBP)] and eight high molecular weight phthalates [monobenzyl phthalate (MBzP), mono-2-ethylhexyl phthalate (MEHP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP), mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP)); monocarboxyoctyl phthalate (MCOP), monocarboxy-isononly phthalate (MCNP), and mono(3-carboxypropyl) phthalate (MCPP). Maternal urine samples were collected, aliquoted, barcoded, and stored at −80°C at the University of California, Berkeley until samples were shipped on dry ice to the CDC for measurement of phthalate metabolites. Phthalate metabolite concentrations were measured using solid phase extraction coupled with isotope dilution high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry.[31] Phenol and phthalate concentrations below the limit of detection (LOD) with no corresponding instrumental signal were imputed from a log-normal distribution with the fill-in method described by Lubin et al.[32]

Phenol and phthalate measures were corrected for urinary dilution by calculating specific gravity (SG)-standardized measures, using the formula: $P_c = P[(1.024 - 1)]/\ (SG - 1)$. In this formula, P is the unadjusted metabolite concentration, SG is the specific gravity of the individual sample, and P_C is the SG-adjusted measure. SG was measured with a refractometer (National Instrument Company Inc., Baltimore, MD). Measurements were then log2 transformed to normalize distributions of the concentrations. A total of 421 participants had each phenol and phthalate measurement available in at least one timepoint. We excluded 14 participants with outlier BPA measurements, defined as greater than 1.5 interquartile ranges beyond the first or third quartile, prior to analysis. No outliers were observed for Triclosan, BP3, or Paraben measurements. Pregnancy-averaged measurements, calculated as the average of the two individual timepoint SG-adjusted measurements, were used for all subsequent analysis if both timepoint measurements were available, and single timepoint measures were used in cases with only one available timepoint. A summary of phenol and phthalate measurements is provided in the supplement. (Table S1).

DNA methylation.

A sample of 373 participants with available cord blood data were selected for DNA methylation analysis. Blood samples were refrigerated and transported to the University of California, Berkeley biorepository where samples without anticoagulant were separated into serum and clot and stored at −80° C until analysis. DNA was isolated from the banked cord blood samples using QIAamp DNA Blood Maxi Kits (Qiagen, Valencia, CA) according to the manufacturer's protocol with minor modifications, as previously described.[33] DNA aliquots of 1 μg were bisulfite converted using Zymo Bisulfite Conversion Kits (Zymo Research, Orange, CA). DNA was amplified, enzymatically fragmented, purified, and applied to the Illumina Infinium HumanMethylation450 (450K) (Illumina, San Diego, CA) according to the Illumina methylation protocol to measure DNA methylation. 450K chips were analyzed using the Illumina Hi-Scan system. Quality control steps included the use of repeats and randomization of samples across chips and plates.[34] Methylation data were imported into R statistical software for preprocessing using *minfi*.[35] Quality control was initially performed at the sample level, excluding samples with overall low intensities (< 10.5) and technical duplicates. Detection P values were computed relative to control probes, and we excluded probes with non-significant detection ($P > 0.01$) for 5%

or more of the samples. Data were preprocessed using functional normalization using the preprocessFunnorm function in the *minfi* R package,[36] adjusted for probe-type bias using the RCP regression on correlated probes method in the *ENmix* R package,[37] and adjusted for sample plate as a technical batch using ComBat from the *sva* package.[38] Visualization of the data using density distributions at all processing steps and PC analyses were used to examine the associations of methylation differences with technical, biological, and measured traits with global DNAm variation using PCA plots. Probes located on sex chromosomes, cross-reactive probes,[39] and CpGs with SNPs at the CpG or single-base extension were removed prior to analysis. DNA methylation beta values were winsorized at 1% on each side to control for extreme methylation values. DNA methylation beta values were converted to M-values using a logit transformation to better fit modeling assumptions. Cord blood cell proportions of CD8 T cells, CD4 T cells, NK cells, B cells, Monocytes, Granulocytes, and nucleated red blood cells were calculated using the Gervin et al reference.[40] For testing the persistence of associations observed in cord blood, DNA methylation of peripheral blood was measured with the 450K array at 9-years of age and with the EPIC array at 14-years of age, with processing and quality control performed the same as described above. Estimated cell proportions for peripheral blood cells (CD4T, NK, Bcell, Mono, Gran) were calculated using EpiDish.[41]

Statistical Analysis.

309 participants had both DNA methylation and phenol measurements available for analysis in cord blood, with 156 and 150 participants available for analysis at the Age 9 and Age 14 year timepoints respectively. A summary of sample size at each stage of the study is provided in Supplemental Table 2. Robust linear models were initially used to examine associations between phenol measures and estimated cell proportions in cord blood, further adjusted for child sex, gestational age at birth, maternal education, maternal BMI, and maternal smoking during pregnancy, using the *rlm* function from the R *MASS* package.[42] To test for differentially methylated probes (DMPs), robust linear regression models of each individual EDC measure as the exposure and DNA methylation M-values as the outcome. Sex-stratified models were adjusted for cell proportions, gestational age at birth, maternal education, maternal BMI, and maternal smoking during pregnancy, while the overall models were adjusted for the same covariate set and child sex. Quantile g-computation was then used to estimate the joint effect of a mixture of environmental phenols (BPA, Triclosan, BP3, and paraben summary measures) and phthalates (MBP, MEP, MiBP, MBzP, MCNP, MCOP, MCPP, MEHP, MEHHP, MEOHP, MECPP) on DNA methylation at each individual CpG site. The primary output from a quantile g-computation model is the psi estimate, which represents the expected change in the methylation M-values for a one quantile simultaneous increase in all the components of the mixture conditional on the same covariate set defined above. A setting of 6 quantiles (q=6) was used for quantile g-computation models. Significance was judged with an FDR-adjusted p-value threshold of 0.05, and all sites with p-values below 10e-06 are discussed as suggestive. Bonferroni-adjusted p-values were additionally used to discuss higher confidence results. For ease of interpretation, all suggestive DMPs were rerun in identical *rlm* models using DNA methylation beta values, allowing interpretation of the model coefficients as expected change in percent methylation at a CpG site. We focus our analysis on individual phenols

as well as a mixture of phenols and phthalates, while a previous analysis from Solomon and colleagues examined differential methylation in response to individual phthalates.[26]

Differentially methylated regions (DMRs) were identified using *comb-p*,[43] using the p-value outputs from the robust linear regression models for the individual pollutants and the p-value outputs from the quantile g-computation models from the mixture analysis. *Comb-p* models were run with a base-pair window of 1000bp, FDR-significance threshold of < 0.001, and no minimum number of CpGs to form a DMR. To better understand the function of the identified DMPs and DMRs, KEGG pathway analysis was performed on results with FDR p-values <0.05 from each DMP model series using *gometh*, as well as on each identified DMR using *goregion*.[44]

To test for persistence of DMPs observed at birth, associations found to be suggestive or significant in cord blood were rerun within the same study population at 9- and 14-years of age. Persistence robust linear regression and quantile g-computation models were adjusted for estimated cell proportions and the same covariate set defined above, minus maternal smoking during pregnancy due to limited sample sizes. Some CpG sites could not be characterized at the 14 year timepoint due to differing probes on the EPIC array, totaling up to 3 of 63 sites in the male models, 77 of 915 sites in the female models, and 4 of 40 sites in the overall models. Persistence was defined as exhibiting an unadjusted p-value < 0.05 and same direction of association at either the 9 year or 14 year timepoint. Information on genes identified in our analysis were retrieved from the GeneCards Human Gene Database[45] and the Human Protein Atlas.[46]

Results

Characteristics of the study sample and distributions of the primary EDC measures are presented in Table 1. The primary analysis was focused on cord blood DNA collected at birth, with the 9- and 14-year DNA methylation data used to test for persistence of changes observed in cord blood results. Few mothers (5.5%) reported smoking during pregnancy, and mothers had a mean pre-pregnancy BMI of 26.8. Of the 309 children in the study sample, 154 were assigned male and 155 were assigned female at birth. Pearson correlation coefficients between the phenol and phthalate measures in our study sample are presented in Figure S1. The major clusters of correlation occur between the phthalate metabolites, particularly among the DEHP metabolites, with minimal correlation among the other EDC measures. BPA shows some evidence for minor correlation with several other EDCs, with the correlation coefficients ranging from −0.04 to 0.34. The associations between prenatal EDCs and cell proportions estimated from the Gervin reference panel are presented in Table S3. We found some evidence that prenatal BP3 concentrations are negatively associated with Bcell proportions (beta = −0.001, p = 0.03) and that the whole mixture is negatively associated with NK cell proportions (beta = −0.004, p = 0.02). No other EDC measures showed evidence of associations with estimated cell proportions.

DMP Analysis

Our initial analysis focused on identifying differentially methylated probes (DMPs) associated with the EDC measures in cord blood. Table 2 summarizes the genomic inflation

factor (λ), number of FDR-significant CpG sites, the number of Bonferroni significant CpG sites, as well as the number of Sidak-significant differentially methylated regions (DMRs) from each model series. We observed the highest number of DMPs in the male BPA models (58 FDR-significant hits) and the female paraben models (909 FDR-significant hits), with only suggestive results from the remaining models. In contrast, we observed several significant DMRs in most models. The p-value distributions from the various DMP analyses ranged from lower than expected, λ=0.76 in the male BP3 models and λ=0.67 in the male mixture models, close to the theoretical distribution, λ=1.04 for the male Triclosan models and λ=1.10 for the female BPA models, to higher than expected, λ=1.93 in the male BPA models and λ=1.88 in the female Paraben models. The sex-stratified analyses revealed associations between DNA methylation and BPA in males and Parabens in females, as well as several other suggestive associations. (Table 3 and Table S4)

In the male models, BPA was positively associated with methylation of cg24868359 ($beta_{\%\ change}$ = 0.30, p = 1.11E-07), a CpG site annotated to the *GRIK1* gene that encodes a member of the kainite family of glutamate receptors. Manhattan plots showing the full distribution of p-values across the genome from each of the DMP models are displayed in Supplemental Figure 2. The whole mixture was suggestively positively associated with DNA methylation in cg15756507 among males ($psi_{\%\ change}$ = 2.14, p = 5.91E-07), with MEHHP, Triclosan, and MCNP identified as the top positively weighted mixture components. (Figure S3) cg15756507 is annotated to *PITPNC1*, a gene involved in cell signaling and lipid metabolism.

Paraben exposure was associated with methylation at several sites in the female models, including a negative association with cg09143850 ($beta_{\%\ change}$ = −0.42, p = 1.35E-09), a CpG site annotated to the *ODZ4* gene which encodes a protein involved in establishing neuronal connectivity during development, and cg02881186 ($beta_{\%\ change}$ = −0.27, p = 6.15–09), a CpG site annotated to the *TRAPPC9* gene which codes for an NF-kappa-beta signaling related protein related to cognitive development.

In the overall models, several suggestive and significant associations were observed between BPA and individual CpG sites. BPA was positively associated with methylation at cg11981631 ($beta_{\%\ change}$ = 0.11, p = 1.67E-08), a CpG site annotated to the *ABCC8* gene which is expressed in beta cells of the pancreas and involved in insulin secretion. Other top hits from the BPA models included a positive association with cg06363569 ($beta_{\%\ change}$ = 0.16, p = 5.19E-08), a CpG site in the *RAB18* gene which encodes a ras-related protein highly expressed in the brain, as well as a positive association with cg22384923 ($beta_{\%\ change}$ = 0.10, p = 1.06E-07), a CpG site in the *LHX4* gene which codes for a transcription factor implicated in development of the pituitary gland. Paraben exposure was negatively associated with methylation in cg12492380 ($beta_{\%\ change}$ = −0.24, p = 1.32E-07), a CpG site in *MYH16* which is a myosin heavy chain pseudogene.

Only 5 CpGs appear as at least suggestive (unadjusted p-value < 10e-06) in multiple models, generally for an individual pollutant's overall model and the same pollutant's male or female model. These CpG sites included 3 sites appearing in both the male and overall BPA models (cg24868359, cg21144063, cg15186648), 1 site appearing in both the female

and overall BPA models (cg06363569), and 1 site appearing in both the female and overall Paraben models (cg01491225). The lack of any shared suggestive CpGs between both male and female models suggests that sex-specific associations may be driving the observed associations in the overall models.

DMR Analysis

We then utilized *comb-p* to evaluate associations between the EDC exposures and differentially methylated regions (DMRs) throughout the epigenome, with the top hits summarized in Table 4 and the complete summaries presented in Table S5.

In the male models, the top DMR for BPA contained 4 CpG sites and mapped to the *TTLL10* gene region, which encodes a protein involved in protein-glycine ligase activity that is highly expressed in the testes. Top DMRs for Triclosan included a DMR containing 8 CpG sites mapping to the *C1orf65* gene region and a DMR containing 9 CpG sites mapping to the *FOXR1* gene region. The top DMR for BP3 contained 6 CpG sites and mapped to the *NXPH2* gene region. The top DMR for the whole mixture contained 10 CpGs and mapped to *NHEDC1*, which encodes an Na/H exchange protein highly expressed in the testes.

In the female models, the top DMR for BPA contained 37 CpG sites and mapped to the *RNF39* gene region, a gene in the MHC region of chromosome 6 that plays a role in synaptic plasticity. The top DMR for Triclosan contained 32 CpG sites and mapped to the *PPT2;PRRT1* genomic regions. Top DMRs for Parabens included a DMR containing 4 CpG sites mapping to the *CFDP1* gene region and a DMR containing 27 CpG sites mapping to the *BLCAP;NNAT* gene regions, which encode proteins involved in cell growth regulation and brain development.

In the overall models, the top DMR for Triclosan contained 32 CpG sites and mapped to the *PPT2;PRRT1* gene regions, the same region found in the female Triclosan models. The top DMR for BP3 contained 8 CpG sites and mapped to the *KLK7* gene region. The top DMR for Parabens contained 6 CpG sites and mapped to the *LOC404266;HOXB6* gene regions. Similar to the findings from the DMP analysis, DMRs identified in multiple models generally only appeared in an individual pollutant's overall model and the same pollutant's male or female model. Genes identified in multiple DMRs included *AIRE* (male Triclosan and overall Triclosan models), *CMYA5* (female Triclosan and overall triclosan models), *PPT2;PRRT1* (female Triclosan and overall Triclosan), *NXPH2* (male BP3 and overall BP3 models), *KLK7* (male BP3 and overall BP3 models), and *TNXB* (female Paraben and overall Paraben models).

KEGG Pathway Enrichment

To better understand the functions of associations found in our analysis, CpG sites with FDR values < 0.05 and CpG sites from Sidak-significant DMRs were used for KEGG pathway enrichment analysis, the results of which are shown in Tables S6 and S7. From the DMP models, some of the top enriched pathways among males in response to BPA included motor proteins (p=2.42e-03), cytokine-cytokine receptor interaction (p=1.92e-02), taurine/hypotaurine metabolism (p=3.61e-02), and thiamine metabolism (p=4.64e-02). Some of the top enriched pathways among females in response to Parabens

included oxytocin signaling (p=7.67e-04), gastric acid secretion (p=7.62e-04), long-term potentiation (p=8.50e-03), aldosterone synthesis/secretion (p=1.97e-02), and polycomb repressive complex (p=5.25e-03).

From the DMR models, some of the top enriched pathways among males in response to BP3 included glucagon signaling (p=1.11e-04) and HIF-1 signaling (p=1.66e-04). The top enriched pathways among males in response to Triclosan included primary immunodeficiency (p=8.90e-03) and ubiquitin mediated proteolysis (p=4.53e-02). Some of the top enriched pathway among females in response to BPA was one carbon pool by folate (p=5.36e-03). Some of the top enriched pathways among females in response to Triclosan included fatty acid elongation (p=5.97e-03) and fatty acid metabolism (p=1.38e-02). Some of the top enriched pathways among females in response to Parabens included ECM-receptor interaction (p=2.44e-02) and chemical carcinogenesis - DNA adducts (p=4.25e-02). Only glucagon signaling and HIF-1 signaling pathways in response to BP3 exposure in males remained significant after FDR-adjustment.

Persistence

To test for the persistence of changes in DNA methylation observed at birth, we reran models for all suggestive hits from the DMP models using participants with available data at age 9 and 14. Model coefficients and 95% confidence intervals for all CpG sites showing evidence for persistence are presented in Figure 1, with model summaries also highlighted in Table 5. Most associations tended to attenuate towards the null with widened confidence intervals by age 9 and 14, with a few notable exceptions. In the male models, 3 CpGs located in unannotated regions of chromosome 7 remained associated with BPA exposure at 9 and 14 years, 1 CpG in *RFPL2* remained associated with BPA at 9 and 14 years, and 1 CpG in *PITPNC1* remained associated with the whole mixture at 14 years. In the female models, 1 CpG located in *CFDP1* remained associated with Parabens at 9 and 14 years and 1 CpG in *PDE7A* remained associated with Parabens at 14 years. In the overall models, 1 CpG located in an unannotated region of chromosome 7 remained associated with BPA at 9 and 14 years, 1 CpG located in *TRIM26* remained associated with BPA at 14 years, and 1 CpG in *SDK1* remained associated with BPA at 14 years.

Discussion

Our study systematically characterized associations between prenatal exposure to environmental phenols and phthalates and DNA methylation at birth in cord blood, including an examination of both differentially methylated CpGs and differentially methylated regions (DMRs), across the epigenome. We also examined associations between a phenol/phthalate mixture and DNA methylation using an EWAS application of quantile g-computation. Within our study sample, we found evidence for sex-specific associations between phenol exposure and DNA methylation. Namely, differential methylation in response to prenatal BPA exposure in males and Parabens in females. Additionally, we were able to examine the persistence of changes in DNA methylation within our study sample in peripheral blood at ages 9 and 14, finding several CpGs that maintained associations with prenatal

EDC exposure into adolescence, which might be relevant for developmental and disease biomarkers.

The top DMP in the male BPA models was a Bonferroni-significant positive association with a CpG site in *GRIK1*, which encodes a subunit of a member of the kainate family of glutamate receptors. DNA methylation in *GRIK1* has been previously found to be positively associated with BMI in an EWAS of peripheral blood in an older adult population with an average age of greater than 60 years.[47] BPA exposure has also been found to be associated with higher risk of obesity,[48] which may help explain the shared positive associations between BPA and BMI with methylation in *GRIK1*. The top DMR among females in response to BPA was located in *RNF39*, which was also previously found to be differentially methylated in response to prenatal MEHP exposure in the CHAMACOS cohort by Solomon et al[26] as well as in response to high molecular-weight phthalate exposure in the APrON study cohort.[49] Other FDR-significant CpG sites in the male BPA models included greater methylation in a CpG site in *CXCL14*, a gene involved in immunoregulatory processes, greater methylation in a CpG in *AK7*, a gene involved in energy homeostasis and ciliary function, and greater methylation in a CpG in *OXTR*, which encodes an oxytocin receptor. *CXCL14* has been suggested to act as a tumor suppressing chemokine, with increased methylation in the promoter region being linked to progression of oral carcinoma cells.[50] BPA has also been suggested to influence oxytocin signaling in the hypothalamus in rodent models, with higher susceptibility in males.[51] Oxytocin signaling was additionally found to be among the top enriched KEGG pathways in the female Paraben models in our analysis. Our findings of increased methylation in *OXTR* in response to BPA exposure in males and enrichment of the oxytocin signaling pathway in response to Parabens in females warrants further examination of the potential influences of BPA on oxytocin signaling in human populations.

Bonferroni-significant DMPs in the female Paraben models included CpG sites in *ODZ4* (also known as *TENM4*), a gene involved in established neuronal connectivity, and *TRAPPC9*, an NF-kappa-B signaling-related protein involved in cognitive development. The potential neurocognitive impacts of parabens remains poorly understood, but previous studies have highlighted potential associations between prenatal paraben exposure and lower mental developmental index scores among girls,[52] as well as between prenatal Phenol/Paraben mixtures and increased risk of non-typical development in children.[53] Another Bonferroni-significant site in the female Paraben models was located in the *PDE8A* gene, which encodes a cyclic nucleotide phosphodiesterase that has also been previously found to be differentially methylated in response to prenatal BPA exposure.[22] We identified a DMR associated with Paraben exposure among females in the *TNXB* gene region, which encodes a glycoprotein that mediates interactions between cells and the extracellular matrix. DNA methylation in *TNXB* has been previously found to be associated with prenatal Triclosan exposure in placental tissue in the EDEN cohort,[27] social anxiety disorder in a population of young adults,[54] prenatal per- and polyfluoroalkyl substance exposure in the Michigan Mother Infant Pairs cohort,[55] and with prenatal manganese/magnesium exposure in the Project Viva cohort.[56] One of the top enriched KEGG pathways associated with Paraben exposure among females was aldosterone synthesis and secretion, which may have relevant implications for blood pressure regulation in response to Paraben exposure. The potential

influence of Parabens on aldosterone signaling remains a minimally understood topic, but a previous study utilizing male rats found that Methyl and Propyl Paraben exposure starting at 42 days of age was associated with increased serum aldosterone 30 days later.[57]

The top DMR for the whole EDC mixture in males mapped to *NHEDC1* (also known as *SLC9B1*), which encodes a Na/H exchange protein. Altered methylation patterns in *NHEDC1* have been previously found to be predictive of fetal intolerance of labor, a common indication for emergency Caesarean section.[58] Disruptions of the intrauterine environment and negative birth outcomes are commonly reported mechanisms related to the EDCs examined here,[59,60,61,62] so our finding of differential methylation in *NHEDC1* in association with the whole EDC mixture may reflect an epigenetic response of the infants to alterations in the intrauterine environment resulting from EDC exposure. One of the top DMRs among males in response to Triclosan was located in *PRDM8*, which encodes a histone methyltransferase that was also previously found to be differentially methylated in response to prenatal MBzP exposure in the CHAMACOS cohort.[26] BP3 was associated with a DMR in the *RWDD3* gene region among males, which was also found to be associated with BPA exposure among females in the Hokkaido Study on Environment and Children's Health cohort.[20] Among males, a suggestive association was found between BP3 and a CpG in *TCERG1L*, which encodes a regulator of transcription. *TCERG1L* has previously been found to exhibit hypomethylation and increased expression in spermatozoa from infertile male patients compared to fertile controls.[63] Interestingly, we also observed a negative association between prenatal BP3 exposure and estimated Bcell proportions, as well as a negative association between exposure to the whole EDC mixture and estimated NK cell proportions, which suggests potential influence of prenatal EDC exposure on immune cell proportions in cord blood, warranting further research. The only KEGG pathways found to be enriched after FDR adjustment, glucagon signaling and HIF-1 signaling, were found with DMRs in response to BP3 exposure among males. The potential impacts of BP3 on these pathways remains a relatively unexplored topic, but prior research has revealed inhibition of glucagon signaling in response to related compounds like triclosan in mouse cell lines,[64] as well as the triggering of ferroptosis via HIF-1a related mechanisms in response to DEHP exposure in mouse Leydig and Sertoli cells.[65]

We found that some changes in DNA methylation detected in cord blood persisted into adolescence. cg25125450, located in *RFPL2*, retained a negative association with BPA exposure into age 14 in males. Methylation in *RFPL2*, which encodes a protein involved in transcriptional regulation, has also previously been found to be associated with prenatal docosahexaenoic acid (DHA) supplementation.[66] cg15756507, the top hit in the male mixture models, is located in *PITPNC1* and retained a positive association with exposure to the whole EDC mixture into age 14 in males. *PITPNC1* encodes a protein involved in cell signaling and lipid metabolism, and *PITPNC1* overexpression has been observed in metastatic breast, melanoma, and colon cancer tissue.[67] cg03011941, located in *CFDP1*, and cg10252135, located in *PDE7A*, both retained positive associations with Paraben exposure into age 14 in females. *CFDP1* encodes a protein involved in cell adhesion and regulation of cell shape, and *PDE7A* encodes a protein involved in cAMP messaging. cg21144063, cg03873153, and cg00749672, all located in intergenic regions of chromosome 7, remained associated with BPA exposure into age 14 in males. Our study utilized cord blood at birth

for the primary analysis and peripheral blood obtained during childhood to test for the persistence of differential methylation. These two sample types can exhibit fairly distinct DNA methylation profiles, and cord blood samples routinely contain measurable portions of nucleated red blood cells that are normally not found in older samples. Despite differences between cord blood and child blood, previous research has found that DNA methylation levels generally tend to stay stable between birth and within the first two years of life.[68]

Our findings are subject to a few relevant limitations. First, our power was limited by relatively modest sample size for an EWAS. This reduced power was especially apparent in the mixture analysis, likely due to the quantization of exposures. Despite this limitation, we were still able to find several associations between environmental phenol exposure and DNA methylation. Relatedly, small sample size may also lead to a higher proportion of false positives, highlighting the need to study the impact of EDC exposure on DNA methylation in larger study samples and additional cohorts. Second, our study sample captures exposures among a predominantly Latino farmworker community in California, which may influence the generalizability of our results to the general population. However, our work contributes to the ongoing necessity of improving the diversity of environmental epigenetics research study populations. Thirdly, our analysis suffered from relatively high deviation of observed p-values from the theoretical p-value distribution for several EDCs. Interestingly, substantial deviation of the observed genomic inflation factor from 1.0 appears to be common among epigenome-wide association studies of EDCs.[20,22,25,27,69,70] This might stem from limited sample size in each study, or point to widespread weak or correlated associations across the epigenome which meta-analyses might be able to clarify. Additionally, we were unable to test for potential mediators, like gestational age at birth or oxidative stress, in the relationship between EDC exposure and epigenetic changes in children, which provides an interesting direction for future research. Lastly, our reliance of quantile g-computation for the evaluation of the mixture effect can be considered a limitation. Several other mixture methods, including Bayesian Kernal Machine Regression, utilize more nuanced definitions of a mixture effect and are capable of modeling more complex exposure-response relationships. However, the primary advantage of quantile g-computation in this context is the provision of a highly computationally efficient and readily interpretable mixture method that can be applied on an epigenome-wide scale.

Our study also features several strengths. Firstly, modeling of individual pollutants, although informative, may not represent true exposure scenarios where individuals are simultaneously exposed to several related pollutants. To address this, we complemented our individual pollutant analysis with an application of mixture methods to an EWAS analysis, which allowed us to examine the joint impact of a phenol/phthalate mixture on DNA methylation. Second, urinary levels of phenols and phthalates can vary greatly over time, and our use of two urinary measures may have provided a more accurate measurement of average pregnancy exposure relative to analyses relying on only a single exposure measurement. Thirdly, the longitudinal nature of the CHAMACOS cohort also allowed us to examine the persistence of observed changes in DNA methylation at birth into adolescence within the same study sample, something that is rarely assessed in other epigenetics studies.

Prenatal exposure to several environmental phenols has been linked to pregnancy and childhood health outcomes. Epigenetic marks provide a key biomarker capable of linking prenatal exposures to outcomes later in life, as well as clarifying biological mechanisms of action. We identified associations of prenatal exposure to four major environmental phenols (BPA, Triclosan, BP3, and Parabens) and a phenol/phthalate mixture with DNA methylation at birth, finding evidence of sex-specific associations of prenatal BPA exposure in males and Parabens in females, as well as several associations that persisted into adolescence. These findings contribute to the ongoing necessity of characterizing the impact of EDCs on health and development in human populations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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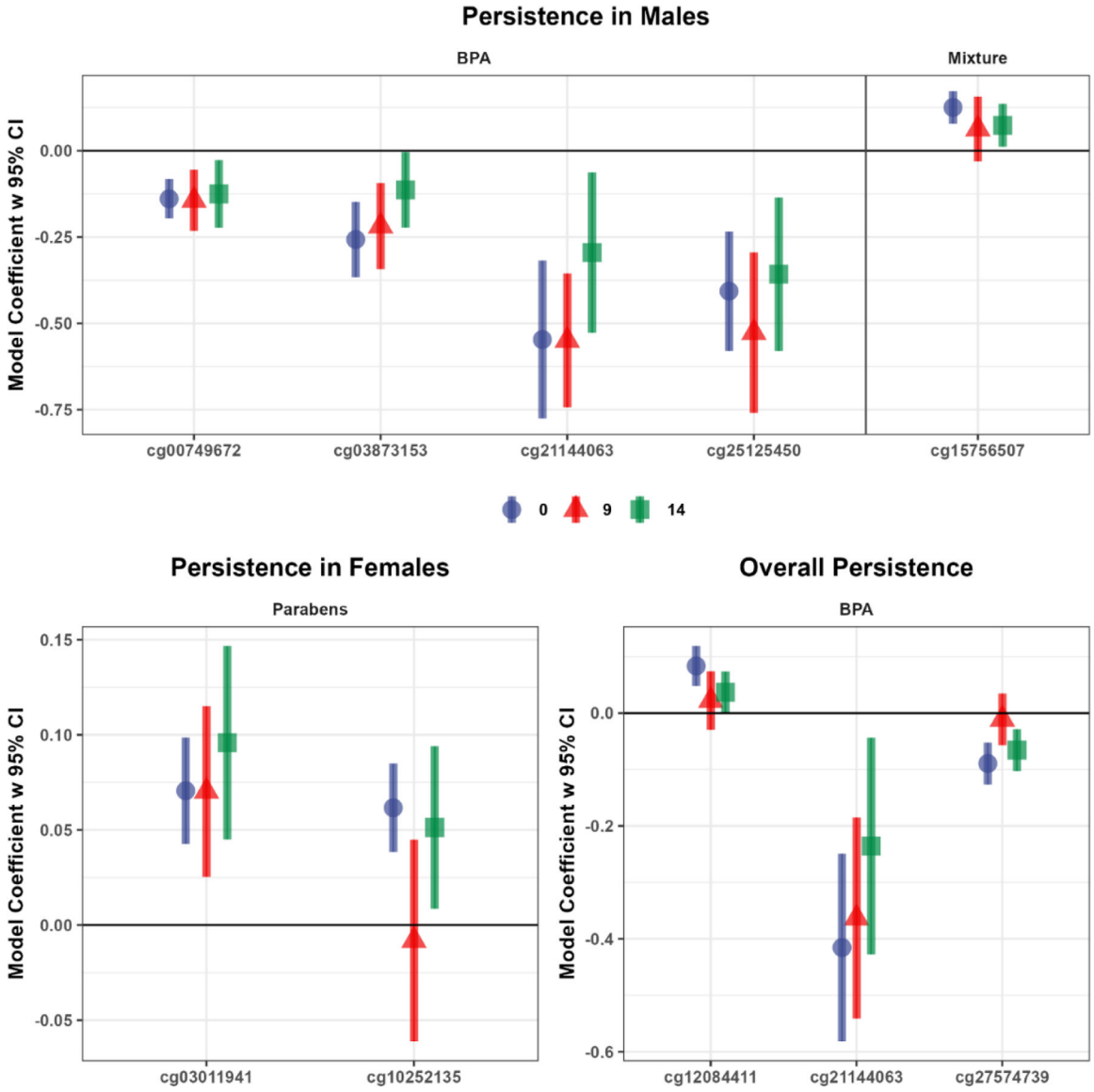

Figure 1:

Persistence of DNA Methylation Changes at Age 9 and 14. Cord blood beta estimates (blue), 9-year estimates (red) and 14-year estimates (green) from individual pollutant models are shown. Effect estimates correspond to beta coefficients for individual pollutant models and psi estimate for mixture model, reflecting expected change in methylation M-value.

Table 1:

Study Sample Characteristics. Mean (standard deviation) reported for continuous demographic variables and count (percentage) reported for categorical demographic variables.
Median and interquartile range (IQR) reported for EDC measures. Primary analysis utilized the Birth timepoint. The Age 9 and Age 14 timepoints were used to test for persistence of differential methylation observed at birth, and their corresponding columns display sample characteristics for the subsets of the Birth timepoint with data available at the specified timepoints.

Study Characteristics	Birth	Age 9	Age 14
Sample Size (N)	309	156	150
Child Sex			
Male	154 (49.8%)	70 (44.9%)	67 (44.7%)
Female	155 (50.2%)	86 (55.1%)	83 (55.3%)
Maternal Education			
≤ 6th grade	125 (40.5%)	61 (39.1%)	58 (38.7%)
7th-12th grade	113 (36.6%)	62 (39.7%)	58 (38.7%)
>=High School Graduate	71 (23.0%)	33 (21.2%)	34 (22.7%)
Pregnancy Smoking			
Yes	17 (5.5%)	5 (3.2%)	6 (4.0%)
No	292 (94.5%)	151 (96.8%)	144 (96.0%)
Gestational Age (days)	276.4 (10.4)	276.2 (9.7)	275.6 (10.2)
Maternal BMI	26.8 (5.3)	27.2 (5.6)	27.5 (5.8)
DNAm Array	450K	450K	EPIC
BPA (ng/mL)	1.32 (0.93, 2.14)	1.31 (0.93, 2.16)	1.35 (0.92, 2.06)
Triclosan (ng/mL)	24.11 (6.40, 123.52)	26.64 (5.84, 167.36)	23.89 (6.17, 161.10)
BP3 (ng/mL)	15.17 (4.62, 135.45)	15.71 (4.60, 121.35)	14.71 (4.38, 99.46)
Parabens (nmol/L)	1746.93 (619.03, 3924.34)	1627.03 (520.43, 3964.29)	1826.58 (624.45, 3912.98)

Table 2:

Differentially methylated probes and regions summary. Number of CpG sites with FDR values < 0.05, number of Bonferroni significant hits, and the genomic inflation factor (λ) from each DMP model series are presented. The number of Sidak-significant differentially methylated regions (DMRs) for each model series is also presented.

	Male				Female				Overall			
	λ	**FDR<0.05**	**Bonferroni**	**DMRs**	**λ**	**FDR<0.05**	**Bonferroni**	**DMRs**	**λ**	**FDR<0.05**	**Bonferroni**	**DMRs**
BPA	1.93	58	1	1	1.10	0	0	7	2.48	36	4	0
Triclosan	1.04	0	0	5	1.47	0	0	4	0.78	0	0	13
BP3	0.76	0	0	19	0.96	0	0	0	0.77	0	0	5
Parabens	0.93	0	0	0	1.88	909	10	41	2.04	3	0	4
Mixture	0.67	0	0	4	2.12	0	0	1	1.34	0	0	3

Table 3:

Top differentially methylated probes. Effect estimates are presented as expected change in percent methylation to ease interpretation. Original M-value based beta coefficients and psi coefficients are presented in Supplemental Table 4. All models were adjusted for cell proportions, gestational age at birth, maternal education, maternal BMI, maternal smoking during pregnancy, as well as child sex in the overall models. Unadjusted p-values (P Value) and FDR adjusted p-values (FDR) are presented. * Sites with Bonferroni p-value < 0.05

EDC	probeID	Adjusted % Change	P Value	FDR Value	Gene
Male					
BPA	cg24868359*	0.30	1.11E-07	3.75E-02	*GRIK1*
BPA	cg06897282	1.09	3.29E-07	3.75E-02	
BPA	cg01821923	0.52	4.64E-07	3.75E-02	*CXCL14*
BPA	cg17504464	0.20	4.67E-07	3.75E-02	*AK7*
Triclosan	cg23831217	0.13	9.92E-07	2.72E-01	*TEX12*
BP3	cg11903920	0.64	5.55E-07	2.16E-01	*TCERG1L*
Parabens	cg13379794	0.16	3.76E-07	9.72E-02	
Mixture	cg15756507	2.14	5.91E-07	2.30E-01	*PITPNC1*
Female					
BPA	cg05149307	0.22	3.43E-07	1.33E-01	*RASAL1*
Triclosan	cg06905499	-0.07	1.44E-07	5.60E-02	*MCF2L*
BP3	cg04773926	-0.14	3.23E-07	1.26E-01	*TTYH3*
Parabens	cg18254183*	-0.55	5.46E-12	2.12E-06	
Parabens	cg26723624*	-0.12	7.46E-10	1.45E-04	
Parabens	cg09143850*	-0.42	1.35E-09	1.76E-04	*ODZ4*
Parabens	cg02881186*	-0.27	6.15E-09	5.98E-04	*TRAPPC9*
Parabens	cg09425164*	-0.46	9.72E-09	7.57E-04	*PDE8A*
Parabens	cg23313005*	-0.18	3.15E-08	2.05E-03	*FAM193B*
Overall					
BPA	cg11981631*	0.11	1.67E-08	6.49E-03	*ABCC8*
BPA	cg02360980*	0.05	4.84E-08	6.73E-03	*LTK*
BPA	cg06363569*	0.16	5.19E-08	6.73E-03	*RAB18*
BPA	cg22384923*	0.10	1.06E-07	1.03E-02	*LHX4*
Parabens	cg12492380	-0.24	1.32E-07	3.93E-02	*MYH16*
Parabens	cg09032010	0.07	2.95E-07	3.93E-02	*RP5-1022P6.2*
Parabens	cg04807855	0.04	3.03E-07	3.93E-02	*SPR*
Parabens	cg01491225	0.02	8.61E-07	8.38E-02	*RNU5E;RNU5D;ZCCHC9*

Table 4:

Top Differentially Methylated Regions (DMR) Summary. Location (chromosome, start position, and end position) for each DMR, along with the sidak p-value, number of probes in the DMR, and overlapping genes within the DMR from each model series.

	Chr.	start	end	Sidak	N Probes	Overlapping Genes
Male						
BPA	1	1108819	1109012	6.14E-07	4	*TTLL10*
Triclosan	1	223566446	223567002	1.52E-10	8	*C1orf65*
Triclosan	11	118842229	118842650	4.82E-06	9	*FOXR1*
BP3	2	139538221	139539181	<2.2E-16	6	*NXPH2*
BP3	6	33872205	33873732	1.13E-13	9	
Mixture	4	103940710	103941205	1.61E-09	10	*NHEDC1*
Mixture	8	19459995	19460243	1.49E-07	6	*CSGALNACT1*
Female						
BPA	6	30038753	30039600	1.07E-09	37	*RNF39*
BPA	6	30419490	30419612	1.09E-06	6	
Triclosan	6	32120622	32121555	<2.2E-16	32	*PPT2;PRRT1*
Triclosan	12	1772229	1772504	3.79E-09	3	
Parabens	16	75468056	75468318	1.74E-09	4	*CFDP1*
Parabens	20	36148374	36149121	1.32E-09	27	*NNAT;BLCAP*
Parabens	6	32064205	32064785	4.12E-08	23	*TNXB*
Mixture	6	30038858	30039442	1.07E-06	30	*RNF39*
Overall						
Triclosan	6	32120622	32121555	<2.2E-16	32	*PPT2;PRRT1*
Triclosan	5	78985424	78985900	3.36E-12	10	*CMYA5*
BP3	19	51487108	51487478	1.41E-06	8	*KLK7*
BP3	8	144120398	144120706	1.94E-05	6	*C8orf31*
BP3	2	139538600	139539001	1.52E-05	3	*NXPH2*
Parabens	17	46682307	46682413	1.19E-06	6	*HOXB6;LOC404266*
Parabens	8	514735	514821	2.33E-05	3	
Mixture	13	113296191	113296447	1.14E-05	4	
Mixture	16	1583809	1584118	3.95E-05	6	*IFT140;TMEM204*

Table 5:

Persistence Summary Table. CpG sites that remain significant (unadjusted p-value <0.05) with effect estimates in the same direction at Age 9 or Age 14. Effect estimates correspond to beta coefficients reflecting expected change in methylation M-value from the individual pollutant models and psi estimates from the mixture models. Birth models were adjusted for cell proportions, gestational age at birth, maternal education, maternal BMI, maternal smoking during pregnancy, as well as child sex in the overall models, while childhood models were adjusted for cell proportions, gestational age at birth, maternal education, maternal BMI, as well as child sex in the overall models.

			Birth		Age 9		Age 14	
probeID	**Gene**	**EDC**	**Effect**	**p-value**	**Effect**	**p-value**	**Effect**	**p-value**
Male								
cg21144063		BPA	**-0.55**	**2.76E-06**	**-0.55**	**2.75E-08**	**-0.29**	**1.29E-02**
cg25125450	*RFPL2*	BPA	**-0.41**	**3.94E-06**	**-0.53**	**8.77E-06**	**-0.36**	**1.61E-03**
cg03873153		BPA	**-0.26**	**3.69E-06**	**-0.22**	**5.74E-04**	**-0.11**	**4.16E-02**
cg00749672		BPA	**-0.14**	**1.93E-06**	**-0.14**	**1.44E-03**	**-0.13**	**1.23E-02**
cg15756507	*PITPNC1*	Mixture	**0.13**	**5.91E-07**	0.06	1.96E-01	**0.07**	**2.47E-02**
Female								
cg03011941	*CFDP1*	Parabens	**0.07**	**7.42E-07**	**0.07**	**2.16E-03**	**0.10**	**2.24E-04**
cg10252135	*PDE7A*	Parabens	**0.06**	**2.14E-07**	-0.01	7.62E-01	**0.05**	**1.87E-02**
Overall								
cg21144063		BPA	**-0.42**	**9.75E-07**	**-0.36**	**6.44E-05**	**-0.24**	**1.62E-02**
cg12084411	*TRIM26*	BPA	**0.08**	**4.19E-06**	0.02	4.02E-01	**0.04**	**4.90E-02**
cg27574739	*SDK1*	BPA	**-0.09**	**2.35E-06**	-0.01	6.37E-01	**-0.07**	**5.03E-04**