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Prenatal phthalate exposure and altered patterns of DNA methylation in cord blood

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Abstract

Epigenetic changes such as DNA methylation may be a molecular mechanism through which environmental exposures affect health. Phthalates are known endocrine disruptors with ubiquitous exposures in the general population including pregnant women, and they have been linked with a number of adverse health outcomes. We examined the association between *in utero* phthalate exposure and altered patterns of cord blood DNA methylation in 336 Mexican-American newborns. Concentrations of 11 phthalate metabolites were analyzed in maternal urine samples collected at 13 and 26 weeks gestation as a measure of fetal exposure. DNA methylation was assessed using the Infinium HumanMethylation 450K BeadChip adjusting for cord blood cell composition. To identify differentially methylated regions (DMRs) that may be more informative than individual CpG sites, we used two different approaches, *DMRcate* and *comb-p*. Regional assessment by both methods identified 27 distinct DMRs, the majority of which were in relation to multiple phthalate metabolites. Most of the significant DMRs (67%) were observed for later pregnancy (26 weeks gestation). Further, 51% of the significant DMRs were associated with the di-(2-ethylhexyl) phthalate metabolites. Five individual CpG sites were associated with phthalate metabolite concentrations after multiple comparisons adjustment (FDR), all showing hypermethylation. Genes with DMRs were involved in inflammatory response (*IRAK4* and *ESM1*), cancer (*BRCA1* and *LASP1*), endocrine function (*CNPY1*), and male fertility (*IFT140, TESC*, and *PRDM8*). These results on differential DNA methylation in newborns with prenatal phthalate exposure provide new insights and targets to explore mechanism of adverse effects of phthalates on human health.

Keywords

in utero exposure; phthalates; children; epigenetics; 450K

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Statement of author contributions

Drs. Holland and Huen conceived and designed the study. Dr. Yousefi and Ms. Escudero-Fung prepared biological samples and conducted experiments. Ms. Solomon and Dr. Yousefi performed the data analysis. Ms. Solomon prepared the manuscript with important intellectual contribution from Drs. Holland, Huen, Eskenazi, Barcellos and Gunier. Dr. Eskenazi is the principle investigator for the CHAMACOS cohort. All authors approved the final manuscript.

Conflict of interest

All authors declare no conflict of interest.

INTRODUCTION

Phthalates are widely used in plastics, personal care products, and other household items, leading to widespread exposure to these chemicals through diet, inhalation, and dermal absorption [CDC 2009]. Phthalates have been previously determined to be endocrine disruptors, and exposure has been found to be associated with a number of adverse health outcomes. In particular, *in utero* exposure assessed in prospective studies has been linked with pre-term delivery [Ferguson et al. 2014], pre-eclampsia [Cantonwine et al. 2016], decreased birth size [Whyatt et al. 2009], sex-specific changes to childhood growth and blood pressure [Valvi et al. 2015], poorer neurodevelopment [Engel et al. 2010; Kim et al. 2011; Factor-Litvak et al. 2014], and decreased male reproductive health [Cai et al. 2015; Swan et al. 2015] . Moreover, maternal and paternal preconception exposure to phthalates has been associated with poorer birth outcomes [Smarr et al. 2015], and exposure in 6-8 year old girls has shown a relationship with BMI and waist circumference increase at 7-13 years [Deierlein et al. 2016]. Cross-sectional studies also demonstrated an association between phthalate exposure and thyroid hormone levels during pregnancy[Huang et al. 2016] and with adult body composition [Corbasson et al. 2016]. Overall, prenatal and lactational exposure to phthalates have been associated with endocrine disrupting effects in animals and adverse birth outcomes in humans, indicating that early life phthalate exposures may contribute to the fetal origins of disease.

Epigenetic modification may be a potential biological mechanism through which exposures can affect health outcomes later in life [Bakulski and Fallin 2014; Breton et al. 2017]. Several animal studies have demonstrated relationships between phthalate exposure and changes to DNA methylation. One study found that *in utero* exposure to di-(2-ethylhexyl) phthalate (DEHP) affected testicular function in rats through changes in DNA methylation [Sekaran and Jagadeesan 2015]. Another study found *in utero* exposure to DEHP was associated with both transgenerational DNA methylation in rat sperm and testicular and prostate diseases [Manikkam et al. 2013], while another study found that *in utero* exposure to DEHP alters DNA methylation throughout the epigenome, particularly in CpG islands [Martinez-Arguelles and Papadopoulos 2015].

Few data are available on the relationship between phthalate exposure and epigenetic modifications in humans. One cross-sectional study showed that mono(2-ethylhexyl) phthalate (MEHP) exposure in children may increase asthma risk through changes to DNA methylation in several candidate genes [Wang et al. 2015]. *In utero* phthalate metabolite concentrations of high molecular weight (HMW), low molecular weight (LMW), and DEHP have been associated with differential methylation of *H19* and *IGF2* imprinted genes and LINE-1 repetitive elements [LaRocca et al. 2014]. Previously we reported that *in utero* exposure to mono-ethyl phthalate (MEP) was related to hypomethylation of Alu and LINE-1 repeats in cord blood. Additionally, higher levels of DEHP metabolites were also associated with hypomethylation of Alu repeats in 9 year old children [Huen et al. 2016].

The purpose of this study using 450K BeadChip analysis is to determine whether *in utero* phthalate exposure is associated with differential methylation in cord blood collected from the participants of the Center of Health Assessment of Mothers and Children of Salinas

(CHAMACOS) with a primary focus on regional changes that are recently being recognized as potentially more informative than individual CpG sites.

METHODS

Study participants

Subjects were participants in the CHAMACOS longitudinal birth cohort study examining the impact of pesticide and other environmental exposures on the health and development of Mexican-American children living in the Salinas Valley, an agricultural region in California. A detailed description of the CHAMACOS cohort has previously been published [Eskenazi et al. 2003]. Women were eligible for study if they were Spanish or English speaking, at least 18 years of age, less than 20 weeks gestation, and were receiving prenatal care at community clinics. From 1999-2000, 601 pregnant women were enrolled and 526 women were followed to their delivery of a liveborn singleton. Women were interviewed at two visits during pregnancy at an average of 13.4 (SD=5.2) and 26.0 (SD=2.7) weeks gestation, and after delivery. For this study, 336 mother-child pairs with both prenatal phthalate metabolite and Illumina HumanMethlyation 450K BeadChip data available in cord blood were included. Study protocols were approved by the University of California, Berkeley Committee for Protection of Human Subjects and written informed consent was obtained from all mothers.

Blood collection and processing

Cord blood specimens were collected by hospital staff at time of delivery. Blood samples were collected in vacutainers both with and without heparin (green- and red-top), and separated into clots, serum, buffy coats, and stored at −80°C until analysis.

Phthalate metabolite measurements

Eleven phthalate metabolites were quantified in prenatal urine samples collected from mothers at the two pregnancy visits as previously described [Holland et al. 2016; Huen et al. 2016]. They included three low molecular weight (LMW) metabolites (MEP, MBP, MiBP), four high molecular weight (HMW) metabolites of DEHP (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)) and four additional HMW metabolites of other parent phthalates (MBzP, mono(3-carboxypropyl) phthalate (MCPP), monocarboxyoctyl phthalate (MCOP), monocarboxynonyl phthalate (MCNP)). Measurements were performed using online solid phase extraction coupled with isotope dilution high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry [Silva et al. 2007]. Quality control procedures included the use of laboratory blanks, calibration standards, and spiked controls with high and low concentrations. The limits of detection (LOD) for all metabolites were previously reported [Silva et al. 2007; Parlett et al. 2013; Holland et al. 2016; Huen et al. 2016]. Concentrations below the LOD with no corresponding instrumental signal were imputed from a log-normal distribution using the “fill-in” method described in Lubin et al. [Lubin et al. 2004]. For other concentrations below the LOD, the actual instrument reading value was used (Table SI). Summary measurements (e.g., ∑LMW, ∑HMW and ∑DEHP) were created as described

elsewhere [Zota et al. 2014]. Briefly, molar concentrations were calculated by dividing the concentration of each metabolite by its molecular weight. For each summary measure, the molar concentrations for each group were summed and then multiplied by the average molecular weight of the metabolites in that group to yield measurements expressed in μg/L. To account for urinary dilution, urinary creatinine was determined using a commercially available diagnostic enzyme method (Vitros CREA slides; Ortho Clinical Diagnostics, Raritan, NJ). Phthalate metabolite concentrations (μg/L) were divided by creatinine levels (g/L) to yield creatinine adjusted phthalate metabolite concentrations expressed in μg/g creatinine that were used for descriptive analyses. Previous studies have shown that the use of creatinine or specific gravity adjusted phthalate metabolite concentrations in regression models may introduce bias [Barr et al. 2005]. Therefore, in the separate linear models for 13 and 26 week time points, we used unadjusted phthalate metabolite concentrations and included maternal creatinine levels as a covariate to adjust for urinary dilution. As previously reported for global DNA methylation in CHAMACOS newborns, we also explored using specific gravity for adjustment and the findings did not change [Huen et al. 2016].

DNA preparation

DNA was isolated from the banked non-heparinized umbilical cord blood clot samples using QIAamp DNA Blood Maxi Kits (Qiagen, Valencia, CA) according to the manufacturer's protocol with minor modifications as previously described [Holland et al. 2006].

450K BeadChip DNA methylation analysis

DNA aliquots of 1μg were bisulfite converted using Zymo Bisulfite conversion Kits (Zymo Research, Orange, CA). DNA was whole genome amplified, enzymatically fragmented, purified, and applied to the Illumina Infinium HumanMethylation450 BeadChips (Illumina, San Diego, CA) according to the Illumina methylation protocol [Bibikova et al. 2011; Sandoval et al. 2011] . 450K BeadChips were handled by robotics and analyzed using the Illumina Hi-Scan system. DNA methylation was measured at 485,512 CpG sites. Samples were run on 36 distinct BeadChips across 5 plate batches and were assigned randomized locations across chips and plates.

Probe signal intensities were extracted by Illumina GenomeStudio software (version XXV2011.1, Methylation Module 1.9) methylation module and background subtracted. Extensive quality assurance and control measures were performed, including assessment of assay repeatability and batch effects using 38 technical replicates as previously described [Yousefi et al. 2013]. The All Sample Mean Normalization (ASMN) algorithm was used to adjust for color channel bias, batch effects, and difference in Infinium chemistry [Yousefi et al. 2013]. This was followed by Beta Mixture Quantile (BMIQ) normalization for differences between type I and type II probes [Teschendorff et al. 2013]. Samples were retained only if 95% of sites assayed had detection P-values > 0.01. Sites with annotated probe SNPs (n=65) and with common SNPs (minor allele frequency >5%) within 50bp of the target identified in the MXL (Mexican ancestry in Los Angeles, California) HapMap population (n=49,748), cross-reactive probes identified by Chen et al. [Chen et al. 2013] (n=25,248), and additional probes mapped to the X (n=10,708) and Y (n=95) chromosomes

were removed [Joubert et al. 2016]. Probes where 95% of samples had detection P-values >0.01 were also dropped (n= 460). This left 400,129 CpGs for analysis. Methylation beta values at all sites were logit transformed to the M-value scale to better comply with modeling assumptions [Du et al. 2010]. The methylation data used in this study has been uploaded to Gene Expression Omnibus (GSE97628).

To reduce the potential of extreme outlying methylation values to drive associations in statistical models, we removed methylation observations that were greater or less than three times the interquartile range for a given probe prior in all analyses of differential methylation. The majority of probes (54.3%) had no observations removed and the remaining mean number of samples per probe was 375 (range from 288 to 376).

Statistical analysis

Analysis of differential methylation at individual CpG sites associated with each of the 11 individual phthalate metabolite measurements and sums at 13 weeks gestation, 26 weeks gestation, and average across both time-points, was performed by fitting a *limma* linear model with empirical Bayes variance shrinkage, adjusting for parity, years in the United States, pre-pregnancy BMI, creatinine (13 week and 26 week models), batch, and cell-type proportions [Smyth 2004]. Adjustment covariates were selected from factors previously reported to be associated with child DNA methylation level. For results of these bivariate analyses by linear regression, Student's T-test, and ANOVA where appropriate. Phthalate metabolite measurements were $\log_{10}$ transformed to reduce the influence of outliers, and subjects with $\log_{10}$ transformed phthalate measurements greater or less than three times the interquartile range for a given phthalate were removed from respective analyses.

Cell-type proportions were estimated using combined cord blood reference datasets from Johns Hopkins that included nucleated red blood cells [Bakulski et al. 2016], Norway [Gervin et al. 2016 Aug 5], and Canada [Cardenas et al. 2016]. We conducted sensitivity analyses using the adult reference data set from Houseman et al.[Houseman et al. 2012] implemented in the R-package *minfi* [Aryee et al. 2014] and also using differential cell count (DCC) measurements for CHAMACOS subjects with available cord blood data [Yousefi et al. 2015]. P-values were adjusted for multiple hypothesis testing using the Benjamini-Hochberg (BH) method for false discovery rate (FDR) with an FDR $q<0.05$ threshold for significance [Benjamini and Hochberg 1995].

While there are a number of DMR finding methods, they each make different underlying assumptions and use a wide-array of statistical methods to call DMRs [Breton et al. 2017]. Since no single method has been widely accepted as the optimal way to interrogate DMRs, we selected two different methods, *DMRcate* and *comb-p* [Pedersen et al. 2012; Peters et al. 2015]. We required DMRs to be identified by both methods to be considered significant. Analysis of differentially methylated regions (DMRs) associated with each of the 11 metabolites and their sums at the two exposure measurements and average pregnancy exposure was performed using both methods. *DMRcate* first fits a *limma* linear model with empirical Bayes adjustment for each individual CpG site. This model was identical to the model implemented for the single CpG site analysis. *DMRcate* smoothed the individual CpG test statistics across their genomic position and compared those to modeled values. CpG

sites with a significant FDR value within a given base pair (bp) window (λ) are then grouped, and a DMR is called for each group containing at least two CpG sites. We implemented the default smoothing parameters with bandwidth λ = 1,000bp and scaling factor C=2. The minimum FDR p-value of CpG sites in a DMR was required to be < 0.001 for the DMR to be considered significant.

The *comb-p* algorithm utilizes a different approach to DMR finding. *Comb-p* adjusts p-values from the site specific model for genomic autocorrelation (ACF), identifies enriched regions of low p-values, and then performs inference on putative DMRs using Sidak multiple testing correction [Sidak 1967]. Settings were chosen to be consistent with those used in *DMRcate*—the ACF distance was set to 1,000bp, the p-value threshold required for a DMR < 0.001, and site specific p-values were generated from the same linear model. As with *DMRcate*, the region needed to have two or more CpGs to be considered a DMR. Those DMRs detected by both *DMRcate* and *comb-p* were considered significant in our analysis.

RESULTS

Characteristics of CHAMACOS children and their mothers are described in Table I. Most women were young, living within 200% of the poverty level, and few smoked during pregnancy. The majority of mothers were born in Mexico and lived in the United States for less than 5 years when their children were born. More than half of the mothers were overweight or obese prior to conceiving. There were few pre-term births and few newborns with low birthweights. This sub-cohort did not differ from the entire CHAMACOS cohort in demographic or exposure characteristics.

Phthalate metabolite concentrations in CHAMACOS mothers [Holland et al. 2016; Huen et al. 2016] were comparable to those of pregnant women from the National Health and Nutrition Examination Survey [Woodruff et al. 2011]. The distribution of creatinine-adjusted urinary phthalate metabolite concentrations measured in the subset of subjects included in the current study are shown in Table II, and did not differ from the total cohort [Holland et al. 2016]. MEP was the metabolite with the highest concentrations while MCNP had the lowest concentrations among 11 phthalates metabolites that were assessed.

Initially, we identified multiple differentially methylated CpG sites associated with phthalate metabolites, and five remained significant after adjustment correction for multiple hypothesis testing (FDR) (Table III). Our final model adjusted for cell-heterogeneity using a combined cord-reference data set that included nucleated red blood cells [Bakulski et al. 2016]. A CpG site in the TSS1500 region of *FKP10* and the 1st exon of *SC65* was hypermethylated in association with an increase in MEHHP at 26 weeks gestation (raw-p=1.8×10^{-8}, FDR-p=0.007) and MEOHP at 26 weeks gestation (raw-p=2.4×10^{-7}, FDR-p=0.048). A CpG site located in the north shore of the TSS1500 region of *ADM* was hypermethylated with both increased MEHHP and MEOHP at 26 weeks gestation (β=0.311, 0.296; raw-p= 4.6×10^{-8}, 1.4×10^{-7} ; FDR-p=0.009, 0.048). We observed hypermethylation in three sites associated with MCNP exposure at 26 weeks—one in the TSS200 region of *IP013* (β=0.22; raw-p= 7.3×10^{-8};FDR-p=0.03), the second in the TSS200 region of *DNM2*

and TSS1500 region of *MIR638* (β=0.29; raw-p= 2.7×10^{-7};FDR-p=0.04), and the third in the TSS200 region of *ZSWIM6* (β=0.18; raw-p= 2.5×10^{-7};FDR-p=0.04). In an initial analysis adjusted using the Houseman reference dataset [Houseman et al. 2012] as has been done so far for the majority of published 450K data, another CpG site located in the south shelf of an island 1.5kb downstream of *CYP11A1* was found to be hypomethylated with a −0.283 fold change in methylation per 10-fold increase in average pregnancy metabolite levels of MBP (raw-p=9.81×10^{-8} , FDR-p=0.04). In our final model we adjusted using a reference dataset generated by flow sorting of cord blood that is more relevant for newborns, and this CpG site remained borderline significant (raw-p=1.52×10^{-7}, FDR-p=0.06). In addition to these CpG sites, there were many sites with p-values approaching significance which may also be biologically relevant (Table SII). The majority of CpG sites (81%), including a significant CpG in *ADM* were hypermethylated with increased MEHHP exposure at 26 weeks gestation. Further sensitivity analyses using an alternative method for cord blood cell count estimation, microscopic differential cell count [Yousefi et al. 2015] did not change results appreciably.

The main focus of our study was on the regional methylation changes. We identified numerous DMRs using two different DMR finding methods, DMRcate and *comb-p*. *Comb-p* found a total of 57 DMR associations, while the *DMRcate* method found 1,421 DMRs with a maximum adjusted p-value < 0.001. The *DMRcate* DMRs encompassed 54 (95%) of the associations found by *comb-p*. For DMRs identified by both methods, the coordinates of the DMR defined by *DMRcate* completely encompassed the coordinates determined by *comb-p*, and included all of the significant CpG sites named by *comb-p*. The average number of CpG sites per DMR as defined by *DMRcate* was 9, and 7 as defined by *comb-p*. The average DMR spanned 900 base pairs in *DMRcate* and 406 base pairs in *comb-p*.

The region level analysis resulted in 27 significant DMRs in common between *DMRcate* and *comb-p*, most of which were associated with multiple (n=54) phthalate metabolite levels and time points (Table IV). Exposure to individual DEHP metabolites as well as the sum of DEHP metabolite concentrations resulted in the highest number of DMRs—12 distinct DMRs with 28 total associations. Most DMRs had multiple DEHP metabolite and time-point associations. The majority of significant DMRs (67%) were related with 26 week gestation metabolite levels. In contrast, only 6 distinct DMRs were associated with exposure at 13 weeks gestation. The metabolite with the highest number of hits was MEHP at 26 weeks (n=7) (Figure 1).

The majority of significant autosomal DMRs (76%) were hypermethylated with increased phthalate metabolite levels (Table V). Among the genes with hypermethylated DMRs were *C5orf63, CNPY1, ESM1, FIBIN, KATNAL2, LASP1, IFT140/TMEM204, LOC101929241, MUC4, PRDM8, BRCA1/NBR2, SEC14L4, SVIL-AS1*, and *ZBED9*. Genes with hypomethylated DMRs included *ATP11A, CFAP161, DPF1, IRAK4, LDHC, LROC1, RNF39*, and *VTRNA2-1*. Most of the DMRs (89%) associated with increased 26 week exposure were hypermethylated, while in contrast, DMR associations at 13 weeks gestation were hypomethylated (71%). Different groups of DMRs in genes were also found for early or late pregnancy. Importantly, for all metabolites that had associations with the same DMR

for both 13 and 26 weeks, the direction of effect was the same, showing consistency of phthalate-induced changes.

Significant DMRs spread over 17 chromosomes: 3-7, 10-19, and 22. Many of the DMRs were located just upstream of the transcription start site for the gene or in a location with markers of a promoter region. Although neither *DMRcate* nor *comb-p* constrain all CpGs within a DMR to have the same direction of association with the predictor of interest, we found that all but four DMRs had 100% concordance across CpGs in the direction of differential methylation. All overlapping DMRs between *DMRcate* and *comb-p* had the same direction of effect. Individually significant CpG sites which showed associations with phthalate exposure were not included in significant DMRs by both *DMRcate* and *comb-p*; however, all individual CpG hits were included in DMRs defined by *DMRcate* alone.

DISCUSSION

We assessed associations of cord blood DNA methylation at 409,961 CpGs and prenatal exposure to phthalates in a cohort of CHAMACOS newborns. To our knowledge, this is the first study to explore regional DNA methylation across the genome in relation to prenatal phthalate exposure in humans. We identified 27 unique DMRs associated with phthalate exposure after adjustment for multiple comparisons and blood cell composition that were concordantly confirmed by two independent methods, *DMRcate* and *comb-p*. Differential methylation was observed in genes related to hormonal balance, male fertility, metabolic health and cancer, demonstrating that epigenetics may be one mechanism through which early life exposure to phthalates can affect health.

Individual CpG sites associated with *in utero* phthalate exposure

Genes with significant CpGs associated with *in utero* phthalate metabolite concentrations were involved in a variety of health outcomes and diseases. For example, a significant CpG site associated with increased phthalate exposure was located in *Adrenomedullin* (*ADM*), a gene which codes for the protein adrenomedullin, involved in hormone secretion and linked with obesity in adults [Nomura et al. 2009]. In our study, the CpG site in the TSS1500 region of *ADM* was hypermethylated in newborns with higher MEHHP and MEOHP. It seems plausible that early life disruption of this protein could have implications for later life metabolic health—and this is a pathway that merits closer scrutiny. Hypermethylation of CpG site located in a CpG island 1.5kb downstream of *Cytochrome P450 Family 11 Subfamily A Member 1* (*CYP11A1*) was associated with exposure to MBP. *CYP11A1* is involved in steroidogenesis and plays a role in metabolic diseases [Tee et al. 2013]. Furthermore, *in utero* exposure to phthalates in rats has been reported to alter expression of *CYP11A1* in Leydig cells and to suppress steroidogenesis [Sekaran and Jagadeesan 2015].

Several significant CpG sites were found in genes linked to congenital abnormalities such as Bruck syndrome and osteogenesis imperfecta (*FK506 Binding Protein 10(FKBP10))* [Alanay et al. 2010; Kelley et al. 2011]*;* and aromelic frontonasal dysostosis (*Zinc Finger SWIM-Type Containing 6* (*ZSWIM6*)) [Smith et al. 2014].

We also observed potential relation of differential methylation induced by phthalate exposure with cancer, including hypermethylation in the TSS1500 region of the *MicroRNA 638* (*MIR638*). This miRNA shows decreased expression in many cancers [Tan et al. 2014]. Its target is *BRCA1*, a well-known gene implicated in breast cancer. Our DMR analysis also showed differential methylation of a region upstream of *BRCA1/NBR2* as described in more detail in the next section. To our knowledge, the relationship of phthalates with any these health outcomes has not been investigated.

Differentially methylated regions (DMRs) and prenatal phthalate exposure

While the majority of published epigenetic studies have thus far focused on individual CpG sites, emerging evidence suggests that regional approaches may be more informative [Vandiver et al. 2015; Yang et al. 2015; Watson et al. 2016, Breton et al. 2017] and could improve statistical power[Jaffe et al. 2012; Dolzhenko and Smith 2014]. Rather than interrogating individual CpG sites, DMR analysis utilizes the patterns of co-correlation between nearby CpG sites to take advantage of the epigenomic structure [Shoemaker et al. 2010; Liu et al. 2014]. We used strict criteria requiring that a significant DMR reach a multiple hypothesis corrected cutoff of $p<0.001$ by two methodologies, *DMRcate* and *comb-p*.

In our study, we observed methylation changes in several genes involved in control of spermatogenesis, testes development, and infertility. For example, a hypermethylated DMR in a CpG island in the body of *Intraflagellar Transport 140* (*IFT140*) and TSS200 region of *Transmembrane Protein 204* (*TMEM204*) was associated with increased levels of several phthalates. A recent study suggests that decreased expression of *IFT140* can inhibit spermatogenesis, and should be considered a candidate gene for male infertility [Herati et al. 2016]. Another gene, *Cilia And Flagella Associated Protein 161* (*CFAP161*), which had a hypomethylated region in a CpG island in the TSS200 region in our study, was previously associated with primary ciliary dyskinesia (PCD), a condition marked by disrupted sperm development and infertility [Austin-Tse et al. 2013]. A DMR associated with increased exposure to MEP was hypomethylated in *Lactate Dehydrogenase C* (*LDHC*). It is expressed exclusively in the testes and one study found decreased expression of *LDHC* in the sperm of men with motility-impaired sperm [Wang et al. 2004]. *Tescalin* (*TESC*) gene where we found hypermethylated DMR with increased MBzP was previously linked with fetal gonad development in mice [Perera et al. 2001].

A number of significant DMRs were found in genes related to endocrine function and metabolic balance. Although genes involved in steroid metabolites are up-regulated with phthalate exposure in rats [Robinson et al. 2012], fetal exposure results in decreased circulating testosterone and aldosterone levels in adult male offspring and estradiol in the female [Martinez-Arguelles and Papadopoulos 2016]. As was shown in zebra fish and in human adrenal cell line, testosterone may decrease with phthalate exposure [Sohn et al. 2016]. A significant hypermethylated DMR in a CpG island upstream of *Canopy FGF Signaling Regulator 1* (*CNPY1*), is responsible for a protein product required for proper fibroblast growth factor (FGF) signaling in zebrafish [Hirate and Okamoto 2006]. In mammals, a subset of secreted FGFs is involved in endocrine function, regulating phosphate,

bile acid, and carbohydrate and lipid metabolism [Ornitz and Itoh 2015]. A hypomethylated region of *Interleukin 1 Receptor Associated Kinase 4* (*IRAK4*) gene involved in inflammatory response was associated with MiBP exposure [Medvedev et al. 2003]. A DMR located in *Endocan* (*ESM1*) has shown a relationship with MEOHP exposure. *ESM1* is also involved in inflammatory disorders and serum levels are associated with type-2 diabetes [Arman et al. 2016]. One recent study has reported urine metabolomics data for a cohort of Chinese men environmentally exposed to phthalates [Zhang et al. 2016]. The findings indicate increased oxidative stress and fatty acid oxidation and decreased prostaglandin metabolism that is consistent with inflammation and metabolic disorders known to be linked with prenatal phthalate exposure.

Multiple genes with DMRs associated with prenatal phthalate exposure in our study are related to different cancers. In particular, we found hypomethylation of DMRs in *Vault RNA 2-1* (*VTRNA2-1*), an imprinted gene which has been identified as a tumor suppressor, and in *ATPase Phospholipid Transporting 11A* (*ATP11A*) gene, and hypermethylated DMR in *Katanin Catalytic Subunit A1 Like 2* (*KATNAL2*). All three genes are associated with cancer and neurodevelopment. One study showed that methylation in *VTRNA2-1* is highly responsive to environment in early embryo development [Silver et al. 2015]. Studies link hypermethylation of *VTRNA2-1* with poor outcome in small cell lung cancer [Cao et al. 2013], esophageal cancer [Lee et al. 2014], and acute myeloid leukemia [Treppendahl et al. 2012]. Increased expression of *ATP11A* is a predictor for metachronous metastasis of colorectal cancer in patients and is associated with worse survival outcomes [Miyoshi et al. 2010].

Among the most significant DMRs cross-validated by both methods was a hypermethylated DMR found in the promoter region of *breast cancer 1, early onset* (*BRCA1*) and *Neighbor of BRCA1 Gene 2* (*NBR2*). *BRCA1* is a tumor suppressor involved in DNA repair, and its mutations are linked to increased risk of breast and ovarian cancer [Miki et al. 1994]. Although phthalates have not been previously linked to changes in *BRCA1* to the best of our knowledge, another endocrine disrupting chemical, bisphenol A (BPA), has been reported to be associated with hypermethylation of *BRCA1* [Qin et al. 2012]. Hypermethylation has the potential to lead to decreased expression of *BRCA1* and increase risk of breast cancer [Rice et al. 2000; Gupta et al. 2014]. *LIM and SH3 Protein 1* (*LASP1*) involved with breast cancer also contained a DMR associated with phthalate exposure [Tomasetto et al. 1995; Frietsch et al. 2010]. While we did not find any publications on the relationship of phthalates with cancer, *in utero* exposure to other endocrine disruptors, such as BPA and DDT, have been linked to cancer in both humans and mice [Doherty et al. 2010; Cohn et al. 2015; Weinhouse et al. 2016]. Overall, our findings underscore the importance of considering the combined impact of nearby CpGs as DMRs when modeling the effect of exposure on DNA methylation.

Strengths, limitations and future directions

Our study has several strengths and limitations. CHAMACOS is a well-characterized prospective birth cohort study that is relatively homogeneous with regard to socioeconomic status, ancestry, and geographic distribution, which helped limit the potential for

confounding bias. The detailed covariate data already available for this cohort further allowed systematic assessment of potential confounders. One limitation of our study that is common for human population studies in general is that DNA methylation was assessed in blood, although there is a growing consensus that blood DNA is an appropriate and informative matrix for research on epigenetic programming of environmental exposures [Wang et al. 2010; Chadwick et al. 2015; Breton et al. 2017]. We also adjusted for blood cell composition and showed in sensitivity analyses that there were only small changes to findings when we used three different reference datasets for cell count estimation [Houseman et al. 2012; Yousefi et al. 2015; Bakulski et al. 2016]. However, some of the genes with regions of differential methylation are not expressed in blood. For example, *CYP11A1* is expressed mainly in the testes, and showed only marginal expression in our blood samples by qPCR (data not shown). In general we observed a low expression of a number of genes that is consistent with other findings for the CHAMACOS children [Holland et al. 2006; Huen et al. 2010; Davé et al. 2015]. Further, we relied on maternal urine samples to assess phthalate exposure to the fetus because urine phthalate concentrations are considered more reliable biomarkers in comparison to serum measurements for non-persistent chemicals such as phthalates [Calafat et al. 2013]. More studies will be needed to determine whether differential methylation persists over time and mediates health risks in older children and adults. Thus far, no other studies examining the association between prenatal phthalate exposure and 450K methylation have been published for cross-validation. We recognize the need to further validate our findings, and a meta-analysis of data from several cohorts is planned in the future by the Pregnancy and Children Epigenetics (PACE) consortium when more data on 450K and phthalate exposure *in utero* from other cohorts become available that should increase statistical power, as recently was demonstrated for effects of maternal smoking on DNA methylation in newborns [Joubert et al. 2016].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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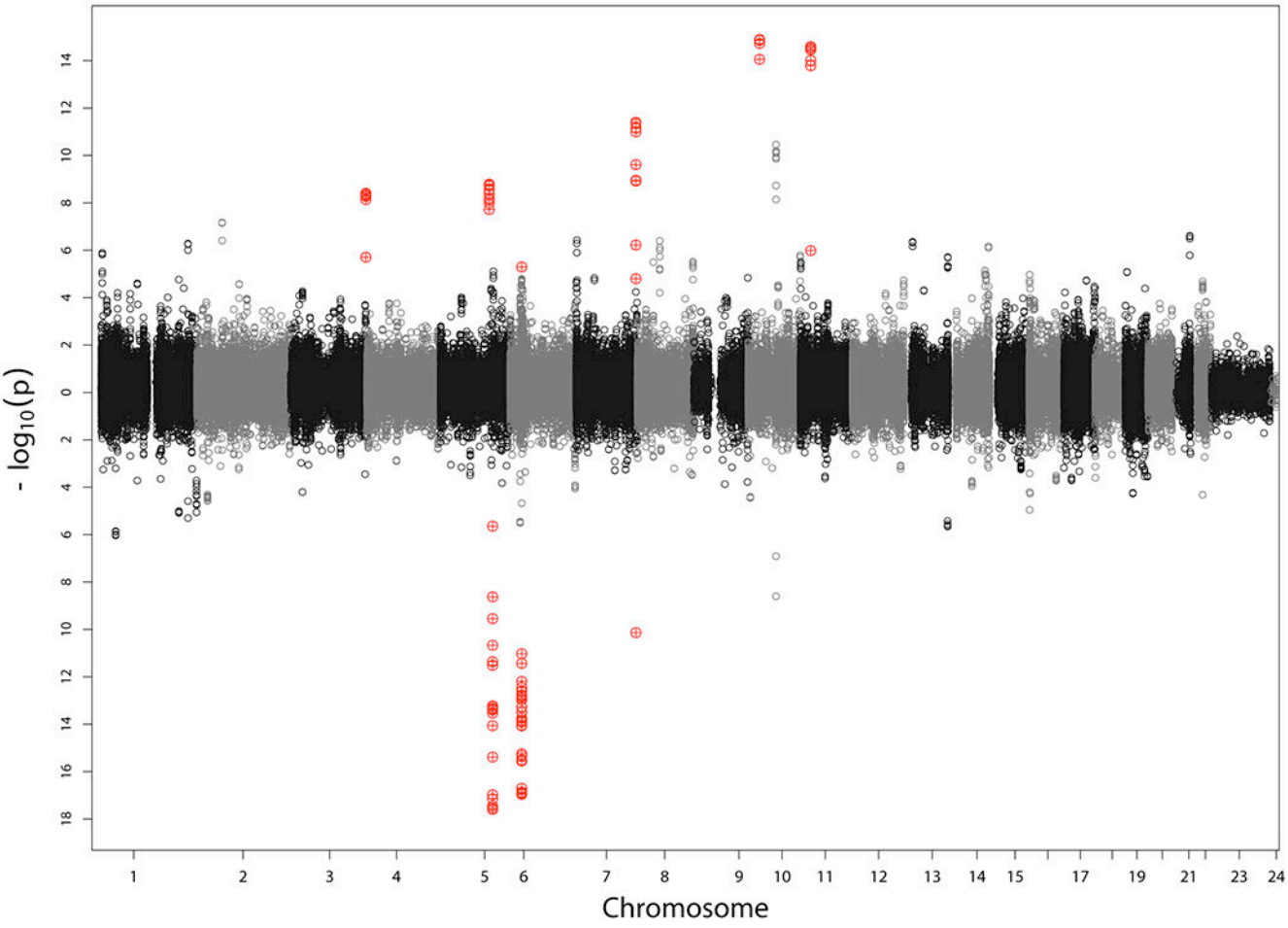

Figure 1.

Manhattan plot of DMRs associated with 26 week MEHP exposure. P-values were produced by the *DMRcate* method, and CpGs in DMRs found by both *DMRcate* and *comb-p* are highlighted in red. Sites which showed hypermethylation in association with phthalate exposure are plotted above the x-axis, and sites showing hypomethylation are plotted below.

Table I.

Demographic characteristics of CHAMACOS newborns and their mothers, N=336.

	N	%
Child sex		
Boy	173	51.5
Girl	163	48.5
Child gestational age at birth		
≥ 37 weeks	317	94.3
34-36 weeks	19	5.7
Child birthweight		
≥2,500 g	327	97.3
< 2,500 g	9	2.7
Maternal country of birth		
Mexico	286	85.1
USA	43	12.8
Other	7	2.1
Number of years mother lived in US at pregnancy		
< 1 years	87	25.9
2-5 years	89	26.5
6-10 years	73	21.7
≥ 11 years	50	14.9
Entire life	37	11.0
Maternal age at delivery (years)		
18-24	155	46.3
25-29	108	32.2
30-34	52	15.5
35-45	20	6
Family income		
At or below Poverty	206	61.3
Poverty-200%	119	35.4
>200% Poverty	11	3.3
Maternal pre-pregnancy BMI (kg/m2)		
Normal	130	39.6
Overweight	121	36.9
Obese	74	22.6
Underweight	3	0.9
Smoking during pregnancy		
No	317	94.3
Yes	19	5.7

Table II.

Distribution of phthalate metabolite concentrations (μg/g-creatinine) at 13 and 26 weeks gestation, and pregnancy average

Exposure	13 Weeks (N=321)				26 Weeks (N=332)				Average (N=336)			
	Median	IQR	Min	Max	Median	IQR	Min	Max	Median	IQR	Min	Max
MEP	173.61	(70.73 , 503.67)	7.21	41791.04	156.32	(66.92 , 389.64)	0.61	9909.71	213.98	(93.85 , 444.74)	7.21	25850.38
MBP	18.88	(9.49 , 39.17)	0.03	428.57	22.62	(11.44 , 41.58)	1.88	423.53	22.96	(13.76 , 43.92)	3.16	228.62
MiBP	2.36	(1.14 , 4.52)	0.01	82.64	2.54	(1.43 , 5.16)	0.02	373.53	2.68	(1.58 , 5.34)	0.06	191.98
∑LMW	225.03	(108.71 , 569.01)	14.15	45920.74	224.63	(114.13 , 481.67)	8.94	10885.36	268.98	(143.48 , 548.28)	14.30	28403.05
MEHP	3.12	(1.50 , 6.52)	0.03	96.70	3.63	(1.90 , 6.82)	0.02	186.24	3.75	(2.23 , 6.51)	0.14	101.57
MEHHP	12.36	(6.79 , 24.65)	0.90	846.73	15.43	(8.33 , 27.66)	0.17	865.69	15.46	(8.97 , 26.81)	1.35	478.56
MEOHP	8.76	(4.96 , 15.98)	0.03	458.74	12.21	(6.77 , 20.85)	0.12	651.60	11.64	(7.01 , 19.21)	0.94	353.43
MECPP	22.03	(13.87 , 37.89)	1.34	977.49	24.82	(16.42 , 42.80)	5.24	1222.07	25.46	(17.34 , 41.51)	5.26	665.24
∑DEHP	46.02	(28.23 , 83.76)	3.53	2280.81	56.06	(33.99 , 96.69)	7.89	2789.15	56.33	(36.59 , 93.94)	8.04	1524.54
MBzP	7.37	(3.36 , 13.48)	0.17	459.87	7.84	(4.44 , 14.49)	0.04	239.34	8.36	(4.74 , 14.14)	0.17	349.61
MCPP	1.73	(1.00 , 2.80)	0.00 *	18.68	2.04	(1.13 , 3.23)	0.00 *	54.12	1.99	(1.25 , 2.94)	0.01	28.26
MCOP	2.77	(1.78 , 4.35)	0.02	162.31	3.12	(2.05 , 5.04)	0.03	33.22	3.26	(2.14 , 4.68)	0.20	82.71
MCNP	1.70	(1.04 , 2.45)	0.03	51.21	1.88	(1.21 , 2.82)	0.01	20.00	1.84	(1.29 , 2.68)	0.03	31.19
∑HMW	63.53	(40.13 , 107.61)	6.90	2300.75	78.18	(46.82 , 126.38)	14.18	2807.64	77.56	(53.07 , 122.06)	13.16	1538.09

MEP, monoethyl phthalate; MBP, mono-n-butyl phthalate; MiBP, mono-isobutyl phthalate; LMW, low molecular weight; MEHP, mono(2-ethylhexyl) phthalate; MEHHP, mono(2-ethyl-5-hydroxyhexyl) phthalate; MEOHP, mono(2-ethyl-5-oxohexyl) phthalate; MECPP, mono(2-ethyl-5-carboxypentyl) phthalate; DEHP, di-2-ethylhexyl phthalate; MBzP, monobenzyl phthalate; MCPP, mono(3-carboxypropyl) phthalate; MCOP, monocarboxyoctyl phthalate; MCNP, monocarboxynonyl phthalate; HMW, high molecular weight

*Small value, rounded to 0.00

Table III.

Differentially methylated CpG sites

CpG Sites	Chromosome	β[a]	FDR P-Value	Significant exposures	Gene(s)	Gene Location	Relation to CpG Island
cg22938275	17	0.155	0.007	26w MEHHP	*FKBP10, SC65*	TSS1500, 1st Exon	Island
		0.141	0.048	26w MEOHP			
cg05149386	11	0.311	0.009	26w MEHHP	*ADM*	TSS1500	N_Shore
		0.296	0.048	26w MEOHP			
cg11332336	1	0.221	0.029	26w MCNP	*IPO13*	TSS200	Island
cg00735591	19	0.286	0.037	26w MCNP	*DNM2, MIR638*	TSS200, TSS1500	Island
cg16159717	5	0.184	0.037	26w MCNP	*ZSWIM6*	TSS200	Island
cg24849517	15	−0.262	0.062[b]	Avg MBP	*CYP11A1*[c]	-	S_Shelf

MEHHP, mono(2-ethyl-5-hydroxyhexyl) phthalate; MEOHP, mono(2-ethyl-5-oxohexyl) phthalate; MCNP, monocarboxynonyl phthalate; MBP, mono-n-butyl phthalate

[a] Fold-change change in DNA methylation M-value per $\log_{10}$ unit increase in phthalate metabolite 10-fold increase in phthalate metabolite concentration

[b] FDR p-value with cell adjustment using the Houseman method was 0.040

[c] cg24849517 is located 1.5kb downstream of *CYP11A1*

Table IV.

Overlap of DMRs between *comb-p* and *DMRcate*

				Comb-p				*DMRcate*			
Gene	**Chr**	**Exposure**	**Max βFC[c]**	**Start**	**End**	**# of Probes**	**P-value[a]**	**Start**	**End**	**# of Probes**	**P-value[b]**
ATP11A	13	26w MiBP	−0.383	113540512	113540632	3	6.09E-08	113539759	113540631	5	7.20E-12
		Avg MiBP	−0.387	113540512	113540632	3	4.80E-05	113539759	113540631	5	9.67E-06
BRCA1/NBR2	17	Avg MBP	0.302	41277974	41278342	9	5.27E-05	41277694	41278906	16	4.29E-08
C15orf26/CFAP161	15	Avg MBzP	−0.428	81426347	81426670	8	4.43E-06	81426347	81426820	9	1.59E-09
		Avg MBP	−0.592	81426347	81426821	9	1.46E-10	81426347	81426820	9	4.72E-13
C5orf63	5	26w MEHP	0.250	126409007	126409311	8	5.97E-06	126408756	126409553	13	8.44E-06
CNPY1	7	26w MEHHP	0.204	155283424	155284356	7	4.84E-05	155283140	155284759	11	3.11E-10
		26w ∑HMW	0.264	155283424	155284449	8	1.97E-07	155283140	155284759	11	3.46E-10
		26w MECPP	0.268	155283424	155284449	8	2.29E-07	155283140	155284759	11	6.36E-11
		26w MEHP	0.171	155284062	155284449	8	5.63E-07	155283233	155284759	10	2.83E-08
DPF1	19	13w MiBP	−0.146	38713242	38713374	3	1.08E-04	38713242	38713373	3	4.43E-04
ESM1	5	26w MEOHP	0.207	54281198	54281479	6	6.37E-05	54281198	54281668	7	8.94E-07
FIBIN	11	26w ∑DEHP	0.319	27015519	27015992	6	1.91E-07	27015519	27016671	7	7.55E-10
		26w MEHHP	0.276	27015519	27015992	6	3.03E-07	27015519	27017005	8	5.21E-10
		26w MEHP	0.231	27015519	27015992	6	7.92E-11	27015519	27016671	7	3.82E-11
		26w MEOHP	0.300	27015519	27015992	6	1.89E-09	27015519	27016671	7	4.89E-12
		26w ∑HMW	0.322	27015592	27015992	5	1.76E-05	27015519	27016671	7	2.13E-07
		26w MECPP	0.328	27015592	27015992	5	4.94E-06	27015519	27017005	8	1.54E-09
IRAK4	12	13w MiBP	−0.440	44152632	44152941	8	3.56E-06	44152117	44153217	14	1.29E-06
KATNAL2	18	13w MiBP	0.368	44562019	44562137	5	1.54E-05	44561718	44562505	9	9.09E-08
LASP1	17	26w MBzP	0.247	37024169	37024626	3	4.62E-04	37024020	37025274	7	1.08E-08
LDHC	11	13w ∑LMW	−0.315	18433500	18433746	6	7.01E-06	18433500	18434354	8	5.20E-09
		13w MEP	−0.377	18433500	18434016	7	1.06E-07	18433500	18434354	8	8.19E-10

				Comb-p				*DMRcate*			
Gene	**Chr**	**Exposure**	**Max βFC[c]**	**Start**	**End**	**# of Probes**	**P-value[a]**	**Start**	**End**	**# of Probes**	**P-value[b]**
ZBED9	6	26w MBP	0.231	28583971	28584289	16	3.06E-05	28583655	28584464	18	1.23E-07
Intergenic	6	26w MBP	0.241	28446794	28447116	5	6.45E-06	28446794	28447115	5	1.34E-05
Intergenic	6	26w MBP	0.318	28601269	28601520	10	1.83E-05	28601269	28601519	10	4.29E-07
		26w MEOHP	0.331	28601269	28601520	10	8.42E-05	28601269	28601519	10	2.01E-08
Intergenic	21	26w MBzP	0.340	44573854	44574023	3	4.68E-04	44573062	44574022	4	2.65E-06

[a] Minimum Sidak P-value for the region

[b] Minimum FDR P-value for the region

[c] Fold change in DNA methylation M-value per $\log_{10}$ unit increase in phthalate metabolite concentration

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Table V.

Differential methylation by phthalate exposure

	Number of DMRs	DMRs with decreased methylation	DMRs with increased methylation	Unique CpGs in all DMRs: DMRcate	Unique CpGs in all DMRs: Comb-p
13w MEP	1	1	1	8	7
26w MBP	3	0	3	33	31
Avg MBP	2	1	1	25	18
13w MiBP	3	2	1	26	16
26w MiBP	2	2	0	31	22
Avg MiBP	2	1	1	11	8
13w ∑LMW	1	1	0	8	6
26w MEHP	7	2	5	95	55
Avg MEHP	2	1	1	39	20
26w MEHHP	4	0	4	32	21
Avg MEHHP	1	0	1	7	7
26w MEOHP	6	0	6	43	37
26w MECPP	4	0	4	36	23
Avg MECPP	1	0	1	7	7
26w ∑DEHP	2	0	2	14	13
Avg ∑DEHP	1	0	1	7	7
26w MBzP	4	0	4	28	14
Avg MBzP	1	1	0	9	8
13w MCPP	1	1	0	6	3
26w MCPP	1	0	1	6	5
Avg MCPP	1	0	1	6	5
13w MCOP	1	0	1	6	4
26w ∑HMW	3	0	3	25	16

MBP, mono-n-butyl phthalate; MiBP, mono-isobutyl phthalate; LMW, low molecular weight; MEHP, mono(2-ethylhexyl) phthalate; MEHHP, mono(2-ethyl-5-hydroxyhexyl) phthalate; MEOHP, mono(2-ethyl-5-oxohexyl) phthalate; MECPP, mono(2-ethyl-5-carboxypentyl) phthalate; DEHP, di-2-ethylhexyl phthalate; MBzP, monobenzyl phthalate; MCPP, mono(3-carboxypropyl) phthalate; MCOP, monocarboxyoctyl phthalate; MCNP, monocarboxynonyl phthalate; HMW, high molecular weight.