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A Comprehensive Assessment of Associations between Prenatal Phthalate Exposure and the Placental Transcriptomic Landscape

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Background: Phthalates are commonly used endocrine-disrupting chemicals that are ubiquitous in the general population. Prenatal phthalate exposure may alter placental physiology and fetal development, leading to adverse perinatal and childhood health outcomes.

Objective: We examined associations between prenatal phthalate exposure in the second and third trimesters and the placental transcriptome at birth, including genes and long noncoding RNAs (lncRNAs), to gain insight into potential mechanisms of action during fetal development.

Methods: The ECHO PATHWAYs consortium quantified 21 urinary phthalate metabolites from 760 women enrolled in the CANDLE study (Shelby County, TN) using high-performance liquid chromatography–tandem mass spectrometry. Placental transcriptomic data were obtained using paired-end RNA sequencing. Linear models were fitted to estimate separate associations between maternal urinary phthalate metabolite concentration during the second and third trimester and placental gene expression at birth, adjusted for confounding variables. Genes were considered differentially expressed at a Benjamini-Hochberg false discovery rate (FDR) $p < 0.05$. Associations between phthalate metabolites and biological pathways were identified using self-contained gene set testing and considered significantly altered with an FDR-adjusted $p < 0.2$.

Results: We observed significant associations between second-trimester phthalate metabolites mono (carboxyisooctyl) phthalate (MCIOP), mono-2-ethyl-5-carboxypentyl phthalate, and mono-2-ethyl-5-oxohexyl phthalate and 18 genes in total, including four lncRNAs. Specifically, placental expression of *NEAT1* was associated with multiple phthalate metabolites. Third-trimester MCIOP and mono-isobutyl phthalate concentrations were significantly associated with placental expression of 18 genes and two genes, respectively. Expression of genes within 27 biological pathways was associated with mono-methyl phthalate, MCIOP, and monoethyl phthalate concentrations.

Discussion: To our knowledge, this is the first genome-wide assessment of the relationship between the placental transcriptome at birth and prenatal phthalate exposure in a large and diverse birth cohort. We identified numerous genes and lncRNAs associated with prenatal phthalate exposure. These associations mirror findings from other epidemiological and *in vitro* analyses and may provide insight into biological pathways affected *in utero* by phthalate exposure. https://doi.org/10.1289/EHP8973

Introduction

Phthalates are ubiquitous chemicals used as plasticizers in numerous consumer products, leading to pervasive human exposure (Ferguson et al. 2014; Sathyanarayana 2008). Parent phthalate compounds undergo hydrolysis to monoesters, which are then are transformed into secondary metabolites depending upon on their chemical structure and molecular weight, overall resulting in a variety of different metabolites that the fetus and placenta are exposed to during pregnancy and are detectable in urine (Domínguez-Romero and Scheringer 2019). Prenatal phthalate exposure is associated with adverse perinatal outcomes and pregnancy complications (Martínez-Razo et al. 2021), including decreased gestational length (Boss et al. 2018; Wolff et al. 2008), decreased anogenital distance, and hydrocoele (Sathyanarayana et al. 2017; Swan et al. 2005). Recent studies also suggest increased odds of negative childhood outcomes, including eczema development, asthma development in males (Adgent et al. 2020), decreased mental and motor development scores and increased internalizing behaviors (Whyatt et al. 2012), social impairment characteristics (Day et al. 2021), and deficits in intellectual development (Factor-Litvak et al. 2014) in relation to prenatal phthalate exposure. In combination, these observational studies suggest that phthalate exposure during the prenatal period may program alterations in the *in utero* environment that have lasting effects on developing children from infancy into middle childhood.

The placenta is a crucial regulator of the *in utero* environment, impacting fetal development and health, as reviewed by Myatt (2006). This ephemeral fetal organ regulates gas exchange, transports nutrients and waste, provides immunological defense, and is involved in maternal–fetal communication via maternal decidual tissue (Burton and Jauniaux 2015). It produces a variety of neuropeptides, growth factors, and steroid hormones, which are released into the maternal circulation (Mesiano 2009). The placenta has a distinct transcriptome that includes genes not expressed in other human organs (Saben et al. 2014). The placental transcriptome is dynamic (Sitras et al. 2012) and responds to

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cues from the maternal environment (Cox et al. 2015). Changes to the placental transcriptome are associated with adverse birth outcomes, including preterm birth (Brockway et al. 2019; Eidem et al. 2015; Paquette et al. 2018) and birthweight (Deyssenroth et al. 2017). The generation and analysis of omics data from key tissues, such as the placenta, is a valuable tool for quantifying how environmental perturbations influence the developing fetus, as reviewed by Everson and Marsit (2018).

Phthalates are endocrine-disrupting chemicals (EDCs) that impact hormone function through direct and indirect mechanisms of action, as reviewed by Mariana et al. (2016). EDCs interact directly with a class of transcription factors known as nuclear hormone receptors, acting as antagonists or agonists, resulting in changes in synthesis and signaling of downstream genes (Hall and Greco 2019). Phthalates may disrupt several nuclear hormone receptors, including peroxisome proliferator-activated receptors (PPARs) (Hurst and Waxman 2003) and sex steroid hormone receptors (estrogen receptors and the androgen receptor) (Takeuchi et al. 2005). PPARS are a family of nuclear transcription factors that play a critical role in regulating lipid metabolism, as reviewed by Gervois et al. (2000). The placenta metabolizes and transfers lipids to the developing fetus, where they are used as essential building blocks for a number of developmental processes (Herrera et al. 2006). Primary phthalate metabolite mono-2-ethylhexyl phthalate (MEHP) can bind to and modulate activity of PPARs (Desvergne et al. 2009). Both MEHP and monobenzyl phthalate, a primary metabolite of butylbenzyl phthalate, induce the expression of PPAR target genes in liver and adipocyte cell lines (Hurst and Waxman 2003). Phthalate metabolites mono-*n*-butyl phthalate (MNBP), monobenzyl phthalate, and MEHP can also induce expression of PPAR-γ in primary placental cells (Adibi et al. 2017). Phthalates can also disrupt immune function by activating pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) in macrophages/monocytes (Hansen et al. 2015). Activation of inflammatory processes is linked to a number of pregnancy complications, including preterm labor (Romero et al. 2014). Although these specific gene and pathway targets have been identified within *in vitro* systems, a broader understanding of how phthalate exposure impacts fetal development is needed.

Phthalates induce molecular changes within the placenta that may alter its function, as demonstrated through a number of *in vitro* and *in vivo* studies, as reviewed by Strakovsky and Schantz (2018). Prenatal exposure to monoethyl phthalate (MEP), a primary metabolite of diethyl phthalate (DEP), has been associated with reduced placental weight (Mustieles et al. 2019). Di-2-ethylhexyl phthalate (DEHP) and its primary metabolite MEHP negatively impact placental differentiation and invasion and impair key placental functions, including nutrient transport and endocrine signaling (Martínez-Razo et al. 2021). Prenatal DEHP treatment has resulted in the down-regulation of genes essential for placental angiogenesis and reductions in microvessel density in the placental labyrinth in mice (Yu et al. 2018). DEHP treatment resulted in significant changes in the expression of 112 genes in trophoblast stem cell lines from rhesus monkey blastocysts, which was quantified through RNA sequencing (Midic et al. 2018). Candidate gene assessments of noncoding genes in human studies revealed associations between *in utero* phthalate exposure and placental microRNAs (miRNAs) (LaRocca et al. 2016) and long noncoding mRNAs (lncRNAs) (Machtinger et al. 2018). These noncoding RNA molecules act as posttranscriptional regulators of gene expression. Phthalates have been shown to induce apoptosis and oxidative stress in placental cell lines through miRNA signaling (Meruvu et al. 2016a, 2016b). Together, the generation and analysis of transcriptomics data through *in vitro* experiments, *in vivo* experiments, and human studies provide a valuable tool for quantifying perturbations that influence genomic regulation and physiological activity, giving insight into underlying biological mechanisms of fetal development.

Transcriptomic studies of phthalate exposure within placental cell lines have primarily focused on assessing gene expression related to known mechanisms, including steroid metabolism (Adibi et al. 2010) and PPAR-γ activity (Adibi et al. 2017; Gao et al. 2017). Only one genome-wide assessment of the first-trimester placental transcriptome and expression patterns associated with total urinary phthalate measurements has been reported (involving 16 individuals) (Grindler et al. 2018). The timing of assessment is critical because the placental transcriptome changes across pregnancy. Herein, we sought to characterize associations between the placental expression of protein-coding genes and lncRNAs at birth and phthalate metabolites measured in urine during the second and third trimester of pregnancy. We elected to analyze the relationship between each individual phthalate metabolite and placental gene expression to gain the most comprehensive understanding of these distinct relationships. This study represents, to our knowledge, the first comprehensive assessment of the placental transcriptome at birth in association with phthalate metabolites quantified in maternal urine during pregnancy.

Methods

Study Participants

Placental tissue was collected during delivery from 937 women enrolled in the Conditions Affecting Neurocognitive Development and Learning in Early Childhood (CANDLE) study. The CANDLE study is a prospective pregnancy cohort study set in Shelby County, Tennessee, which enrolled women between 2006 and 2011, and has been described in detail previously (Sontag-Padilla et al. 2015). This study represents a subset of the CANDLE population ($n = 760$) with complete transcriptomic data, phthalate measurements in the second or third trimester, and childhood health outcomes (criteria from the parent study). Exclusion criteria included confirmed clinical chorioamnionitis, oligohydramnios, placental abruption, infarction or previa, and fetal chromosomal abnormalities. Covariate data—including maternal race, maternal age, and maternal education—were self-reported from a demographic survey conducted during the enrollment visit, and fetal sex and birthing method (labor type) were ascertained from medical record abstraction by a registered nurse, as described in the CANDLE methodological overview (Sontag-Padilla et al. 2015). Data represented in the present study include only participants with no missing data. All research activities for the CANDLE cohort were approved by the institutional review board (IRB) of the University of Tennessee Health Sciences Center. Analyses were conducted as part of the Environmental influences on Child Health Outcomes (ECHO) PATHWAYS study and were approved by the University of Washington IRB.

Collection of Maternal Urine and Quantification of Phthalate Metabolite Concentrations

Detailed methods of phthalate measurements within this cohort have been described previously (Adgent et al. 2020). Maternal urine was collected using phthalate-free polypropylene containers from women during two clinical visits that occurred in the second and third trimester (Sontag-Padilla et al. 2015). Samples were processed and stored at −80°C in the study repository of the University of Tennessee Health Science Center Department of Pathology. Samples were analyzed for 21 metabolites using solid-phase extraction and high-performance liquid chromatography–

tandem mass spectrometry. Process and instrument blanks were included for quality control. Specific gravity was determined using a handheld refractometer. Phthalate measurements were adjusted for specific gravity, and the log concentration (in nanograms per milliliter) was used in our models, as was described previously (Adgent et al. 2020). For samples below the limit of detection (LOD), the concentration was reported as the LOD divided by the square root of 2. We included phthalate metabolites where >70% of samples were above the LOD. Final analyses included 16 metabolites in the second trimester and 14 metabolites in the third trimester, as well as DEHP concentration. DEHP was calculated as the molar sum of five metabolites: MEHP, mono-2-ethyl-5-oxohexyl phthalate (MEOHP), mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP), mono-2-ethyl-5-carboxypentyl phthalate (MECPP), and mono 2-(carboxymethyl) hexyl phthalate (MCMHP). A subset of 155 participants (21% of the third-trimester samples) had two urine measurements within the third trimester (range of visits: 2.5–12.6 wk; median visit difference: 5.2 wk). For these individuals, we calculated the mean value for each phthalate metabolite from both measurements. We identified one participant as an outlier whose urinary measurements of MECPP, MEHHP, MEOHP, and DEHP were six standard deviations (SDs) from the median value and four SDs from the second lowest value. This participant's second-trimester measurements were removed from the analysis. Urine collected from these two clinical visits was also used to quantify urinary cotinine, which was also adjusted by specific gravity, as previously described (Ni et al. 2021). Maternal cotinine >200 ng/dL at either urine collection time point was used as a marker of maternal smoking given that this cutoff is commonly used to define smokers (Schick et al. 2017).

Placental Sample Processing and RNA Sequencing

Within 15 min of delivery, a piece of placental villous tissue in the shape of a rectangular prism with approximate dimensions of $2 \times 0.5 \times 0.5$ cm was dissected from the placental parenchyma and cut into four ~0.5-cm cubes. The tissue cubes were placed in a 50-mL tube with 20 mL of RNAlater and refrigerated at 4°C overnight (≥8 h but ≤24 h). Each tissue cube was transferred to an individual 1.8-mL cryovial containing fresh RNAlater. The cryovials were stored at −80°C, and the fetal villous tissue was manually dissected and cleared of maternal decidua. Following dissection, the fetal samples were placed into RNAlater and stored at −80°C. Approximately 30 mg of fetal villous placental tissue was used for RNA isolation. The tissue was homogenized in tubes containing 600 µL of Buffer RLT Plus with β-mercaptoethanol using a TissueLyser LT instrument (Qiagen). RNA was isolated using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen) according to the manufacturer's recommended protocol. RNA purity was assessed by measuring optical density ratios ($OD_{260/230}$ and $OD_{280/260}$) with a NanoDrop 8000 spectrophotometer (Thermo Fischer Scientific). RNA integrity was determined with a Bioanalyzer 2100 using RNA 6000 Nanochips (Agilent). Only RNA samples with an RNA integrity number (RIN) of >7 were sequenced.

RNA sequencing was performed at the University of Washington Northwest Genomics Center (NWGC). Total RNA was poly A enriched and complementary DNA libraries were prepared using the TruSeq Stranded mRNA kit (Illumina). Each library was uniquely barcoded and subsequently amplified by 13 cycles of polymerase chain reactions. Library concentrations were quantified using Qubit Quant-it dsDNA high sensitivity assay fluorometric quantitation (Life Technologies). Average fragment size and overall quality were evaluated by the DNA1000 assay on an Agilent 2100 Bioanalyzer. Each library was sequenced to an approximate depth of 30 million reads on an Illumina HiSeq 4000 instrument. De-multiplexed BAM files were converted to FASTQ format using Samtools bam2fq. RNA sequencing quality control was performed using both the FASTX-toolkit (version 0.0.13; ILRI Research Computing) and FastQC (version 0.11.2; ILRI Research Computing) (Brown et al. 2017). Transcript abundances were estimated by aligning to the GRCh38 transcriptome (Gencode version 33) using Kallisto (Bray et al. 2016), then collapsed to the gene level using the Bioconductor tximport package, scaling to the average transcript length (Soneson et al. 2015). Only protein-coding genes, processed pseudogenes, and lncRNAs were included in this analysis.

Identification of Differentially Expressed Genes

Differentially expressed mRNAs were identified using the limma-voom pipeline (Law et al. 2014). Gene counts were scaled to library size (normalized using a trimmed mean of M-values) (Robinson and Oshlack 2010) and converted to log counts/million (log CPM). After filtering to remove unreliably expressed genes (defined as average log-CPM <0), observation-level weights were computed based on the relationship between the mean and variance of the log-CPM values. Comparisons were then made using conventional weighted linear models. We adjusted for multiple comparisons using the Benjamini-Hochberg approach (Benjamini and Hochberg, 1995). Genes were considered statistically significant at a false discovery rate (FDR) adjusted $p < 0.05$. We selected potential confounders *a priori* by reviewing covariate data that was associated with phthalates and placental transcriptomics. These models included the following confounding variables: *a*) RNA sequencing batch; *b*) birthing method/labor type (labor vs. no labor); *c*) fetal sex; *d*) maternal race (Black vs. other); *e*) maternal age (continuous); and *f*) maternal education (college or above vs. high school or less). Separate models were run for the second and third trimester. Maternal race was dichotomized because of the small sample size (<5%) of specific race groups (multiple race, Asian, or other). We performed this analysis in all infants with complete data at each trimester, and we also performed a stratified analysis in only female and only male infants. In our stratified analysis, we did not adjust for fetal sex, but we did adjust for all other confounders. A complete overview of the sample collection and a directed acyclic diagram are provided in Figure S1.

Pathway Enrichment Analysis

To identify pathways with significant associations between gene expression and each individual phthalate metabolite, we applied a self-contained gene set test. We specifically used the FRY method (Giner and Smyth 2016), which is a technical improvement over the commonly used Roast method (Wu et al. 2010). The FRY method evaluates whether the average *t*-statistic for each gene set is larger than expected under the null hypothesis. We included all Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathways (Kanehisa et al. 2016) except disease pathways (KEGG release 98.1). Because this was an exploratory analysis, pathways were considered statistically significant at FDR-adjusted $p < 0.2$. Individual pathways were visualized using the Bioconductor Pathview package (Luo and Brouwer 2013).

Results

Maternal urine samples were collected at clinic visits in the second trimester (13–26 wk, $N = 594$ samples) and the third trimester (26–38 wk, $N = 735$ samples) to quantify phthalate metabolites. A total of 570 placental samples collected at birth had matched urinary phthalate measurements at both time points,

Table 1. Continuous and categorical information about CANDLE cohort participants ($N = 760$ total), recruited from Shelby County, Tennessee, from 2006 to 2011.

	Second trimester urine samples with matched placental data ($N = 594$)			Third trimester urine samples with matched placental data ($N = 735$)		
Categorical/Continuous variables	N/Median	%/Range (Min-Max)	Number of DEGs[a]	N/Median	%/Range (Min-Max)	Number of DEGs[a]
Categorical Variables						
Maternal Race (Binary)						
Black	342	57.6%	Ref	408	55.5%	Ref.
Other	252	42.4%	3,725	327	44.5%	4,460
Birthing Method (Labor Type)						
Labor	484	81.5%	Ref	595	81.0%	Ref
No Labor	110	18.5%	1,527	140	19.0%	2,286
Fetal Sex						
Female	296	49.8%	Ref	375	51.0%	Ref
Male	298	50.2%	1,137	360	49.0%	1,319
RNA Sequencing Batch						
Batch 1	120	20.2%	8,211	143	19.5%	8,543
Batch 2	14	2.4%	4,198	22	3.0%	5,928
Batch 3	192	32.3%	7,752	230	31.3%	8,067
Batch 4	268	45.1%	Ref	340	46.3%	Ref
Maternal Education						
High School or Less	271	45.6%	1,934	351	47.8%	2,736
College or Above	323	54.4%	Ref	384	52.2%	Ref
Maternal Smoking Status: Cotinine >200 ng/dL						
No	545	91.8%	Ref	676	92.0%	Ref
Yes	49	8.2%	1	59	8.0%	18
Continuous Variables						
Maternal Age (Years)	26.76	16–40	218	27	16–40	464
Gestational Age at Birth (weeks)	39.28	26.85–41.85	1,496	39.29	31.7–41.9	1,378

Note: This analysis included only participants with complete covariate data, so there are no missing data. Placental expression was quantified in placentas following delivery, but we constructed two data sets based on which samples had matched urine collected from the second or third trimester. The number reported here represents the number of genes that were considered statistically significant with an false discovery rate adjusted $p < 0.05$. CANDLE, Conditions Affecting Neurocognitive Development and Learning in Early Childhood; DEG, differentially expressed gene; max, maximum value; min, minimum value; Ref, reference group.

[a]*p*-Values were derived from conventional univariate logistic regression models (for categorical data) or univariate linear regression models (for continuous data) with observation-level weights for mean/variance relationships and adjusted for multiple comparisons using the Benjamini-Hochberg approach.

and 760 placental samples had urinary phthalate measurements at either time point. The time between the second and third visit ranged from 4 to 20 wk, with a median time between visits of 10 wk. Complete covariate data from individuals at each time point are shown in Table 1. The majority of individuals in this cohort identified as Black (58% of samples with second-trimester phthalate measurements and 56% of samples with third-trimester phthalate measurements). Most of the remaining participants ("other") identified as White (37.5% of participants with urine collected in the second trimester and 38.37% of participants with urine collected in the third trimester). The rest of the participants (<5%, also grouped into the "other" category) identified as either multiple race, Asian, or other. Most participants underwent labor (81%), and the distribution of sex across placental samples was similar. Approximately 8% of participants in this cohort had cotinine measurements >200 ng/dL at either time point and were considered smokers based on this criterion. We identified a number of identification of differentially expressed genes (DEGs) associated with our *a priori*–selected confounding variables, including race, birthing method/labor type, fetal sex, maternal education, maternal age, maternal smoking status, and RNA sequencing batch, using a cutoff of FDR-adjusted $p < 0.05$ (Table 1). Covariates with the highest number of genes associated with placental gene expression at each trimester were maternal race (Black vs. all others) and maternal education (some college education or above vs high school or less).

A summary of phthalate metabolite concentrations is shown in Figure 1 and described in Table 2. A different number of phthalate metabolites were analyzed at each trimester (16 in the second trimester and 14 in the third trimester) due to differences in the number of samples above the LOD at each time point (Table S1). The molar sum of DEHP metabolites was included in both trimesters. The urinary concentrations of five metabolites were significantly correlated between time points, based on a Pearson correlation coefficient >0.4 and $p < 0.05$ (Table S2). The correlations of different phthalate concentrations with each other within each time point are shown in Figure S2. MEHP, MEHHP, MEOHP, MECPP, and MCMHP were strongly correlated given that these phthalates are derivatives of the parent compound, DEHP. We observed other correlations between phthalates that are derived from phthalic acid, including MCMHP. Mono (carboxyisooctyl) phthalate (MCIOP) and mono (carboxyisononyl) phthalate (MCINP) were strongly correlated, which is reflective of the fact that MCINP can be converted to MCIOP, although they are primarily derived from different parent compounds (Saravanabhavan and Murray 2012).

Results from linear models fully adjusted for maternal race, maternal age, maternal education, labor status, fetal sex, RNA sequencing batch, and maternal smoking are presented in Table 3. Second-trimester urinary MEOHP concentrations were associated with increased placental expression of 17 genes, including four lncRNAs. Second-trimester urinary MECPP concentrations were associated with increased expression of one gene (LUC7 like 3 pre-mRNA splicing factor, *LUC7L3*) and two lncRNAs. Second-trimester urinary concentrations of MCIOP were associated with decreased expression of one gene (carboxypeptidase Z, *CPZ*) and one lncRNA (nuclear paraspeckle assembly transcript 1, *NEAT1*). The gene *LUC7L3* and the long noncoding RNAs *NEAT1* and *MUC20-OT1* were positively associated with urinary concentrations of both MECPP and MEOHP (Table 3; Figure S3), which is likely related to the positive correlations between the metabolites and the fact that they are derived from the same parent compound (DEHP). *NEAT1* was also negatively associated with urinary concentrations of MCIOP. Third-trimester urinary concentrations of MCIOP were associated with increased

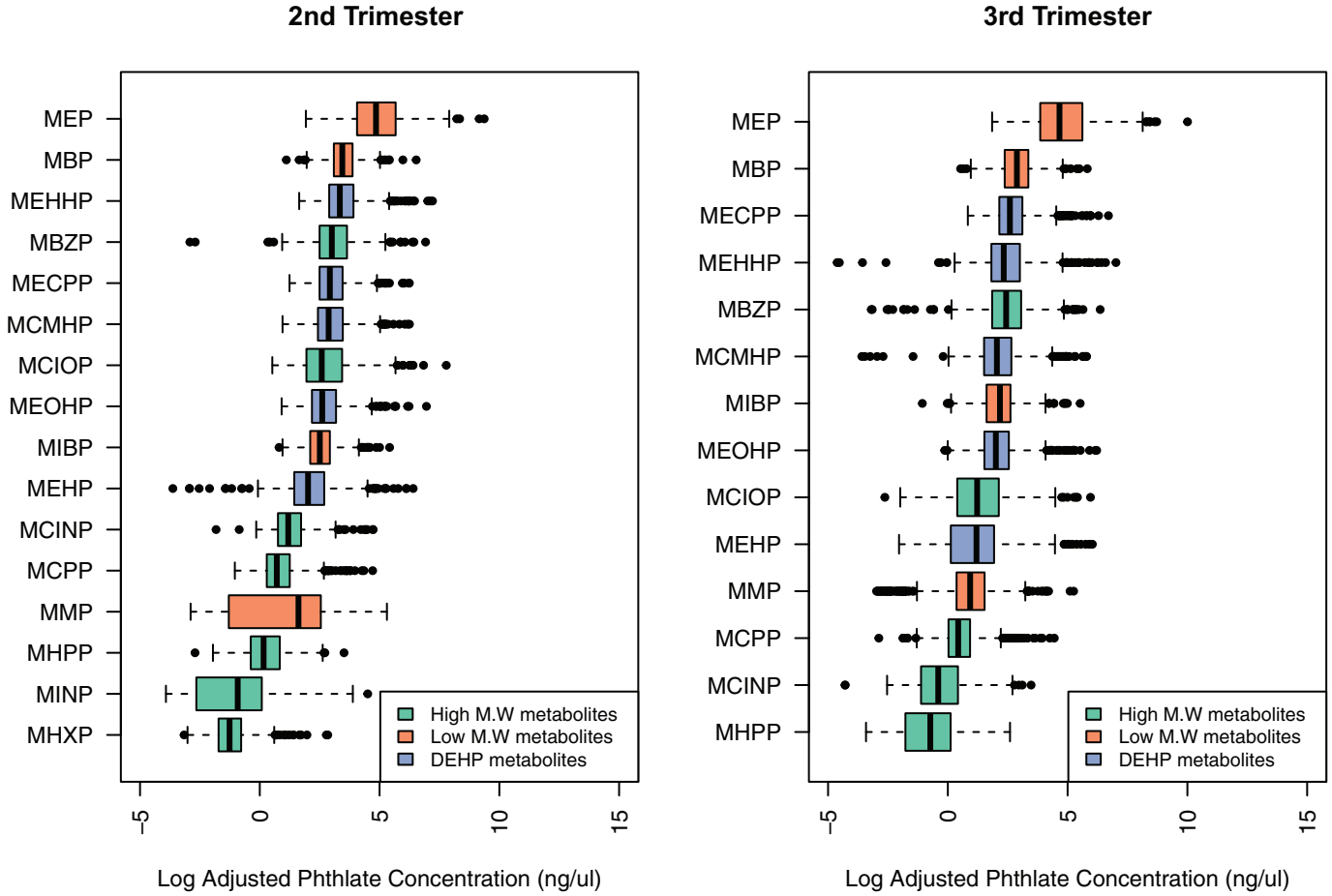

Figure 1. Box plot depicting concentrations of phthalate metabolites in CANDLE participants detectable in urine in the second ($N = 594$) and third trimester ($N = 735$). In this box plot, the box represents the 25th to 27th percentile (i.e., 50% of the data), with the horizontal line in the box representing the median expression (50th percentile). The whiskers represent the minimum and maximum values that do not exceed 1.5 times the interquartile range, with the remaining values plotted as outlier dots. Note: CANDLE, Conditions Affecting Neurocognitive Development and Learning in Early Childhood; MBP, monobutyl phthalate; MBZP, monobenzyl phthalate; MCINP, monocarboxy isononyl phthalate; MCIOP, mono (carboxyisooctyl) phthalate; MCMHP, mono[2-(carboxymethyl) hexyl] 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; MHPP, mono(4-hydroxypentyl) phthalate; MHXP, mono-*n*-hexyl phthalate; MIBP, mono-isobutyl phthalate; MINP, mono-isononyl phthalate; MMP, mono-methyl phthalate; M.W., molecular weight.

placental expression of 13 genes and decreased expression of five genes. Three of these genes were lncRNAs. Mono-isobutyl phthalate (MIBP) concentration quantified in the third trimester was associated with increased expression of one gene (fat storage-inducing transmembrane protein 2, *FITM2*) and decreased expression of one gene (Golgin A8 family member B, *GOLGA8B*).

We performed a stratified analysis of male and female placental samples and prenatal phthalate exposure in the second and third trimester (Table S3). In males, we identified positive associations between the expression of 14 genes and urinary phthalate concentrations of DEHP and its metabolites MECPP, MEHHP, MEHP, and MEOHP. Most of the DEGs (12) were associated with the primary DEHP metabolite, MEHP. Three genes [*NEAT1*, pleckstrin homology like domain family B member 2 (*PHLDB2*), and long intergenic non-protein coding RNA 2327 (*LINC02327*)] were associated with multiple metabolites of DEHP (Figure S4). In female samples, 12 genes were associated with prenatal phthalate metabolite concentrations in the second trimester. Ten of these genes were associated with urinary concentrations of MIBP. We observed decreased expression of two genes in males [melanophilin (*MLPH*) and alkB homolog 1, histone H2A dioxygenase (*ALKBH1*)] in association with urinary concentrations of MCIOP in the third trimester. In female samples, we observed a positive association between the expression of GATA zinc finger domain containing 2B (*GATAD2B*) and urinary concentrations of MCIOP, and a positive association between tRNA methyltransferase 12 homolog (*TRMT12*) and urinary concentrations of MECPP. Of all the genes significantly associated with phthalate exposure in this stratified analysis, only one [MORC family CW-type zinc finger 4 (*MORC4*)] was located on the X or Y chromosome, and the rest were autosomal. There were no overlapping DEGs associated with any phthalate metabolite between male and female placental samples, or any overlaps between sex-specific DEGS associated with phthalate metabolites in either male or female samples between the second and third trimester. Of the 30 total DEGs identified in this sex-stratified analysis, 2 were also statistically significant in our main model, where males and females were combined. *NEAT1* was significantly positively associated with MEHP expression in the main model, with stronger and more significant relationships in male placentas. MCIOP concentrations in the third trimester were significantly negatively associated with placental *MLPH* expression in our main model, but this effect was stronger and more statistically significant in male placentas. Overall, we observed fewer sex-specific relationships between placental

Table 2. Distribution of phthalate metabolite concentrations at each trimester (ng/mL) in CANDLE participants in the second ($N = 594$) and third trimester ($N = 735$), all with complete data.

				Second trimester						Third trimester					
Name	Parent compound(s)	Primary or secondary metabolite	Mol. wt.	Range (min-max)	25th percentile	50th percentile	Mean	75th percentile	SD	Range (min-max)	25th percentile	50th percentile	Mean	75th percentile	SD
Mono-methyl phthalate (MMP)	Di-methyl phthalate (DMP)	Primary	194.18 (low)	0.06–201.78	0.28	5.03	8.80	12.77	13.6	0.05–190.86	1.44	2.54	4.91	4.64	11.41
Monoethyl phthalate (MEP)	Diethyl phthalate (DEP)	Primary	222.24 (low)	6.85–11,710.34	58.00	128.08	299.37	289.92	743.14	6.35–22,148.62	47.31	105.7	326.49	275.03	1,003.47
Monobutyl phthalate (MBP)	Dibutyl phthalate (DnBP) (primary); butylbenzyl phthalate (BzBP)	Primary	278.34 (low)	3.03–685.03	22.03	31.34	40.90	47.97	41.24	1.70–337.26	10.74	17.86	23.85	28.81	24.88
Mono-isobutyl phthalate (MIBP)	Diisobutyl phthalate (DiBP)	Primary	278.35 (low)	2.23–225.84	8.21	12.26	16.80	18.64	17.56	0.34–250.80	5.04	8.8	12.06	13.66	15.95
Mono(4-hydroxypentyl) phthalate (MHPP)	Dipentyl phthalate (DPeP)	Secondary	306.4 (high)	0.07–33.67	0.69	1.17	1.82	2.30	2.26	0.03–13.40	0.17	0.48	0.90	1.12	1.37
Monobenzyl phthalate (MBZP)	Butylbenzyl phthalate (BzBP)	Primary	312.36 (high)	0.03–1,013.05	12.14	20.24	35.87	38.08	66.25	0.04–578.00	6.35	11.4	20.60	21.33	35.93
Mono-*n*-hexyl phthalate (MHXP)	Di-*n*-hexyl phthalate (DnHP)	Primary	334.4 (high)	0.04–17.07	0.18	0.28	0.50	0.46	1.13	—	—	—	—	—	—
Mono-3- carboxypropyl phthalate (MCPP)	Dioctyl phthalate (DnOP (primary); di-*n*-butyl phthalate (DnBP); other HWM phthalates	Secondary	390.56 (high)	0.35–111.23	1.34	2.03	4.05	3.49	8.09	0.06–85.14	1.03	1.54	2.77	2.53	5.7
Mono (carboxyisooctyl) phthalate (MCIOP)	Diisononyl phthalate (DiNP) (Primary); di-iso-decylphthalate (DiDP)	Secondary	418.61 (high)	1.69–2,405.66	7.02	13.28	42.08	31.07	126.96	0.07–385.46	1.48	3.4	9.44	8.39	23.47
Mono-isononyl phthalate (MINP)	DiNP	Primary	418.61 (high)	0.02–90.56	0.07	0.4	1.41	1.08	4.93	—	—	—	—	—	—
Monocarboxy isononyl phthalate (MCINP)	DiDP	Secondary	446.67 (high)	0.16–113.44	2.14	3.28	5.65	5.57	10.01	0.01–32.30	0.33	0.67	1.39	1.52	2.42
Mono-2-ethylhexyl phthalate (MEHP)	Di-2-ethylhexyl phthalate (DEHP)	Primary	390.56 (high)	0.03–603.39	4.22	7.5	17.06	14.71	44.27	0.13–417.27	1.13	3.3	9.21	6.89	30.49
Mono-2-ethyl-5-carboxypentyl phthalate (MECPP)	DEHP	Secondary	390.56 (high)	3.46–514.66	12.13	18.65	32.31	31.65	49.18	2.30–816.26	8.57	13.35	26.32	22.46	53.75
Mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP)	DEHP	Secondary	390.56 (high)	5.18–1,353.05	18.15	28.27	53.82	49.78	108.12	0.01–1,116.75	6.16	10.31	25.41	20.22	70.44
Mono[2-(carboxymethyl) hexyl] phthalate (MCMHP)	DEHP	Secondary	390.56 (high)	2.58–516.49	11.33	17.7	31.42	31.98	46.83	0.03–327.87	4.54	7.75	16.22	14.18	32.07
Mono-2-ethyl-5-oxohexyl phthalate (MEOHP)	DEHP	Secondary	390.56 (high)	2.49–1,050.13	8.83	13.56	26.38	24.08	59.28	0.88–498.92	4.60	7.42	16.07	12.78	37.97
Mono esters of DEHP[a]	DEHP	—	—	0.05–12.51	0.20	0.3	0.54	0.53	0.94	0.02–9.97	0.09	0.14	0.31	0.25	0.7

Note: —, not applicable; CANDLE, Conditions Affecting Neurocognitive Development and Learning in Early Childhood; HWM, high-molecular weight; max, maximum value; min, minimum value; SD, standard deviation.
[a]DEHP concentration is reported in micromoles per liter because it is calculated as the molar sum of MEHP, MEOHP, MEHHP, MECPP and MCMHP.

Table 3. Significant associations between phthalate metabolites in the second ($N = 594$) and third trimester ($N = 735$) and placental gene expression in CANDLE participants.

Phthalate	Gene	Ensembl gene ID	Complete gene name (type)	Effect estimate	FDR-adjusted p-value
Second trimester					
MCIOP	*CPZ*	ENSG00000109625	Carboxypeptidase Z	−0.165	4.15×10^{-2}
	NEAT1	ENSG00000245532	Nuclear paraspeckle assembly transcript 1 (*LncRNA*)	−0.087	4.15×10^{-2}
MECPP	*LUC7L3*	ENSG00000108848	LUC7 like 3 pre-mRNA splicing factor	0.052	4.38×10^{-2}
	MUC20-OT1	ENSG00000242086	MUC20 overlapping transcript (*LncRNA*)	0.131	4.38×10^{-2}
	NEAT1	ENSG00000245532	Nuclear paraspeckle assembly transcript 1 (*LncRNA*)	0.226	4.38×10^{-2}
MEOHP	*ACIN1*	ENSG00000100813	Apoptotic chromatin condensation inducer 1	0.045	4.69×10^{-2}
	AJ009632.2	ENSG00000229425	*LncRNA*	0.207	2.14×10^{-2}
	ANKRD10	ENSG00000088448	Ankyrin repeat domain 10	0.121	3.60×10^{-2}
	AP1G2	ENSG00000213983	Adaptor related protein complex 1 subunit gamma 2	0.082	2.56×10^{-2}
	ARGLU1	ENSG00000134884	Arginine and glutamate rich 1	0.069	2.14×10^{-2}
	CCNL1	ENSG00000163660	Cyclin L1	0.086	2.14×10^{-2}
	CLK1	ENSG00000013441	CDC like kinase 1	0.095	2.95×10^{-2}
	LENG8	ENSG00000167615	Leukocyte receptor cluster member 8	0.091	3.82×10^{-2}
	LUC7L3	ENSG00000108848	LUC7 like 3 pre-mRNA splicing factor	0.055	7.34×10^{-3}
	MUC20-OT1	ENSG00000242086	MUC20 overlapping transcript (*LncRNA*)	0.121	2.56×10^{-2}
	NEAT1	ENSG00000245532	Nuclear paraspeckle assembly transcript 1 (*LncRNA*)	0.228	2.14×10^{-2}
	NPIPB4	ENSG00000185864	Nuclear pore complex interacting protein family member B4	0.121	2.56×10^{-2}
	PSMA3-AS1	ENSG00000257621	PSMA3 antisense RNA 1	0.074	4.85×10^{-2}
	RBM6	ENSG00000004534	RNA binding motif protein 6	0.060	2.95×10^{-2}
	SON	ENSG00000159140	SON DNA and RNA binding protein	0.053	4.95×10^{-2}
	SRSF11	ENSG00000116754	Serine and arginine rich splicing factor 11	0.050	4.39×10^{-2}
	TUBGCP6	ENSG00000128159	Tubulin gamma complex associated protein 6	0.077	2.14×10^{-2}
Third trimester					
MCIOP	*MLPH*	ENSG00000115648	Melanophilin	−0.121	4.47×10^{-2}
	AC018638.5	ENSG00000243679	*LncRNA*	−0.071	4.47×10^{-2}
	KRT10	ENSG00000186395	Keratin, type I cytoskeletal 10	−0.056	4.47×10^{-2}
	ADPRM	ENSG00000170222	Manganese-dependent ADP-ribose/CDP-alcohol diphosphatase	−0.041	4.47×10^{-2}
	LINC01578	ENSG00000272888	*LncRNA*	−0.040	4.47×10^{-2}
	RNF4	ENSG00000063978	E3 ubiquitin-protein ligase RNF4	0.021	4.47×10^{-2}
	NOP9	ENSG00000196943	Nucleolar protein 9	0.022	4.47×10^{-2}
	CTDSP2	ENSG00000175215	Carboxy-terminal domain RNA polymerase II polypeptide A small phosphatase 2	0.034	4.47×10^{-2}
	IP6K1	ENSG00000176095	Inositol hexakisphosphate kinase 1	0.035	4.47×10^{-2}
	RAPGEF1	ENSG00000107263	Rap guanine nucleotide exchange factor 1	0.039	4.47×10^{-2}
	BCL9L	ENSG00000186174	B-cell CLL/lymphoma 9-like protein	0.040	4.47×10^{-2}
	ZNF616	ENSG00000204611	Zinc finger protein 616	0.044	4.47×10^{-2}
	KIAA1522	ENSG00000162522	Uncharacterized protein KIAA1522	0.046	4.47×10^{-2}
	PRRC2B	ENSG00000130723	Protein PRRC2B	0.047	3.29×10^{-2}
	AMOTL1	ENSG00000166025	Angiomotin-like protein 1	0.047	4.44×10^{-2}
	SOCS7	ENSG00000274211	Suppressor of cytokine signaling 7	0.056	4.47×10^{-2}
	MT-ND5	ENSG00000198786	NADH-ubiquinone oxidoreductase chain 5	0.058	4.47×10^{-2}
	AP003119.3	ENSG00000261578	*LncRNA*	0.082	4.47×10^{-2}
MIBP	*GOLGA8B*	ENSG00000215252	Golgin subfamily A member 8B	−0.210	3.40×10^{-2}
	FITM2	ENSG00000197296	Fat storage-inducing transmembrane protein 2	0.063	3.40×10^{-2}

Note: The log fold change and p-values were derived from conventional linear models with observation-level weights for mean/variance relationships, and adjusted for multiple comparisons using the Benjamini-Hochberg approach. All models were adjusted for confounders including RNA sequencing batch, labor method, fetal sex, maternal race, maternal age, and maternal education. We included only participants with complete data in this analysis. Genes were considered statistically significant with an false discovery rate adjusted $p < 0.05$. CANDLE, Conditions Affecting Neurocognitive Development and Learning in Early Childhood; ID, identifier; FDR, false discovery rate; lncRNA, long noncoding ribonucleic acid; MCIOP, mono (carboxyisooctyl) phthalate; MECPP, mono-2-ethyl-5-carboxypentyl phthalate; MEOHP, mono-2-ethyl-5-oxohexyl phthalate; MIBP, mono-isobutyl phthalate; NADH, nicotinamide adenine dinucleotide plus hydrogen.

expression and metabolite concentrations in the third trimester vs. the second trimester.

Gene set testing of KEGG pathways was performed for exploratory pathway analysis, revealing a total of 27 biological pathways that were perturbed in association with phthalate metabolites, using a threshold of FDR-adjusted $p < 0.2$, as shown in Table S4 and in Figure 2. Second-trimester maternal urinary MCIOP concentrations were associated with increased gene expression within seven biological pathways. Third-trimester MCIOP concentrations were associated with decreased expression of one pathway and increased expression of six biological pathways. MCIOP concentration in both trimesters was associated with overall increased expression of genes within the adherens junction pathway (Figure S5) and the sphingolipid signaling pathway (Figure S6). Second-trimester urinary concentrations of mono-methyl phthalate (MMP) were associated with the increased expression of three pathways and the decreased expression of 16 pathways. The cortisol synthesis and secretion pathway, adherens junction pathway, longevity regulation pathway (multiple species), and growth hormone synthesis, secretion, and action pathway were all significantly associated with MCIOP and MMP, but in opposing directions (Figure 2). Third-trimester

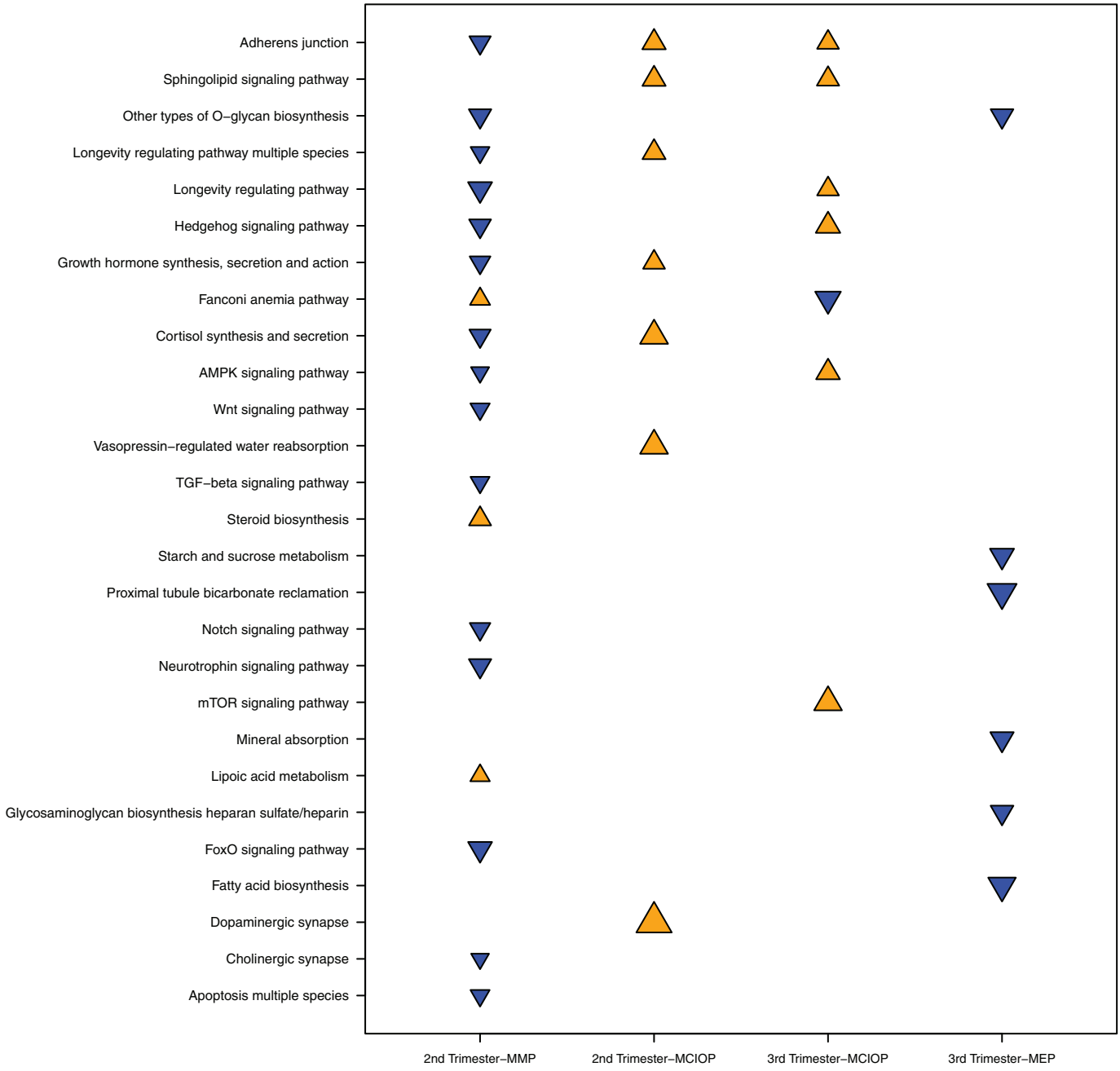

Figure 2. Modified dot plot of KEGG pathways that were significantly enriched for genes associated with maternal urinary concentrations of phthalate metabolites in CANDLE participants in the second ($N = 594$) and third trimester ($N = 735$). p-Values were derived using FRY gene set testing, which evaluates whether the t statistic for a gene set (i.e., KEGG pathway) is larger than expected under the null using the t-statistics derived from the conventional linear models. These models were adjusted for confounders including RNA sequencing batch, labor method, fetal sex, maternal race, maternal age, and maternal education. We only included participants with complete data in this analysis. We adjusted for multiple comparisons at the pathway level using the Benjamini-Hochberg approach, and pathways were considered statistically significant with an false discovery rate adjusted $p < 0.2$ value. Blue downward pointing triangles indicate pathways with decreased expression, and orange upward pointing triangles indicate pathways with increased expression. The size of the dot corresponds to the $-\log p$-value, (i.e., a larger point represents greater significance). Full results are presented in Table S4. Note: AMPK, AMP-activated protein kinase; CANDLE, Conditions Affecting Neurocognitive Development and Learning in Early Childhood; KEGG, Kyoto Encyclopedia of Genes and Genomes; MCIOP, mono (carboxyisooctyl) phthalate; MEP, monoethyl phthalate; monoethyl phthalate; MMP, mono-methyl phthalate; mTOR, mammalian target of rapamycin; TGF, transforming growth factor.

maternal urinary concentrations of MEP were associated with the decreased expression of genes within six pathways.

Discussion

The main findings of the present study were *a*) identification of 42 significant associations between urinary concentrations of four phthalate metabolites and placental expression of 38 genes; *b*) unique associations identified in relation to phthalate metabolites MCIOP and MIBP, concentrations of which have increased in the general population in recent years and are not well studied in previous literature; and *c*) identification of 27 KEGG pathways with altered placental gene expression in association with phthalate metabolites MCIOP, MMP, and MEP. These findings provide unique insight into how prenatal phthalate exposure might influence placental function, potentially impacting fetal development. To our knowledge, this study represents the most comprehensive

analysis of the relationship between urinary phthalate metabolites and the placental transcriptome within a large and diverse birth cohort ($N = 760$), enhancing generalizability beyond that of previous studies. To our knowledge, this is also the first study to perform transcriptome-wide analysis of maternal phthalate exposure in relation to placental gene expression at birth, a critical time point for perinatal outcomes, with adjustment for many key covariates. Our findings (such as associations with phthalate metabolites and *NEAT1* expression) mirror those of previous epidemiological studies and other *in vitro* analyses (Grindler et al. 2018; Machtinger et al. 2018), providing data supporting proposed mechanisms of action linking prenatal exposures with fetal development.

The present study stands apart from other placental transcriptomics investigations because of the comprehensive quantification of multiple phthalate metabolites derived from different parent compounds. We observed the largest number of associations between placental gene expression and prenatal exposure to MEOHP (a secondary metabolite of DEHP) and MCIOP [a secondary metabolite of diisononyl phthalate (DINP)]. Both MCIOP and MEOHP are secondary oxidized metabolites of their parent compound, and for high-molecular weight phthalate monoesters with five or more carbons, the oxidized metabolite is the main metabolite detectable in urine because the primary metabolite is rapidly metabolized during phase 1 metabolism and does not bioaccumulate, as reviewed by Saravanabhavan and Murray (2012). Thus, we may detect associations within the placental transcriptome related to these specific phthalate metabolites because the secondary oxidized metabolites are more likely to bioaccumulate compared with the primary metabolite. Previous studies have mainly investigated DEHP and its metabolites, or lower-molecular weight compounds, including monobutyl phthalate (MBP) or MEP (Adibi et al. 2010, 2017; Gao et al. 2017; LaRocca et al. 2016; Machtinger et al. 2018; Meruvu et al. 2016a, 2016b; Wang et al. 2013). The present analysis includes many metabolites not previously investigated in prior studies in animals or *in vitro* as reviewed by Strakovsky and Schantz (2018). We observed the largest number of associations between placental gene expression and maternal urinary concentrations of the phthalate metabolite MCIOP quantified during the second and third trimester. MCIOP is a secondary oxidative metabolite of DINP (Silva et al. 2006) but may also be formed by the metabolism of di-iso-decylphthalate (see Table 2). To our knowledge, only this study and one other (Grindler et al. 2018) have examined the influence of MCIOP on the placental transcriptome. Although the levels of the phthalate derivatives of DEHP in the US population decreased between 2001 and 2010, the levels of DINP metabolites increased within the same time frame, based on data collected within five cycles of the National Health and Nutrition Examination Survey—a representative survey of the civilian, noninstitutionalized population of the United States that is conducted by the Centers for Disease Control and Prevention (Zota et al. 2014). This analysis suggests that DINP metabolites influence the placental transcriptome, and more mechanistic analysis of the influence of DINP and its metabolites on placental function is warranted. In addition, future studies may consider applying summarized measures of phthalate metabolite concentrations, which may reveal additional insight.

We examined associations between the placental transcriptome and phthalate exposure quantified in mid and late pregnancy, contributing to the understanding of phthalate disruption of the placental transcriptome in these specific developmental windows. We observed a larger number of more unique genes associated with phthalates measured at the third trimester (20 genes) compared with the second trimester (18 genes), and there was no overlap between genes whose placental expression was altered at each trimester. In the second trimester, most genes were associated with DEHP and its metabolite MECPP, but in the third trimester, genes were associated with the phthalate metabolites MIBP and MCIOP. Other studies have found similar patterns following phthalate exposure later in pregnancy and fetal developmental outcomes. For example, maternal urinary DEHP levels in the third trimester (but not the first) were associated with preterm birth in The Infant Development and the Environment Study (TIDES) cohort—a prospective birth cohort conducted within San Francisco, California; Rochester, New York; Minneapolis, Minnesota; and Seattle, Washington, and this relationship was modified by whether a mother was exposed to one or more psychosocial stressors during pregnancy (Ferguson et al. 2019). This may be reflective of developmental processes happening within these specific windows. The significance of these windows of susceptibility to exposure is particularly relevant based on the link between phthalate exposure and neurodevelopmental changes in early childhood. Studies linking prenatal phthalate exposure to neurodevelopment have revealed associations between urinary phthalate metabolites measured during the third trimester (Factor-Litvak et al. 2014; Whyatt et al. 2012). There are distinct neurodevelopmental events occurring between the second trimester (neurite outgrowth, synaptogenesis) and the third trimester (neural network formation and functioning), which suggests that the timing of exposure to phthalates may differentially impact these processes (Courchesne et al. 2019). Associations between the placental transcriptional landscape and phthalates may serve as a reflection of perturbations in other fetal tissues. Animal models have revealed that the timing of exposure to phthalates is significant because it relates to placental function itself, particularly when assessing epigenetic end points, such as imprinting in early pregnancy (Strakovsky and Schantz 2018). Herein, we collected data from the placenta at the end of gestation, hence our transcriptome profiling may not reflect the gene expression levels of the placenta in mid pregnancy. In the human body, phthalates have a relatively short half-life of ~ 12 h (Hoppin et al. 2002), so our measurements may not be reflective of a woman's longer-term chronic or cumulative exposure. Ongoing work in experimental systems may reveal more insight into the role of timing during sensitive developmental windows.

We identified seven lncRNAs associated with different phthalate metabolites. LncRNAs are untranslated mRNA molecules <200 nucleotides in length that may be involved in a variety of transcriptional and posttranscriptional gene regulation functions through binding to DNA, RNA, and proteins (Wang and Chang 2011). These mRNA molecules can act as both regulators of health and disease but also as biomarkers associated with a variety of environmental exposures in different tissues, and they functionally interact with a number of different environmental factors (Karlsson and Baccarelli 2016). A pilot study of candidate lncRNAs in 20 placental samples identified positive associations between prenatal urinary phthalate concentration and placental expression of a number of lncRNAs (Machtinger et al. 2018). This study reported that *NEAT1* placental expression was negatively associated with urinary MMP concentrations. In our analysis, *NEAT1* placental expression was positively associated with urinary concentrations of MEOHP and MECPP, and negatively associated with MCIOP concentration in the second trimester. *NEAT1* is involved the formation of paraspeckles (Clemson et al. 2009), which could lead to retention of mRNAs and perturbed transcriptional regulation within the placenta. *NEAT1* has also been positively associated with intrauterine growth restriction (Gremlich et al. 2014), suggesting that it may be related to functional changes in the placenta that influence

developing infants. Differences in placental lncRNA concentrations have been associated with other prenatal exposures and outcomes, such as prenatal cadmium concentrations and birthweight (Hussey et al. 2020), implicating lncRNA perturbations as a potential molecular mechanism for toxicity. More work is needed to understand how changes in lncRNA expression may impact placental function and fetal development.

We identified a number of genes whose placental expression was only associated with phthalate exposure in male or female samples. We observed the largest number of associations between DEHP and the metabolites MECPP, MEHHP, MEHP, and MEOHP in the second trimester and gene expression in male placentas. We observed significant associations between MIBP metabolite concentration in the second trimester and gene expression in female placentas. This suggests that individual phthalate metabolites may influence placental gene expression of male and female placentas differently. Sex differences in the relationship between prenatal phthalate exposure and pregnancy-related variables are consistent with previous work. We previously identified a negative association between MBP concentrations during pregnancy and total free testosterone in women carrying female fetuses as a significant positive association between total testosterone and MBP in women carrying male fetuses (Sathyanarayana et al. 2014). These molecular differences in relation to phthalate exposure may be reflective of the different growth strategies of male and female fetuses. Male fetuses grow faster and have a relatively smaller placental size, which is thought to be more efficient but also leaves them less able to adapt to perturbations (Eriksson et al. 2010). Fetal sex may serve as a moderator of the relationship between exposure to environmental toxins and developmental defects (DiPietro and Voegtline 2017). The differences in placental expression in association with phthalate exposure observed in male and female placentas may reflect increased signaling required for adaptation and responses to environmental changes.

A number of the genes associated with prenatal phthalate exposure in females are important regulators of placental growth, and they can be modified by estrogen signaling, including signal transducer and activator of transcription 3 (*STAT3*), tumor protein p63 (*TP63*), and DNA (cytosine-5-)-methyltransferase 1 (*DMNT1*). *STAT3* is a component of the STAT protein family that is activated after phosphorylation by Janus kinases. In the placenta, *STAT3* promotes the invasive phenotype of trophoblast cells (Poehlmann et al. 2005). Activation of STAT3 signaling can be suppressed by 17-beta-estradiol (E2) in breast cancer cells (Yamamoto et al. 2000), but this has not been established in the placenta. TP63 is also a transcription factor involved in placental growth given that TP63 inhibits the invasion of trophoblasts and promotes a proliferative state (Li et al. 2014). Estradiol decreases *TP63* expression in breast cancer cells via miRNA signaling (Kim et al. 2013). In the present study, MIBP was also associated with decreased expression of *DNMT1* in females, which encodes an important enzyme responsible for catalyzing the transfer of methyl groups in the process of DNA methylation. Appropriate placental DNA methylation is essential for fetal and placental growth (Koukoura et al. 2012). *DNMT1* expression has been positively associated with placental and birth weight in female placentas, but not male placentas, indicating a sex-specific role in fetal growth (Mukhopadhyay et al. 2016). Dibutyl phthalate (DnBP) caused increased liver expression of *DNMT1* in rats (Urbanek-Olejnik et al. 2016). Changes in expression of DNA methyltransferases may be related to downstream perturbations in DNA methylation induced by phthalates (Grindler et al. 2018). More work is needed to understand the complex relationship between phthalates modulation of estrogen signaling in the placenta and placental gene expression.

Pathway enrichment analysis was used for exploratory, hypothesis-generating purposes and to contextualize these differences in gene expression associated with phthalate exposure because many of the individual genes that were statistically significant in our work have not been extensively investigated. Different phthalate metabolites (i.e., MCIOP, MMP, and MEP) were associated with distinct biological pathways, which may reflect differing biological mechanisms. We observed broad associations within the adherens junction, with decreased expression of these genes associated with MMP in the second trimester, but increased expression in relation to MCIOP in the second and third trimester. Human placental vesicles contain tight junctions throughout their structure, with the exception of terminal villus microvesicles (Leach et al. 2000), which form a crucial component to the maternal fetal barrier and are involved in the transfer of key nutrients and signaling molecules. The adherens junction is influenced by phthalates given that exposure to MEHP caused gaps in this junction within rat Sertoli cells (Yao et al. 2010), and DnBP treatment during pregnancy altered the formation of Sertoli cell tight junctions in offspring in mice (Hutchison et al. 2008). The effect of phthalates and their metabolites on the adherens junction in the developing placenta remain unclear, but given the crucial role of the maternal–fetal interface in the transport of nutrients and signals between the mother and fetus, phthalate-induced alterations of genes within this pathway could impact fetal development. Moreover, we hypothesize that compromises in tight junctions may allow other exogenous factors to enter the placenta more easily. In this way, pathway enrichment analysis has generated new hypotheses related to phthalate exposure *in utero*.

We observed associations in placental gene expression and phthalate exposure in pathways that have been previously shown to be influenced by phthalate exposure. In the present study, genes within the forkhead box O1 (FOXO1) signaling pathway and apoptosis pathway had decreased expression in association with MMP. Treatment with other phthalate metabolites has been shown to induce oxidative stress and apoptosis in placental cell lines (Meruvu et al. 2016a, 2016b). We observed associations in placental gene expression related to phthalate metabolites in pathways related to PPAR-γ, including the sphingolipid metabolism pathway (in association with MCIOP) and the fatty acid biosynthesis pathway (in association with MEP). PPAR-γ regulates the initial steps in sphingolipid generation by transcriptional regulation of serine palmitoyl transferase and by initiating the synthesis of acyl-CoA synthesis (Wang et al. 2020). The placenta transports essential long-chain fatty acid derivatives (including those from sphingolipids) from the maternal to the fetal circulation and helps metabolize these enzymes along the way. Fatty acids are critical to fetal development and growth because they are essential components of membrane lipids and intracellular mediators of gene expression, and they are also major contributors of energy required for the placenta itself, as reviewed by Duttaroy (2009). Differences in specific fatty acid profiles have been identified in umbilical venous plasma from infants experiencing intrauterine growth restriction (Alvino et al. 2008; Gomez-Lopez et al. 2021). The developing fetus may be particularly vulnerable to perturbations in lipid levels during late pregnancy given that this stage involves rapid brain growth (Schepanski et al. 2018), which is highly dependent upon the availability of lipid precursors (Clandinin et al. 1980). Prostaglandin, which is produced by the placenta and play key roles in parturition (Thorburn, 1991), is also formed from lipid precursors. Previous studies on candidate phthalate-related genes have primarily focused on PPARs (Adibi et al. 2017; Gao et al. 2017; Huang et al. 2018) because they are known targets of EDCs. PPARs are nuclear hormone receptors

that respond to fatty acids and other lipophilic hormones, and they are considered primary sensors of lipid signaling. Binding of phthalate metabolites to the PPAR receptor leads to the activation of genes involved in lipid transport, lipogenesis, fatty acid oxidation, and fatty acid transport (Maradonna and Carnevali 2018). Although we did not observe significant associations between phthalate exposure and expression of genes within the KEGG PPAR signaling pathway itself, we did observe significant gene associations within pathways that are directly downstream. Overall, our work indicates that phthalates may alter fatty acid synthesis and metabolism within the placenta, which may disrupt the placentas key function of transporting and providing these essential building blocks for fetal development, thus potentially impairing long-term fetal growth.

Our results should be interpreted in light of limitations in RNA sequencing analysis. We captured the placental transcriptome at birth, which is a snapshot of a highly regulated and temporal process. Expression was quantified using bulk RNA sequencing data, so our findings may be confounded by different cell types collected within each sample, which is a well-established challenge in this field (Breton et al. 2017). Alternatively, differences in cellular proportions could be influenced by phthalates, thus the associations we observed related to phthalate exposure may not reflect gene expression differences but, instead, indicate differences in the number and type of cells. Our ability to adequately address this was limited by a lack of single cell data available for the placenta and a lack of reference-free approaches for transcriptomic analyses (Konwar et al. 2019). Nevertheless, there is a long-standing precedent for using bulk RNA sequencing data sets in the context of environmental exposures research (Clarkson-Townsend et al. 2020; Everson and Marsit 2018; Hussey et al. 2020; Lesseur et al. 2014). Based on the limited number of DEGs and the fact that most of the top DEGs were not well studied, we performed pathway analysis to identify associations between phthalate exposure and placental gene expression in established KEGG biological pathways using self-contained gene set testing. Because this was meant to be a hypothesis-generating approach, we used a less stringent statistical cutoff (FDR-adjusted <0.2) to identify associations in biological pathways. Our analysis is also limited by our understanding of the true concentrations of prenatal phthalate exposure because we collected single spot samples of maternal urine, as previously discussed (Adgent et al. 2020). We elected not to adjust for gestational length in our analyses, which could confound our results, given associations between the placental transcriptome and preterm birth (Brockway et al. 2019; Eidem et al. 2015; Paquette et al. 2018). There is emerging evidence from human studies that prenatal phthalate exposure is associated with preterm birth (Boss et al. 2018; Ferguson et al. 2014, 2019), indicating a complex relationship between prenatal phthalate exposure, the placental transcriptome, and gestational length that is beyond the scope of this present work. Finally, we observed confounding by maternal race because maternal race was associated with a high number of differentially expressed genes in our data set (Table 1). It is challenging for us to further address this confounding because self-reported race reflects both genetic ancestry and social factors and is associated with differences in many exposures and experiences, including socioeconomic adversity, differences in environmental exposures, and experiences of racism, and it is challenging to capture the antecedents of the biological differences we observe (Borrell et al. 2021). The vast majority of CANDLE participants ($\sim 95\%$) reported their race as either Black or White, so this cohort is not diverse enough to adjust for race in a nondichotomous way or to analyze exposure–outcome relationships of other racial groups. Future research thoughtfully designed to study these issues may advance understanding of the relations studied here.

Our study has a number of unique strengths. We quantified placental gene expression at the end of gestation, whereas prior transcriptome-wide studies have investigated this association in placental tissue collected solely during the first trimester (Grindler et al. 2018). We identified more associations between urinary phthalate measurements in the third trimester and the placental transcriptome at birth, revealing the importance of the timing of exposure–outcome relationships. To our knowledge, this is the largest placental transcriptomic data set published to date, containing data from a diverse population of women, and our detailed covariate data allowed adjustment for confounding variables. In addition, our cohort of subjects was more diverse, with $\sim 50\%$ Black participants, and is thus more generalizable to the overall population. We used a self-contained gene set test, which is an improvement over first generation overrepresentation analyses (Khatri et al. 2012) that require an input list of genes based on a predefined threshold or competitive tests such as gene set enrichment analyses (Subramanian et al. 2005), for which statistical significance is derived through permutation testing or ranked genes and which can result in bias related to intergene correlations. Finally, our study is among the most comprehensive studies in terms of the number of metabolites quantified, including several phthalate metabolites that have not yet been studied in relation to the placental transcriptome.

Prenatal phthalate exposure has been linked to pregnancy complications and longer-term pediatric health outcomes. Our work in this large human cohort suggests that phthalates may disrupt placental function via the placental transcriptome, which may represent a mechanism by which phthalates could influence fetal development. We identified a number of genes, including lncRNAs, that were significantly associated with the maternal urinary concentration of specific phthalate metabolites across the second and third trimester. These genes were enriched within pathways important to placenta function, such as the adherens junction, sphingolipid metabolism, and fatty acid biosynthesis. MCIOP, a secondary oxidative metabolite of DINP, had the strongest associations with the placental transcriptome in our study, based on the overall number of significant genes and pathways. More work is needed to understand the potential underlying mechanisms, including how phthalate exposure might disrupt the underlying transcription and how changes in the placental transcriptome disrupt placental function.

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