Research Article

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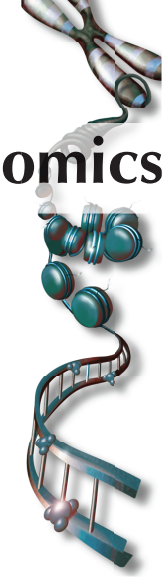
Epigenomics



DNA methylation of imprinted genes in Mexican–American newborn children with prenatal phthalate exposure

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Aim: Imprinted genes exhibit expression in a parent-of-origin-dependent manner and are critical for child development. Recent limited evidence suggests that prenatal exposure to phthalates, ubiquitous endocrine disruptors, can affect their epigenetic dysregulation. **Materials & methods:** We quantified DNA methylation of nine imprinted gene differentially methylated regions by pyrosequencing in 296 cord blood DNA samples in a Mexican–American cohort. Fetal exposure was estimated by phthalate metabolite concentrations in maternal urine samples during pregnancy. **Results:** Several differentially methylated regions of imprinted genes were associated with high molecular weight phthalates. The most consistent, positive, and false discovery rate significant associations were observed for *MEG3*. **Conclusion:** Phthalate exposure *in utero* may affect methylation status of imprinted genes in newborn children.

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Epigenetic influences on gene expression have been implicated as a mediator of the relationship between environmental exposures and health status. Early-life adverse environments can cause epigenetic shifts, establishing disease trajectories into adulthood, an idea described by the Developmental Origins of Health and Disease hypothesis [1,2]. Epigenetic modifications can alter gene expression without changing the underlying nucleotide sequence. DNA methylation is the epigenetic mechanism most often studied [1,3,4]. It is simultaneously heritable and susceptible to environmental insults, and is able to reveal cumulative effects of environmental exposures throughout the life course [5,6].

Genomic imprinting involves the expression of only one allele of the gene and is dependent on the parental origin of the expressed allele [7]. The determination of active versus inactive allele is regulated by DNA methylation that is established during the process of epigenetic reprogramming that occurs in the developing gametes [8]. The majority of the known imprinted genes within the human genome have differentially methylated regions (DMRs) whose methylation is also parent-of-origin-dependent [9,10]. Methylation patterns in the DMRs are remodeled and established prior to germ layer specification, and are maintained in somatic tissues throughout life [8]. A number of human studies have demonstrated the effects of environmental exposures on imprinted gene DNA methylation, including the influence of diet [11–14], cigarette smoke [15], maternal antibiotic use [16], metal exposure [17–22] and maternal stress [23,24]. Differentially methylated regions of imprinted genes represent a unique opportunity to assess the effects of *in utero* exposure since they influence development and growth in early life [18].

Limited but growing evidence indicates that exposure to phthalates is associated with DNA methylation changes of imprinted genes [25–27]. Phthalates, diesters of phthalic acid, are a family of chemicals often found in consumer

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products, resulting in common exposure in the USA [28,29]. Some phthalates, such as di-(2-ethylhexyl) phthalate (DEHP), are added to plastics to increase flexibility and can be found in toys, plastic containers and medical supplies. Other phthalates, such as diethyl phthalate, are used as solvents in personal care products such as perfumes and lotions [30]. Common routes of exposure to phthalates, which are not chemically bound to their substrates and leach into the environment, include ingestion, dermal absorption and inhalation. Previous studies in humans have demonstrated associations between phthalate exposure during or prior to pregnancy and adverse impacts on health outcomes in children, including altered birth weight [31–33], gestational age [34,35], preterm birth [36,37], child growth and development [38,39], child behavior [40] and asthma [41].

Research in animals [42–45] has shown associations between phthalates and global and site-specific methylation; however, there is a paucity of information on imprinted gene DMR methylation in relation to phthalate exposure *in utero*. Only two human studies are available at this time that have examined the relationship between phthalate exposure during pregnancy and placental imprinted gene methylation (*H19* and *IGF2*) [25,27]. Currently there are no data available on the relationship between cord blood imprinted gene methylation profiles and prenatal exposure to phthalates.

Previously, we reported on the prenatal phthalate metabolite concentrations in participants of the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) study and observed inverse associations of MEP concentrations with DNA methylation of repetitive elements in newborns and children [46]. We have also assessed the relationship between prenatal phthalate exposure in CHAMACOS mothers and both genome-wide site-specific and region DNA methylation as assessed by the 450K array in newborns, identifying 27 different regions within the human genome associated primarily with DEHP metabolites [47]. The objective of the present study is to assess the impact of prenatal phthalate exposure on DNA methylation of imprinted genes in CHAMACOS newborns.

Materials & methods

Study subjects

CHAMACOS is a longitudinal birth cohort study assessing neurological, developmental and respiratory health effects of environmental exposure in pregnant Mexican–American women and their children residing in the agricultural region of Salinas Valley, CA, USA [48]. From 1999 to 2000, a total of 601 pregnant women were enrolled in the study and 527 women delivered live, singleton newborns. At the time of enrollment, CHAMACOS mothers were at least 18 years of age, Spanish or English speaking, eligible for low-income health insurance, and were receiving prenatal care at one of several participating clinics. During pregnancy, CHAMACOS mothers were interviewed twice (13.2 $\pm$ 5.1 and 26.0 $\pm$ 2.7 weeks gestation) by trained bilingual, bicultural staff regarding reproductive and medical history, sociodemographic factors and pregnancy-specific lifestyle and environmental exposures [48,49]. The pregnancy visits will subsequently be referred to as either the early or late pregnancy visits.

Immediately following delivery, whole cord blood was collected from the umbilical vein in BD vacutainers (Becton, Dickinson and Company, NJ, USA) without anticoagulant. Blood specimens were allowed to clot for at least 30 min, centrifuged at 1200 rpm for 10 min, divided into serum and blood clot aliquots, and stored at -80°C. The QIAamp Blood DNA Maxi kit (Qiagen, Inc., CA, USA) was used to isolate genomic DNA from clots as previously described [50]. Methylation of nine imprinted gene DMRs was measured in 296 newborn children (148 girls and 148 boys) that had sufficient DNA available for analysis. The subset was not significantly different from the main CHAMACOS cohort in many demographic and exposure characteristics (child sex, poverty index, education, gestational age and parity). However, mothers in this sample tended to be slightly younger and more obese, lived fewer years in the USA and gave birth to children that were less likely to have a low birth weight. The number of CHAMACOS participants with pyrosequencing data is approximately double the subjects included in existing literature assessing the relationship between phthalate exposure and imprinted genes [25,27] and we anticipate that we will have sufficient power to detect a minimum of a 0.05 linear correlation between phthalates and imprinted genes.

CHAMACOS study protocols were approved by the University of California (UC), Berkeley Committee for Protection of Human Subjects. All mothers provided written informed consent at the time of enrollment.

Phthalate metabolite measurements

Maternal urine samples were collected during the early and late pregnancy visits. The urine samples were aliquoted, barcoded, and stored at -80°C in the UC Berkeley School of Public Health Biorepository. Eleven phthalate

metabolites were quantified using online solid phase extraction coupled with isotope dilution high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry, as previously described [46,49,51,52]. The metabolites measured included three low molecular weight (LMW) metabolites (monoethyl phthalate [MEP], mono-n-butyl phthalate [MBP], mono-isobutyl phthalate [MiBP]), four DEHP metabolites (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 high molecular weight (HMW) metabolites (monobenzyl phthalate [MBzP], mono[3-carboxypropyl] phthalate [MCPP], monocarboxyoctyl phthalate [MCOP], monocarboxynonyl phthalate [MCNP]). Quality control procedures comprised incorporation of laboratory and field blanks, calibration standards and spiked controls with low and high concentrations into the experimental runs.

The limits of detection (LOD) for all the phthalate metabolites have been previously described [46,51,53]. The instrumental reading values were used when the measured phthalate metabolite concentrations fell below the LOD. In instances, where the measured concentrations were below the LOD and instrumental signals were unavailable, the 'fill-in' method, described in Lubin *et al.* [54] was used to impute phthalate metabolite concentrations from a log-normal distribution. Summary measurements of the DEHP metabolites in units of micrograms per liter were generated as described previously [55]. Specifically, molar concentrations of each DEHP metabolite were calculated by dividing the concentration of the metabolite by its molecular weight. The molar concentrations for each DEHP phthalate metabolite were summed and then multiplied by the average molecular weight of the metabolites. All phthalate metabolites, with the exception of the DEHP metabolites, were analyzed individually and HMW and LMW sum variables were not generated since values are largely driven by DEHP and diethyl phthalate metabolites, respectively. Phthalate metabolite concentrations (micrograms per liter) were divided by creatinine concentrations (grams per liter), measured using a commercially available diagnostic enzyme method (Vitros CREA slides; Ortho Clinical Diagnostics, NJ, USA) concurrently with the phthalate metabolites, to generate values (micrograms per gram creatinine) adjusted for urinary dilution as previously described [49].

Since, we anticipate that average phthalate metabolite concentrations during pregnancy will be a more stable indicator of pregnancy exposure, we assessed *in utero* phthalate exposure as the average of the $\log_{10}$ transformed concentrations of creatinine-corrected phthalate metabolites from the two prenatal visits [52]. Participants with measurements greater or less than three-times the interquartile range for a particular phthalate were removed from analyses. All descriptive analyses, figures and regression models included creatinine adjusted phthalate metabolite concentrations averaged across pregnancy. Additionally, we performed a sensitivity analysis that included adjusting for urinary dilution using specific gravity instead of creatinine, and we observed that it did not affect the findings. Specific gravity was quantified with a refractometer (National Instrument Company, Inc., MD, USA), and urinary phthalate metabolites were adjusted for specific gravity as previously described [46]. The results of the principal component and mean methylation analyses of the imprinted gene and average phthalate exposure during pregnancy when adjusting for specific gravity were similar to the creatinine adjusted models.

DNA methylation analyses

The EZ DNA Methylation kit (Zymo Research, CA, USA) was used for bisulfite conversion of 800 ng of DNA, following the manufacturer's instructions. Treating DNA with sodium bisulfite converts unmethylated cytosines to uracils and leaves methylated cytosines unchanged.

The PyroMark PCR Kit (Qiagen, CA, USA) was used to prepare bisulfite-treated DNA for PCR amplification, using published primer sequences for nine imprinted gene DMRs and assays previously developed and validated at Duke University [8,56]. These imprinted genes were selected based on their biological significance and availability of reliable assays [25,57–61]. Pyrosequencing of the amplified and bisulfite converted DNA was achieved using the Pyromark Q96 MD system (Qiagen), yielding percent methylation estimates for the CpG sites within the sequence analyzed for each DMR. Methylation was measured for the following imprinted genes: *H19*, *IGF2*, *MEG3* and *MEG3-IG*, *MEST*, *NNAT*, *PLAGL1*, *PEG3* and *SGCE*/*PEG10*. Specifically, methylation was measured at the DMRs upstream of *IGF2* exon 3 (chr 11p15.5; three CpG sites), upstream of the *H19* gene (chr 11p15.5; four CpG sites), two DMRs involved in regulating the *DLK1*/*MEG3* imprinted domain (chr 14q32.2; *MEG3-IG*: four CpG sites; *MEG3*: seven CpG sites), the *MEST* promoter (chr 7q32.2; four CpG sites), the *NNAT* locus (chr 20q11.23; three CpG sites), the *PEG3* promoter (chr 19q13.43; ten CpG sites), the *PLAGL1* locus (chr 6q24.2; six CpG sites) and the *SGCE*/*PEG10* promoter (chr 7q21.3; six CpG sites). The paternal allele is expressed for the genes *IGF2*, *DLK1*, *MEST*, *NNAT*, *PEG3*, *PLAGL1* and *SGCE*; whereas the maternal allele is expressed in

MEG3 and *H19*. The selected imprinted genes are critical in the regulation of early growth [61], and alterations of DNA methylation by environmental insults, such as phthalate exposure, could have implications for imprinted gene expression and downstream effects on growth trajectories. In the CHAMACOS cohort, we have observed that *in utero* exposure to MEP is associated with increased odds of being overweight or obese that persists from age 5 to 12 [52]. Quality assurance measures involved inclusion of technical repeats and positive and negative controls. Samples whose methylation values exceeded two standard deviations from the mean for the plate were reanalyzed. The CV of intraplate repeat measures ranged from 1–3%. Using mixtures of DNA with fully methylated and unmethylated sequences [8,61], we have previously shown that pyrosequencing allows for the detection of 0.5–5% methylation differences.

Three approaches were used to account for cell composition in the analysis based on cytological differential cell counts (DCC) [62] and 450K-array estimates [47]. The methods included adjusting for cytological DCC in a subset of cord blood samples [46,62] and estimating cell-type proportions from CHAMACOS 450K cord blood DNA methylation data [47] using adult [63] and cord blood [64] flow sorted reference panels. The cord blood reference panel includes estimates of nucleated red blood cells (nRBCs).

Gene expression analysis

Validation of significant hits of the relationship between average phthalate metabolites during pregnancy and imprinted gene methylation in newborns was performed using a two-step RT-PCR in a subset of the CHAMACOS participants with imprinted gene data and available isolated RNA. Specifically, we assessed the overall expression of *MEG3* in 119 CHAMACOS children with imprinted gene data, since methylation changes due to environment exposure could lead to alteration of gene expression. As a negative control, we also examined overall expression patterns in *DLK1* and its relationship with *MEG3-IG* methylation, an imprinted gene DMR where we did not expect to see changes in gene expression since the mean methylation across the DMR was not significantly associated with phthalate exposure. Expression of the reference gene *GAPDH* was used as a control. SuperScript™ IV VILO™ Master Mix was used to convert RNA to cDNA. Predesigned TaqMan Gene Primer Assays for *MEG3, MEG3-IG* and *GAPDH*, as well as, TaqMan Fast Advanced Master Mix were used in the RT-PCR analysis according to the manufacturer's protocols. Reactions were performed on a Rotor-Gene 6000 (Qiagen, formerly Corbett Life Science). Amplicon specificity was verified by reviewing melt curves. Ct values were calculated for each gene, and ΔCt values, calculated as the difference between the imprinted genes (*MEG3* or *MEG3-IG*) and *GAPDH* Ct values, were used in statistical analysis. Interplate replicates and negative and positive controls were included in the expression analysis for quality control.

Statistical analyses

Covariates included in the regression models were selected from factors related to phthalate metabolite levels in the CHAMACOS cohort, after performing bivariate analyses (i.e., linear regressions, Student's t-test, ANOVA) and important covariates identified in previous studies examining the relationship between imprinted genes and prenatal phthalate exposure. The relevant covariates include prepregnancy body mass index (BMI), years in the USA, parity and child sex. Prepregnancy BMI and years in the USA were coded as continuous variables, while parity and sex were coded as shown in Table 1. Additionally, a plate (batch) variable was included in the regression models to adjust for technical effects.

Prior to performing regression analyses, we logit-transformed the methylation fractions to 'M-values' to reduce dependence of their variance on their mean levels. Methylation fractions have more intuitive biological interpretation, but the M-value is more statistically valid for the differential analysis of methylation levels [65,66]. Methylation outliers, specifically M-values that were below the 25th percentile minus three-times the interquartile range and values greater than the 75th percentile plus three-times the interquartile range, were designated as not available in the dataset, resulting in one to four substitutions for all imprinted genes, with the exception of the *MEST* and *PLAGL1* DMRs. As a result of the correlation between CpG sites within a DMR, we decided to conduct DMR-level principal components (PCs) analyses of the data to determine the independent M-value methylation signals, represented as linear combinations of CpGs that explain at least 95% of variability at the locus. DMR-level PC analyses yielded a total of 26 PCs across the nine investigated imprinted gene DMRs (number of PCs: *H19* = 2, *IGF2* = 2, *MEG3* = 1, *MEG3-IG* = 3, *MEST* = 2, *NNAT* = 2, *PLAGL1* = 4, *PEG3* = 6 and *SGCE/PEG10* = 4). The first PCs for all imprinted gene DMRs demonstrated similar contributions for each CpG to the variance explained by PC1. We fit separate regression models for each combination of the 26 PCs and 12 phthalate metabolite variables (11

Table 1. Demographic characteristics of Center for the Health Assessment of Mothers and Children of Salinas mothers and children (1999–2000).

Characteristic	CHAMACOS mothers and their children with imprinted gene DMR methylation data (n = 296) n (%)
Child sex:	
– Boy	148 (50.0)
– Girl	148 (50.0)
Child gestational age at birth:	
– 34–36 weeks	20 (6.8)
– ≥37 weeks	276 (93.2)
Child birth weight:	
– Low birth weight (<2500 g)	10 (3.4)
– Normal birth weight (≥2500 g)	286 (96.6)
Maternal age at pregnancy:	
– 18–24	137 (46.3)
– 25–29	101 (34.1)
– 30–34	46 (15.5)
– 35–45	12 (4.1)
Number of years mother lived in USA at pregnancy:	
– Less than 1 year	55 (18.6)
– 1–5 years	102 (34.5)
– 6–10 years	70 (23.6)
– 11 or more years	69 (23.3)
Maternal prepregnancy BMI:[†]	
– Underweight (<18.5 kg/m^2)	2 (0.7)
– Normal (18.5–24.9 kg/m^2)	118 (40.7)
– Overweight (25–29.9 kg/m^2)	115 (39.7)
– Obese (≥30 kg/m^2)	55 (19.0)
Parity:	
– 0	106 (35.8)
– ≥1	190 (64.2)
Education:	
– ≤6th grade	125 (42.2)
– 7–12th grade	114 (38.5)
– ≥High school graduate	57 (19.3)
Poverty status:	
– ≤Poverty level	184 (62.2)
– Poverty level	112 (37.8)

[†]Total number of observations for maternal prepregnancy BMI varies due to missing data.
BMI: Body mass index; CHAMACOS: Center for the Health Assessment of Mothers and Children of Salinas; DMR: Differentially methylated region.

phthalate metabolites and the DEHP summary variable) averaged across pregnancy, with the PCs as the response and the average phthalate metabolite concentrations and confounder variables (mentioned above) as covariates. Since the analysis with the PCs does not provide information regarding direction of association, we also performed separate regression analyses for each imprinted gene DMR using the average M-value methylation across the CpGs within the DMR as the outcome. We confirmed regression findings of the relationship between average phthalate metabolites and mean imprinted gene methylation by implementing a bootstrap analysis, sampling individuals with replacement for 1000 iterations.

Since many imprinted genes impact early growth patterns and *MEG3* has been associated previously with growth outcomes at birth, we performed additional analyses to examine the relationship between average percent methylation of the *MEG3* DMR and birth weight. A dichotomous birth weight variable was generated at a cut-off of 2500 g to distinguish between low and normal/high birth weight newborns. We performed a two-sample t-test

Table 2. Distribution of phthalate metabolite concentrations averaged across pregnancy.

Phthalate metabolite	Pregnancy average (n = 265)			
	Median	IQR	Minimum	Maximum
MEP	214.2	(90.7–444.7)	7.2	6607.7
MBP	24.4	(14.2–45.3)	3.2	228.6
MiBP	2.7	(1.6–5.3)	0.1	192.0
MEHP	3.9	(2.2–7.0)	0.1	101.6
MEHHP	16.1	(9.5–29.2)	1.4	478.6
MEOHP	12.1	(7.0–20.7)	0.9	353.4
MECPP	26.7	(17.7–45.3)	5.3	665.2
Σ DEHP	60.9	(37.1–98.5)	8.0	1524.5
MBzP	8.2	(4.7–13.8)	0.5	97.4
MCPP	2.0	(1.3–2.9)	0.1	28.3
MCOP	3.4	(2.1–4.7)	0.5	82.7
MCNP	1.9	(1.3–2.6)	0.3	17.1

All units are in μg/g creatinine.
DEHP: Di-2-ethylhexyl phthalate; IQR: Interquartile range; MBP: Mono-n-butyl phthalate; MBzP: Monobenzyl phthalate; MCNP: Monocarboxynonyl phthalate; MCOP: Monocarboxyoctyl phthalate; MCPP: Mono(3-carboxypropyl) phthalate; MECPP: Mono(2-ethyl-5-carboxypentyl) phthalate; MEHHP: Mono(2-ethyl-5-hydroxyhexyl) phthalate; MEHP: Mono(2-ethylhexyl) phthalate; MEOHP: Mono(2-ethyl-5-oxohexyl) phthalate; MEP: Monoethyl phthalate; MiBP: Mono-isobutyl phthalate.

to compare mean methylation values in the *MEG3* DMR in the two birth weight groups. We also ran regressions to test the association between *MEG3* mean methylation and birth weight, coded as a continuous or binary variable, adjusting for infant sex, maternal prepregnancy BMI, methylation plate, route of delivery and gestational age.

We controlled for the false discovery rate (FDR). The FDR can be defined as the average number of false rejections of the null hypothesis divided by the total number of rejections [67]. All statistical analyses were performed in R Version 3.2.3 (R Foundation for Statistical Computing, Vienna, Austria, 2013). p-values <0.05 were considered significant, and two-sided statistical tests were used.

Results

CHAMACOS participants

Demographic characteristics of CHAMACOS mothers and children are presented in Table 1. Most mothers were young, living below the poverty level, had previously delivered children, had low levels of education, and were overweight or obese (BMI $\geq$25 kg/m^2) prior to pregnancy. Approximately, half of the women resided in the USA for over 5 years at the time of delivery (mean = 6.9 years, SD = 6.9 years). The majority of the children, equally represented by boys and girls, were born term (93.2%) and with a normal birth weight (96.6%).

Phthalate exposure

Table 2 shows the distributions of average phthalate metabolite concentrations across pregnancy for the CHAMACOS mothers included in this study. Detection frequencies of phthalate metabolites during pregnancy were 90–100%. Compared with ten other phthalate metabolites, MEP had the highest urinary concentrations during pregnancy (interquartile range [IQR]: 90.7–444.7 μg/g creatinine) in the CHAMACOS cohort [49,52], consistent with trends observed in the general US population of adults [28]. Levels of urinary MEHHP (IQR: 9.5–29.2), MEOHP (IQR: 7.0–20.7) and MECPP (IQR: 17.7–45.3) contributed most to the DEHP sum concentration.

DNA methylation of imprinted genes

Distributions of DNA methylation values at individual CpG sites within each DMR and average methylation for each of the nine imprinted gene DMRs are shown in Figure 1. The percentage of DNA methylation varies between imprinted genes and within the individual CpG sites within DMRs. *MEG3* exhibited the highest (73 ± 5%) and *PEG3* had the lowest (35 ± 1%) average methylation compared with the other seven imprinted genes DMRs, which were closer to the expected methylation level of 50% and ranged from 44 to 55%. Moreover, whereas some of the imprinted genes (*H19*, *MEST*, *PLAGL1*, *SGCE*) have relatively similar CpG methylation values across each DMR, the *MEG3-IG* and *NNAT* DMRs demonstrate a wide range of methylation in CpGs from different individuals (*MEG3-IG*: 36–71%; *NNAT*: 42–75%).

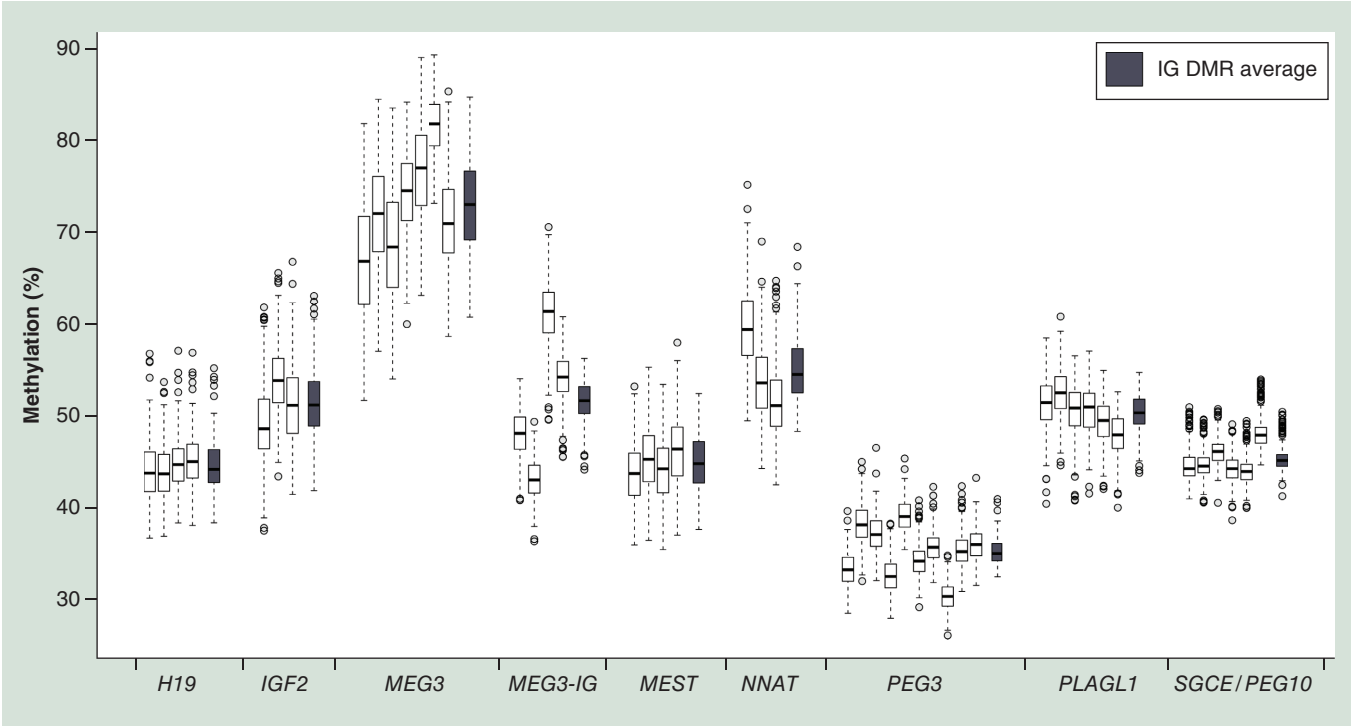

Figure 1. Distribution of average and CpG specific imprinted gene differentially methylated region percent methylation in Center for the Health Assessment of Mothers and Children of Salinas newborns. The white box plots represent distributions of the % methylation of individual CpGs in the nine imprinted gene DMRs in 296 newborns. The leftmost box plot for each imprinted gene DMR represents the first CpG assessed in the DMR. The gray box plots to the right of each group of white boxes represent the average % methylation across all CpG sites within an IG DMR. The total number of observations for each imprinted gene varies slightly due to missing data.
DMR: Differentially methylated region.

Table 3. Regression models of average maternal phthalate metabolite concentrations during pregnancy with principal components of child imprinted gene differentially methylated region M-value methylation at delivery.

Imprinted gene DMR	PC #	Significant exposure	β†	95% CI	p-value
MEG3	PC 1	MEHP	-0.321	(-0.602 to -0.039)	0.03
	PC 1	MEHHP	-0.544	(-0.885 to -0.203)	2E-03
	PC 1	MEOHP	-0.615	(-0.944 to -0.286)	3E-04*
	PC 1	MECPP	-0.812	(-1.211 to -0.412)	9E-05*
	PC 1	Σ DEHP	-0.719	(-1.099 to -0.338)	3E-04*
	PC 1	MBzP	-0.448	(-0.765 to -0.132)	0.01
MEG3-IG	PC 2	MCPP	0.050	(0.013–0.087)	0.01
PEG3	PC 3	MCOP	-0.051	(-0.095 to -0.007)	0.02
	PC 3	MCNP	-0.055	(-0.104 to -0.006)	0.03

Covariates in each regression model included batch, years in the USA, parity, prepregnancy BMI and child sex. Models included $\log_{10}$ transformed concentrations of creatinine-corrected phthalate metabolites.
†β for PCs are not interpretable; for direction of association, refer to mean methylation analysis
*Significant after FDR adjustment (all $p < 0.04$). $p < 0.05$ statistically significant.
BMI: Body mass index; DEHP: Di-2-ethylhexyl phthalate; DMR: Differentially methylated region; MBzP: Monobenzyl phthalate; MCNP: Monocarboxynonyl phthalate; MCOP: Monocarboxy-octyl phthalate; MCPP: Mono(3-carboxypropyl) phthalate; MECPP: Mono(2-ethyl-5-carboxypentyl) phthalate; MEHHP: Mono(2-ethyl-5-hydroxyhexyl) phthalate; MEHP: Mono(2-ethylhexyl) phthalate; MEOHP: Mono(2-ethyl-5-oxohexyl) phthalate; PC: Principal component.

Phthalates & DMR methylation principal component analyses

Significant results of the regression analyses of associations between PCs of imprinted gene DMR methylation in cord blood DNA and phthalate metabolite concentrations during pregnancy are shown in Table 3. After adjusting for years in the USA at the time of pregnancy, parity, prepregnancy BMI, child sex and batch, HMW phthalate metabolites were related to PCs of the *MEG3* DMRs and PC3 of *PEG3*. All DEHP metabolites assessed were

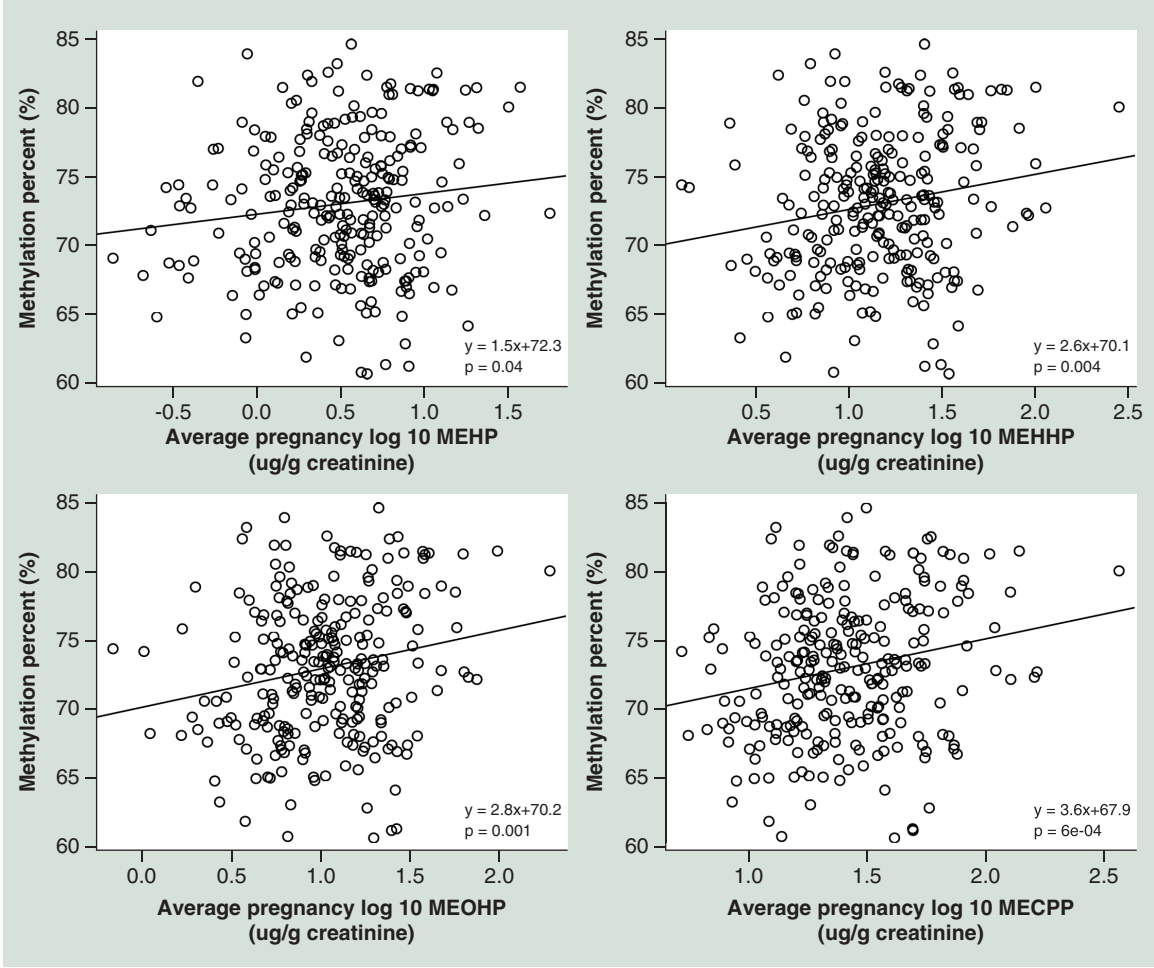

Figure 2. Scatter plots of the crude relationships between prenatal di-2-ethylhexyl phthalate metabolites and mean percent methylation of the *MEG3* differentially methylated region.
MECPP: Mono(2-ethyl-5-carboxypentyl) phthalate; MEHHP: Mono(2-ethyl-5-hydroxyhexyl) phthalate; MEHP: Mono(2-ethylhexyl) phthalate; MEOHP: Mono(2-ethyl-5-oxohexyl) phthalate.

significantly associated with PC1 of the *MEG3* DMR (MEHP: p = 0.03; MEHHP: p = 0.002; MEOHP: p = 0.0003; MECPP: p = 0.0001). MBzP was also related to PC1 of the *MEG3* DMR (p = 0.01). MCPP was associated with PC2 of the *MEG3-IG* DMR (p = 0.01). Average pregnancy levels of MCOP (p = 0.02) and MCNP (p = 0.03) were associated with the third principal component of *PEG3*. Out of the observed associations between the average phthalate metabolites during pregnancy and the *MEG3* DMRs, only the associations between *MEG3* and certain DEHP metabolites (MEOHP, MECPP, Σ DEHP) remained significant after FDR adjustment (all $p < 0.04$).

Phthalates & imprinted gene average DMR methylation

In order to determine directions of association, we analyzed the relationship between phthalate exposure and DNA methylation averaged across all CpG sites within each imprinted gene DMR (Figure 2, Table 4). In crude models without adjustment for confounders, we found a positive association between average pregnancy DEHP metabolites and mean methylation percent at the *MEG3* DMR (Figure 2, all $p < 0.05$). In models adjusting for covariates, *MEG3-IG* and *PEG3*, which were significantly associated with some of the phthalate metabolites in the PC analysis, were no longer significant in the analysis with mean M-value methylation as the outcome (*MEG3-IG* and MCPP; *PEG3* and MCOP; *PEG3* and MCNP; all p-values >0.05). MBzP (β = 0.17; p = 0.01) and DEHP metabolites (MEHP: β = 0.12, p = 0.03; MEHHP: β = 0.21, p = 0.002; MEOHP: β = 0.23, p = 0.0003; MECPP: β = 0.31, p = 0.00007) were positively associated with mean methylation across the *MEG3* DMR. All of the observed relationships remained significant after FDR adjustment (all $p < 0.05$), with the exception of the associations with

Table 4. Regression models of average maternal phthalate metabolite concentrations during pregnancy with mean child imprinted gene differentially methylated region M-value methylation at delivery.

Imprinted gene DMR	Significant exposure	β	95% CI	p-value
MEG3	MEHP	0.120	(0.015–0.225)	0.03
	MEHHP	0.206	(0.078–0.333)	2E-03*
	MEOHP	0.232	(0.110–0.335)	3E-04*
	MECPP	0.306	(0.157–0.455)	7E-05*
	Σ DEHP	0.271	(0.130–0.413)	2E-04*
	MBzP	0.168	(0.050–0.286)	0.01

Covariates in each regression model included batch, years in the USA, parity, prepregnancy BMI and child sex. Models included log10 transformed concentrations of creatinine-corrected phthalate metabolites.
*Significant after FDR adjustment (all $p < 0.05$). $p < 0.05$ statistically significant.
BMI: Body mass index; DEHP: Di-2-ethylhexyl phthalate; FDR: False discovery rate; MBzP: Monobenzyl phthalate; MECPP: Mono(2-ethyl-5-carboxypentyl) phthalate; MEHHP: Mono(2-ethyl-5-hydroxyhexyl) phthalate; MEHP: Mono(2-ethylhexyl) phthalate; MEOHP: Mono(2-ethyl-5-oxohexyl) phthalate.

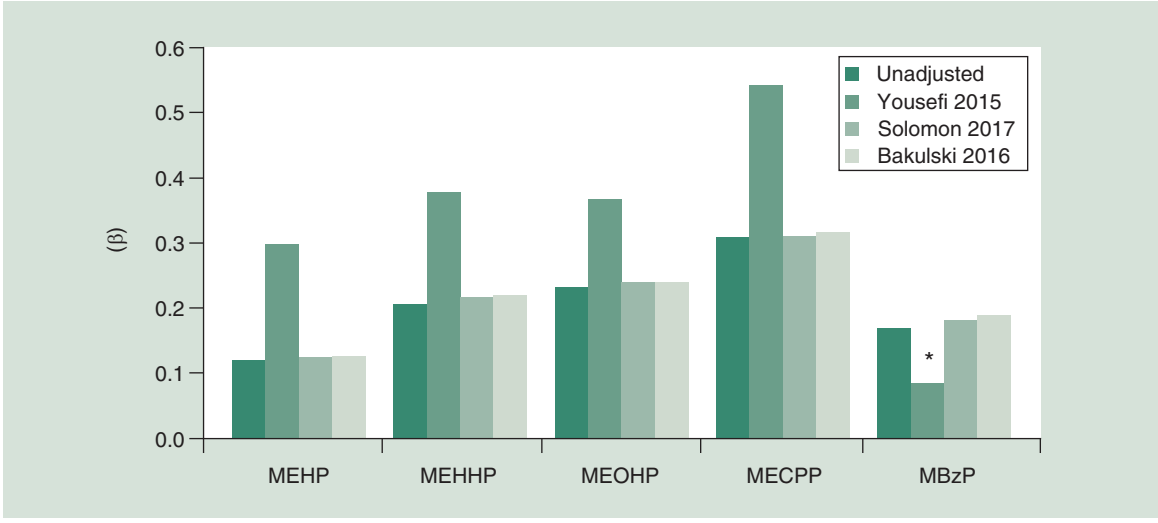

Figure 3. Regression coefficients of the relationships between prenatal di-2-ethylhexyl phthalate metabolites and mean methylation of the *MEG3* DMR with and without cell-type adjustment. The methods included adjusting for cytological differential cell counts in a subset of cord blood samples (Yousefi 2015 [62]) and estimating cell-type proportions from CHAMACOS 450K cord blood DNA methylation data using adult (Solomon 2017 [47]) and cord blood (Bakulski 2016 [64]) flow sorted reference panels. The cord blood reference panel includes estimates of nucleated red blood cells (nRBCs).
*Not statistically significant at $p < 0.05$; all other presented relationships were significant at $p < 0.05$.
MBzP: Monobenzyl phthalate; MECPP: Mono(2-ethyl-5-carboxypentyl) phthalate; MEHP: Mono(2-ethylhexyl) phthalate; MEHHP: Mono(2-ethyl-5-hydroxyhexyl) phthalate; MEOHP: Mono(2-ethyl-5-oxohexyl) phthalate.

MEHP and MBzP. Bootstrap analysis confirmed the findings; specifically, the significant and positive association between HMW phthalates and mean methylation across CpG sites within the *MEG3* DMR.

Adjustment for cell composition

Figure 3 presents the regression estimates for the significant relationships of the mean *MEG3* methylation and prenatal phthalate metabolite concentrations adjusting for cell-type composition using three different methodologies as described in the methods section. The array-based estimates of cell composition [47,64] produced estimates of the regression coefficients similar to the model without cell-type adjustment (unadjusted). Coefficients from models adjusting for cytological DCC [62] showed even stronger relationships between phthalate exposure and *MEG3* methylation in the same direction. Adjusting for white blood cell composition estimates using CHAMACOS 450K array data and the cord blood flow sorted reference panel replicated the crude models of the imprinted gene mean methylation and average pregnancy phthalate metabolites, suggesting limited bias due to cell-type composition.

Gene expression validation

Following identification of significant hits for the association between DEHP and BzBP phthalate metabolites and *MEG3* methylation, we assessed gene expression of the *MEG3* gene in a subset of 119 CHAMACOS participants. Ct values for MEG3 ranged from 22 to 36, with a mean of 27.6. As expected [6], *MEG3* M-value methylation was inversely correlated with *MEG3* expression relative to the housekeeping gene *GAPDH* (Pearson r = -0.10; p = 0.29). As a control, we also assessed the relationship between *DLK1* expression and *MEG3-IG* mean methylation, an imprinted gene DMR that was not significantly associated with phthalate exposure. As expected, we did not observe a significant relationship (Pearson r = -0.05; p = 0.58). Additionally, we tested the relationships between phthalate metabolite concentrations and *MEG3* expression, adjusting for the covariates identified in the methylation analysis. We observed consistent negative associations between DEHP metabolites and *MEG3* expression (MEHP: β = -0.44, MEHHP: β = -1.14, MEOHP: β = -0.63, MECPP: β = -1.21); however, none of the associations reached statistical significance.

Imprinted gene methylation & birth weight

We found a significant difference in mean *MEG3* percent methylation levels comparing low birth weight newborns and newborns with normal to high birth weights (t = -2.35; p = 0.04), with lower average methylation values in the low birth weight group. However, the regression analyses did not show a significant relationship between *MEG3* methylation and continuous and binary birth weight in the CHAMACOS newborns.

Discussion

We examined the association of prenatal phthalate exposure and imprinted gene methylation profiles in newborn children using DNA isolated from cord blood. Previous analysis in the CHAMACOS cohort of repetitive element methylation and array-based DMRs uncovered significant associations of DNA methylation in cord blood with MEP and DEHP metabolites, respectively [46,47]. In the current study of nine imprinted gene DMRs, the most consistent findings were the associations between DEHP and BzBP phthalate metabolites and DNA methylation of the *MEG3* DMR, located within an intron, downstream of the *MEG3* promoter [68]. DNA methylation profiles in the CHAMACOS cohort were similar to values reported for imprinted genes in other cohorts with DNA isolated from cord blood samples [68].

To the best of our knowledge, our study is the first to demonstrate the relationship between *MEG3* DNA methylation and prenatal phthalate exposure. In CHAMACOS newborns, prenatal exposure to several DEHP metabolites was positively and significantly associated with DNA methylation at the *MEG3* DMR. Validation analysis demonstrated an inverse relationship between *MEG3* methylation and expression, which is consistent with expected effects of hypermethylation. However, this result did not quite reach statistical significance. Relatively weak relationships between differential DNA methylation and expression of the same genes is common, as regulatory mechanisms may also include noncoding RNAs and chromatin modifications [1].

Hypomethylation or increased expression of *MEG3* in humans have previously been associated with higher birth weight and large for gestational age status, respectively [69,70]. Conversely, decreased expression of *MEG3* has been observed in human Intrauterine Growth Restriction placenta samples [71]. In the current study, we did not find a significant relationship between *MEG3* methylation and continuous birth weight, which corroborates a previous report by Vidal *et al.* (2013) [16]. However, we did observe a significant difference when we contrasted *MEG3* methylation in low birth weight newborns and normal weight ones. In addition to its relationship with birth outcomes, hypermethylation of the *MEG3* gene in human tumor samples and cell lines has previously been associated with tumorigenesis [57,58,72–76]. Additionally, epigenetic and genetic studies have also uncovered links between *MEG3* variants and methylation patterns and diabetes [77]. Specifically, Kameswaram *et al.* [78] found decreased expression of *MEG3* in human islets from T2D organ donors, which was highly correlated with hypermethylation of *MEG3*. Studies in mice have demonstrated that decreased expression of the *Meg3* gene can impact glucose tolerance and reduce insulin secretion [79]. Modification of epigenetic profiles in *MEG3* following environmental exposure could lead to shifts in hormonal and metabolic markers, as well as early growth.

Only two studies have assessed the relationship between pregnancy phthalate exposure and imprinted gene methylation, both in placenta samples. Zhao *et al.* [27] measured urinary phthalate concentrations of five metabolites (two LMW and three HMW) and DNA methylation of *IGF2* (two CpGs) in 181 placenta samples collected in China. The researchers observed that DEHP metabolites were significantly and negatively associated with *IGF2* DNA methylation. Additionally, LaRocca *et al.* [25] examined the association between phthalate metabolite

concentrations and DNA methylation of the *H19* and *IGF2* imprinted genes in 179 placenta samples from two cohorts, the Harvard Epigenetic Birth Cohort and the Predictors of Preeclampsia Study. The authors found a significant inverse relationship between *H19* methylation in placenta and the sum of the LMW phthalates metabolite concentrations and the sum of all the 11 phthalate metabolites measured. A similar pattern was observed for the *IGF2 DMR0*. They also observed an inverse association between DEHP metabolites and the *IGF2 DMR0*, similar to the results seen in the study by Zhao *et al.* However, both of these studies did not analyze *MEG3* methylation in respect to phthalates.

The number of CpG sites in the studies assessing placenta DNA methylation and the cord blood analysis in CHAMACOS differ between the studies. Additionally, only two imprinted genes were assessed in the placenta studies, while our study involved eight imprinted genes with nine DMRs. We are also mindful that the difference between the tissues used (placenta vs cord blood) may also be the reason for observed differences in methylation results with phthalate exposure.

A recent study by Goodrich *et al.* [26] examined the relationship between prenatal and childhood exposure to heavy metals and endocrine disrupting chemicals, including phthalates and DNA methylation of DMRs of two imprinted genes (*H19*: 4 CpG sites, *IGF2*: 7 CpG sites) in periadolescents. They found that phthalate metabolite levels (MBzP and MIBP) in maternal urine samples were associated with *H19* hypermethylation in blood from 247 8- to 14-year-old children, but this finding was attenuated to nonsignificance after adjustment for multiple testing. Overall, the CHAMACOS results as well as the findings by LaRocca *et al.* [25], Zhao *et al.* [27] and Goodrich *et al.* [26] suggest potential epigenetic modifications in imprinted genes involved in fetal growth, in association with prenatal phthalate exposure from the time of birth that may persist into adolescence.

A hypothesized mechanism by which exposure to environmental chemicals may induce epigenetic shifts involves altered transcription factor occupancy. Martin and Fry [80] performed an analysis across 11 studies that included altered cord blood and placental methylation patterns of CpG sites within 341 genes related to five environmental contaminants (lead, arsenic, cadmium, tobacco smoke and mercury). They found that 56 transcription factor binding sites were enriched in the promoter regions of the genes, which could impact the access of DNA methyltransferase to CpG sites, inhibiting the process of DNA methylation and leading to hypomethylation of the gene. Additionally, the authors provided suggestive evidence that glucocorticoid-receptor dependent signaling may mediate the relationship between environmental chemical exposure and transcription factor activity, since the promoter regions of 38 of the 56 genes encoding the transcription factors contained glucocorticoid-responsive-related sequence elements. It is possible that prenatal exposure to phthalates could initiate a similar response of altered transcription factor activity in the current study in the *MEG3* DMR, located downstream of the promoter region, resulting in methylation shifts. Future studies should further explore the mechanism proposed by Martin and Fry in additional environmental contaminants, including phthalates.

One of the notable strengths of our study involves the novel investigation of DNA methylation profiles of multiple imprinted genes in newborns in relation to prenatal phthalates, a ubiquitous endocrine disruptor. Two previous studies [25,27] assessed the role of pregnancy phthalate levels on DNA methylation in imprinted genes; however, they used placenta samples and only for three or fewer imprinted regions. In addition to the advantages of assessing a greater number of imprinted genes in our study, our analyses also benefited from a larger sample size.

There were also several limitations in our study. In the umbilical cord, nRBCs and leukocytes mixed in the clots may contribute to DNA methylation levels [64]. However, we were able to account for variable leukocyte composition typical in umbilical cord blood in sensitivity analyses using cytological differential cell count [62], as well as a cord blood reference panel including nRBCs [47,64]. The results for the unadjusted and adjusted models were very similar. Another potential limitation involves the applicability of the findings to other populations, given that the CHAMACOS participants are Mexican–Americans from families with low socioeconomic status. Since observed exposure levels to phthalates in CHAMACOS women were similar to the general population of US adults, the data are likely relevant to many other populations. In this study, we have focused on nine imprinted gene DMRs that have been previously established and validated, and we intend to explore other regions with parental-origin-dependent differential methylation in the future.

Conclusion

Several significant associations were observed in CHAMACOS newborns between prenatal phthalate exposure, primarily DEHP metabolites, and DNA methylation in DMRs of *MEG3*. *MEG3* is known for its involvement in early growth, tumorigenesis and metabolic processes. This new data complement our earlier reports on significant

effects of prenatal phthalates on *Alu* and *LINE-1* repeats, as well as differential methylation of several genes revealed by 450K analysis. Our findings would need to be replicated in other cohorts in the future, but they do provide further insight into the complex relationship between environmental exposure to EDCs and biological pathways leading to altered health status in children, including modifications of DNA methylation of imprinted genes.

Future perspective

Understanding the molecular mechanisms that mediate the effects of chemical exposure in the prenatal environment on health is critical for disease prevention. DNA methylation, an epigenetic mechanism that has been shown to relate to both exposures and disease status, may be one pathway through which endocrine disrupting chemicals, including phthalates, exert their effect. There is limited, yet suggestive evidence that prenatal phthalate exposure can impact DNA methylation profiles of imprinted genes, as well as other genes. Future research should focus on understanding the role of imprinted gene methylation on mediating the effects of environmental exposure on health outcomes through adulthood.

Summary points

- Epigenetic mechanisms are possible mediators of the relationship between environmental exposure and disease status.
- DNA methylation is the most widely examined epigenetic mechanism.
- DNA methylation is involved in the regulation of imprinted genes, which are characterized by the expression of one of the two parental alleles.
- In 296 newborns from the Center for the Health Assessment of Mothers and Children of Salinas study, we assessed the relationship between prenatal phthalate exposure and methylation of nine imprinted gene differentially methylated regions.
- Prenatal phthalate exposure averaged across pregnancy was associated with multiple imprinted gene differentially methylation regions.
- Average phthalate metabolites concentrations across pregnancy of several di-2-ethylhexyl phthalate metabolites were significantly and positively associated with methylation at the *MEG3* DMR, a gene associated with early growth, tumorigenesis and diabetes. Many of these relationships remained significant after false discovery rate adjustment.
- Sensitivity analysis, including adjusting for specific gravity instead of creatinine to account for urinary dilution and accounting for cord blood cell composition by multiple methods, yielded similar results to crude models.
- Early-life environmental exposure to endocrine disrupting chemicals, specifically phthalates, may contribute to dysregulation of DNA methylation of imprinted genes.

Author's contributions

G Tindula, N Holland, C Hoyo, B Eskenazi and SK Murphy conceived and designed the study; A Bradman, K Huen and Z Huang provided expert guidance in study design and manuscript review; G Tindula, C Grenier and ME Fung analyzed the data; and G Tindula and N Holland wrote the manuscript. All authors read, edited and approved the manuscript prior to submission.

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Disclaimer

The interpretations of the study findings pertain to the authors alone, and do not reflect the official views of NIEHS, the US EPA, Duke University, NC State University or the University of California, Berkeley.

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Ethical disclosure

The authors state that they have obtained appropriate institutional review board approval for all human experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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- **Martin and Fry presents a novel biological mechanism, transcription factor activity, which can mediate the effect of environmental chemicals on CpG methylation profiles.**