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Assessment on metabolic perturbations associated with maternal exposure to phthalates among pregnant African American women

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Bachelor's Degree of Education
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Abstract

Assessment on metabolic perturbations associated with maternal exposure to phthalates among pregnant African American women
By Xiaoyue Zhang

Background: Phthalates have been linked with numerous harmful health effects. Limited data are available on the molecular mechanism underlying phthalate toxicity on human health. In this analysis, we measured urinary phthalate metabolites and conducted high-resolution metabolomics (HRM) to identify biological perturbations associated with phthalate exposures among pregnant African American (AA) women, who are disproportionately exposed to high phthalates levels.

Methods: We used untargeted HRM profiling to characterize serum samples collected during early (8-14 weeks gestation) and late (24-30 weeks gestation) pregnancy from 73 participants from the Atlanta AA Maternal-Child cohort. We measured 8 urinary phthalate metabolites in early and late pregnancy, including Monoethyl phthalate (MEP), Mono-n-butyl phthalate (MBP), Mono(2-ethlyhexyl) phthalate (MEHP), and Mono (2-ethyl-5-hydroxyhexyl phthalate (MEHHP), to assess maternal exposures to phthalates. Metabolite and metabolic pathway perturbation were evaluated using an untargeted HRM workflow.

Results: Geometric mean creatinine-adjusted levels of urinary MEP, MBP, MEHP, and MEHHP were 67.3, 6.6, 1.4, and 4.1 μg/g creatinine, respectively, with MEP and MEHP higher than the mean levels of Non-Hispanic blacks in the general US population (2015-2016). There were 814 and 1,435 metabolic features significantly associated with at least one phthalate metabolites during early and late pregnancy, respectively. Metabolic pathway enrichment analysis revealed perturbations in four inflammation- and oxidative stress-related pathways associated with phthalate metabolite levels during both early and late pregnancy, including glycerophospholipid, urea cycle, arginine, and tyrosine metabolism. We confirmed 10 metabolites associated with urinary phthalates, including thyroxine and thiamine, which were negatively associated with MEP, as well as tyramine and phenethylamine, which were positively associated with MEHP and MEHHP.

Conclusion: Our results demonstrate that urinary phthalate levels are associated with perturbations in biological pathways connected with inflammation and oxidative stress. The findings support future hypothesis-testing investigations on potential molecular mechanisms underlying the impact of maternal phthalates exposure on adverse health outcomes.

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Introduction

Phthalates, a group of plasticizers used in hundreds of products, [1] have very large consumption around the world. As of 2016, Di(2-ethylhexyl) phthalate (DEHP) was the most frequently used plasticizer, with annual consumption at 3.07 million tons globally [2]. Given that there is no chemical bound with polymer system, phthalates would leak, migrate, or vaporize easily from plastic embodiment into the surrounding environments, when they undergo slight environmental changes [1]. With phthalates' huge production and demands, wide range of uses, and easy leakage from plastic to the environment, humans are frequently exposed to phthalates through ingestion, inhalation, and dermal pathway. In particular, communities of color and the poor, especially African American (AA), are disproportionately exposed to high phthalates exposures, where the geometric mean levels of several urinary phthalate metabolites in Non-Hispanic black group were higher than other race groups[3].

Phthalate exposures have been associated with numerous negative health effects, including adverse reproductive and birth outcomes [4,5], neurodevelopment [6], obesity [7], diabetes [8], and asthma [9]. Given that phthalates can penetrate the placenta [10], many studies have reported a wide range of phthalate-related adverse birth outcomes, including alteration of steroid hormones [11], decreased anogenital distance (AGD) [12], preterm birth (defined as gestational age <37 weeks) [5], altered gestational age [13], change in body size at birth (birth weight, length, head circumference) [14,15], and clinical pregnancy loss [16]. Prenatal exposures to phthalates have been linked to systemic inflammation [17], enhanced oxidative stress [18], and endocrine disruption [19], with studies observing increased levels of inflammation biomarker [17] and oxidative stress related factors [18], enhanced generation of reactive oxygen species, [20], as well as perturbations in steroid hormones metabolite levels [19], ultimately contributing to pregnancy loss, preeclampsia, preterm birth, and fetal growth restriction [18].

Despite these epidemiological observations and limited mechanistic studies, the detailed biological mechanisms and endogenous pathways underlying the toxicity of phthalate exposures on pregnancy and birth outcomes are complex and remain largely unknown, thus limiting the development of interventions. With the advancement in the omics-based technologies, metabolomics, a high-throughput analytical platform capable of quantifying a large number of metabolites from exogenous and endogenous sources, has emerged as a powerful tool to improve internal biological effect and metabolic perturbation estimation to complex environmental exposure. To address these knowledge gaps, we designed and conducted this study, where we measured 8 urinary phthalate metabolites and conducted high-resolution metabolomics (HRM) to identify biological perturbations associated with phthalate exposures among pregnant African American women, who are disproportionately exposed to high phthalates levels. We hypothesized that maternal exposures to phthalates would be associated with altered maternal metabolome, with perturbations centering around endogenous endocrine disruption, inflammation- and oxidative stress-related pathways.

Methods

Study participants

In this analysis, study participants were from the Atlanta African American Maternal-Child Cohort, where pregnant women who self-identify as African American were recruited from the prenatal care clinics of two metropolitan hospitals in Atlanta, GA, including the Grady Memorial Hospital and Emory University Hospital Midtown [21,22]. The cohort inclusion criteria are: 1) self-reported African American; 2) 8-14 weeks' gestation with a singleton pregnancy verified by clinic record and/or ultrasound; 3) 18-40 years old; 4) without current chronic medical condition [21]. Biological samples, including urine for phthalates exposure assessment and serum for metabolomics profiling were collected at the first clinical visit (early pregnancy: 8-14 weeks' gestation) and second clinical visit (late pregnancy: 24-30 weeks' gestation). Socio-demographic data, including age, education, and socioeconomic status, were collected at the first

clinical visit. Health survey data on medication and substance use like tobacco, alcohol and drugs were collected at both clinical visits. Clinic data on parity, pre-pregnancy body mass index (BMI), gestational weeks and baby's gender were collected at both clinical visits and post-delivery [21]. We assembler the final analytic database by obtaining the phthalate exposure data, maternal metabolic profiles, sociodemographic, health and clinical data from the existing database generated from the parent study. 73 participants who had both phthalate exposure and metabolic profiling were included in the final analysis. The study was approved by the Emory University Internal Review Board.

Phthalate exposure assessments in maternal urine samples

Maternal urinary phthalate exposure levels were analyzed by tandem mass spectrometry on urine samples collected at both clinical visits. We measured eight urinary phthalate metabolites: Monoethyl phthalate (MEP), Mono-n-butyl phthalate (MBP), Monoisobutyl phthalate (MiBP), Monobenzyl phthalate (MBzP), Mono(2-ethlyhexyl) phthalate (MEHP), Mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), Mono (2-ethyl-5-hydroxyhexyl phthalate (MEHHP), and Mono(2-ethly-5-carboxypentyl) phthalate (MECPP). The limits of detection (ng/mL) for these phthalates metabolites were 1 (MEP), 4 (MBP), 2 (MiBP), 0.2 (MBzP), 0.2 (MEHP), 0.4 (MEOHP), 0.4 (MEHHP), and 5.25 (MECPP). The values below the limit of detection were assigned the limit of detection divided by the square root of two [23]. Urinary creatinine concentration (mg/dL) was also measured and urinary phthalate levels were divided by creatinine level, adjusting for variability due to urinary dilution [24]. And the molar sum of DEHP metabolites (∑DEHP) were calculated by the following formula: [(MEHP*(1/278.34)) + (MEHHP*(1/294.34)) + (MEOHP*(1/292.33)) [25]. Although phthalates have short half-live less than 12hr, existing study showed that women's pattern of phthalate exposure may be sufficiently stable to estimate an short-term exposure level based on a single urine measurement [26].

High-resolution metabolomics profiling

We conducted high-resolution metabolomics profiling on the maternal serum samples collected at both clinical visits using liquid chromatography coupled with high-resolution metabolomics (LC-HRMS) with established protocol [27-31]. Hydrophilic interaction liquid chromatography (HILIC) column with positive electrospray ionization (ESI) and C18 hydrophobic reversed-phase chromatography column with negative ESI were used to enhance the coverage of metabolic signal detection. We included two quality control pooled reference plasma samples, the pooled human plasma purchased from Equitech Bio and NIST 1950[32], at the beginning and end of each analytical batch for normalization, batch evaluation, retention time alignment, and *post hoc* quantification. We used ProteoWizard to convert raw LC-HRMS data files to .mzML files, and apLCMS were used to extract metabolic features with modifications of xMSanalyzer [33,34], which performed peak detection, noise filtering, m/z and retention time alignment, feature quantification, and data quality filtering. Metabolic features were characterized according to their mass-to-charge-ratio (m/z), retention time, and intensity. The intensities of metabolic features were $\log_2$ transformed for the following analysis.

Untargeted metabolome-wide association study (MWAS)

In the untargeted MWAS analysis, we examined the association between maternal urinary phthalate metabolites (continuous variable) and metabolic feature intensities (continuous variable) using general linear models, controlling for subject-specific and biological covariates. Because 23.3% MECPP level at the first clinical visit and 39.7% at the second clinical visit could be not detected, we only analyzed other seven phthalate metabolites and sum of DEHP metabolites. The MWAS models had the following form:

$$\begin{aligned}\mathrm{Log}_2 Y_{ij} \sim \mu + \beta_{1j} Phthalate_{ik} + \beta_{2j} Age_i + \beta_{3j} BMI_i + \beta_{4j} Education_i + \beta_{5j} Parity_i + \beta_{6j} Tobacco_i + \\ \beta_{7j} Alcohol_i + \beta_{8j} Marijuana_i + \beta_{9j} Insurance\_type_i + \beta_{10j} Baby\_sex_i + \beta_{11j} Gestational\_age_i + \varepsilon_{ijk.} \quad (1)\end{aligned}$$

Where $Log_2 Y_{ij}$ refers to the log intensity of metabolic feature *j* for participant *i*. Separate models were conducted for each urinary phthalate metabolites, each metabolic feature detected from each technical column (serum HILIC positive ESI, and Serum C18 negative ESI). We conducted the MWAS model on features detected during early and late pregnancy separately. In these models, μ is the intercept and $Phthalate_{ik}$ refers to maternal urinary phthalate metabolites *k* for each participant *i*, representing participants' short-term exposures to phthalates during early or late pregnancy. We controlled for potential confounding factors, including age, pre-pregnancy body mass index (BMI), education (categorical), parity (categorical), tobacco, alcohol and drug use withing 1 month before each clinical visit (categorical), insurance type (categorical), sex of baby, as well as gestational age at each clinical visit. ε_{ijk} represents residual random normal error. We identified significant features using two different levels of statistical significance (p-value: <0.05, and <0.005). Given the multiple testing nature of metabolomics analysis, we also corrected these raw p-values for multiple comparisons using Benjamin-Hochberg false discovery rate (FDR) procedure at two thresholds (q-value: <0.2 and <0.05). The results were presented in Manhattan plots, where x-axis is the retention time of each metabolic feature and y-axis is −log10(p) for β_1 from Equation (1). All analyses were performed using R 4.0.2.

Metabolic pathway enrichment analysis

We performed pathway identification and module analysis using mummichog[35] (v. 1.0.5) with the cutoff raw p<0.005. Pathway analyses were conducted separately for each set of significant features from each of the urinary phthalate metabolites MWAS models on features detected in the HILIC and C18 columns during early and late pregnancy. An adjusted p for each pathway were calculated from resampling the reference input file in mummichog using a gamma distribution. we classified pathways with adjusted p <0.05 with at least four of the phthalate metabolites models. The results were presented by heat map, where each cell in heat map represented the p-value of associations between each metabolic pathway and each phthalate metabolite. We utilized a reference file for each technical column (C18 negative and

HILIC positive) with file consisting of features with a raw p-value <0.05 as sensitivity analysis. FDR-corrected q-values could not be used due to null findings in the shorter-term exposure windows.

Chemical annotation and metabolite confirmation

Metabolic features significantly associated with urinary phthalate metabolites and also enriched in a relevant pathway ($p<0.005$) were annotated by matching mass m/z value for adducts commonly formed to the METLIN, HMDB, and KEGG databases, using a mass error threshold of 10 ppm. To further minimize false positive match, tentative matches were further screened on their retention time, isotope patterns, and peak quality by examining the extracted ion chromatograph (EIC plots). Finally, we confirmed the chemical identity of the annotated metabolites with level one evidence [36] by comparison of accurate mass m/z, retention time and ion dissociation patterns to the analytical standards.

Results

The demographic characteristics of participants are shown in Table 1. Mean age and BMI at first clinical visit were 25.96 ± 4.86 years old and 28.24 ± 6.65 kg/m^2. More than 20% of the study participants smoked, drank or used drug during pregnancy. Nearly 40% of them were nulliparous. More than half of them were poorly educated and used Medicaid insurance. And 53% of infants were female. Mean gestational age of newborns were 38.65 ± 1.55 weeks. Comparing with those recruited from Emory University Hospital Midtown, the participants from Grady Memorial Hospital were significantly younger, with lower education and economic levels (p-values<0.001).

Maternal urinary phthalate metabolites

The detectable rates and the levels of maternal urinary phthalate metabolites at both clinical visits were presented in Table 2. Most of phthalate metabolites had high detectable rates other than MECPP. Geometric mean creatinine-adjusted levels of urinary MEP, MBP, MiBP, MBzP, MEHP, MEOHP,

MEHHP and MECPP at first clinical visit were 72.52, 5.98, 5.79, 4.05, 1.33, 2.62, 4.77, and 6.68 μg/g creatinine, respectively; those at the second clinical visit were 62.45, 7.31, 5.58, 4.27, 1.46, 2.74, 3.55, and 5.61 μg/g creatinine, respectively. For MEP, its geometric mean levels at both clinical visits were higher than the geometric mean levels of people age 20 and older as well as the geometric mean levels of Non-Hispanic blacks investigated by U.S. Centers for Disease Control and Prevention between 2015 and 2016[3]. For MBzP, its geometric mean levels at both clinical visits were higher than the geometric mean levels of people age 20 and older between 2015 and 2016. As for MEHP, its geometric mean levels at both clinical visits were higher than the geometric mean levels of Non-Hispanic blacks between 2015 and 2016.

Association of phthalate exposure and metabolic profiles in participants

Using LC-HRMS, we detected a total of 29,524 metabolic features in serum samples. Specifically, for serum samples collected at the first clinical visit, 15,310 features were detected in HILIC ESI and 12,812 were detected in C18 ESI; for serum samples collected at the second clinical visit, 15,167 and 12,754 features were detected in HILIC ESI and C18 ESI, respectively. We conducted 32 sets of MWAS models, with 8 maternal urinary phthalate metabolites analyzed among serum metabolic features detected in 2 chromatography columns on samples collected from 2 clinical visits. Only a few metabolic features remain statistically significant after FDR correction, and therefore, we select raw $p<0.005$ as the significance cutoff for the main statistical analysis. In total, 507 and 337 unique metabolic features were statistically significantly related to at least one or more of phthalate metabolites using HILIC ESI and C18 ESI on samples collected at the first clinical visit, respectively (p-value<0.005). For samples collected at the second clinical visit, the corresponding numbers of significant metabolic features were 801 and 634 in HILIC ESI and C18 ESI, respectively (Table 3).

Perturbated metabolic pathways associated with phthalate exposure

Metabolic pathway enrichment analysis revealed 14 metabolic pathways associated with at least four phthalate metabolites on samples collected at the first clinical visit (Figure 1), whereas perturbations in 18 metabolic pathways were observed on features detected at the second clinical visit (Figure 2). Seven pathways consistently appeared to be associated with varying phthalate metabolites models at both clinical visits, including glycerophospholipid metabolism, urea cycle/amino group metabolism, tyrosine metabolism, lysine metabolism, arginine and proline metabolism, aspartate and asparagine metabolism, as well as butanoate metabolism (Figure 3). In addition, 7 pathways were uniquely identified during early pregnancy, with 11 unique pathways observed during late pregnancy.

Chemical annotation and confirmation on metabolic features significantly associated with phthalate exposure

Finally, we matched the samples with authentic reference standards, verified by tandem mass spectrometry, to confirm the chemical identity of metabolic features that were both associated with the measured phthalates as well as those enriched within phthalate exposure-relevant metabolic pathways. In total, we confirmed the chemical identity of ten metabolic features with level 1 evidence and they were shown in Table 4, with their EIC plots shown in Figure 4. All of these confirmed chemicals were found in samples collected at the second clinical visit during late pregnancy. Specifically, we confirmed five chemicals associated with MEP, where we found negative associations between MEP and 5-oxo-l-proline, thiamine, thyroxine, and pyruvate (β= -0.00006, -0.0004, -0.00008, -0.00010); and positive association between MEP and 5'-deoxyadenosine (β= 0.00019). Octanoylcarnitine was founded to be negatively associated with MBP (β= -0.061), whereas 3-Methoxytyramine was negatively associated with MBzP. Besides, bilirubin was solely positively associated with MEHP (β= 0.079). For DEHP metabolites, phenethylamine and phenylethanolamine-tyramine were found to be associated with increased levels of DEHP metabolites, including hydrolyzed primary DEHP metabolite (MEP), oxidative secondary DEHP

metabolites (MEHHP and MEOHP) as well as the molar sum of DEHP metabolites. What's more, we also found a consistent pattern on the coefficients of these DEHP metabolites with the following trend: β ($\sum$DEHP) > β (MEHP) > β (MEHHP) > β (MEOHP).

Discussion

In this analysis, we used targeted phthalates exposure assessment and untargeted MWAS study to examine metabolic perturbations associated with prenatal exposures to phthalates during early and late pregnancy among pregnant African American women. We identified numerous biological pathways and metabolites significantly associated with maternal phthalate exposure. Many of these metabolic signals were closely related to systemic inflammatory response and oxidative stress. Collectively, these findings provide insights on potential molecular mechanism underlying phthalate toxicity on adverse birth outcome. To our knowledge, this constitutes the first untargeted MWAS study examining the biological perturbation associated with prenatal phthalate exposures in maternal serum metabolome.

Among the pathways significantly associated with phthalates exposures during early and late pregnancy, many were closely involved in oxidative stress, acute inflammatory response, and chronic low-grade inflammation, including purine, urea cycle, glycerophospholipid, cytochrome P450, caffeine, pentose phosphate, nicotinate and nicotinamide, arginine and proline, and tyrosine metabolisms[37-47]. Our findings were also consistent with previous study reporting the association between phthalate exposure and biomarkers of inflammation and oxidative stress [17,18], suggesting that phthalate exposure would disrupt the biological pathways connected with inflammation and oxidative stress. Taken together, these cumulative evidence indicated a causative role of phthalate exposure in inducing systemic inflammation and enhancing oxidative stress, which ultimately lead to preterm birth, pregnancy loss, preeclampsia, and fetal growth restriction [18,48].

We found that tyrosine metabolism, a pathway closely related to systemic inflammation, was consistently associated with numerous phthalate exposures during both early and late pregnancy, including MBP, MiBP and MEHP during early pregnancy, and DEHP metabolites during late pregnancy. Specifically, four metabolic features in this pathway were confirmed with level 1 evidence, including phenethylamine, tyramine, 3-methoxytyramine and thyroxine, indicating that the phthalate exposures-related metabolic perturbations in maternal metabolome may center around tyrosine metabolism (Figure 5). Among these metabolites, thyroxine was significantly negatively related to MEP. Thyroxine is one of thyroid hormones secreted into the bloodstream by the thyroid gland, which plays important roles in heart and digestive function, metabolism, muscle control, bone health, and brain development. In the early stage of pregnancy, the embryo is completely dependent on maternal thyroid hormones that pass through the placenta, and till about 12-14 weeks gestation, the fetal thyroid function begins [49]. Even after the fetal thyroid gland begins to secrete thyroxine, the thyroxine transferred from the mother still constitutes a part of circulating fetal thyroxine and continues to protect the fetal neurodevelopment until birth [49]. The negative association between MEP and maternal serum level of thyroxine in our study suggests that MEP may have adverse effect on fetal neurodevelopment by decreasing the maternal serum level of thyroxine during late pregnancy. Previous study has reported the endocrine disruption effect of phthalate on pregnant women regarding steroid hormones metabolite including cortisol, a stress hormone, estradiol and testosterone, hormones [50]. And our result revealed another new perspective of endocrine disruption effect of phthalate on thyroid hormones. Besides, we observed a significantly positive association between tyramine and DEHP metabolites. Tyramine is an amino acid related to increasing cardiac output, respiration and blood glucose, releasing norepinephrine as well as peripheral vasoconstriction [51]. Obayomi's animal study reported that tyramine and its specific receptor TAAR1 are located in the uterus of mice, and this monoamine can induce uterine contractions, and its effect is similar to oxytocin [51]. In an epidemiologic study, Ferguson et al. found that ∑DEHP were associated with increased odds of preterm birth [5]. These previous findings, together with our observations, indicated a potential mechanistic pathway underlying how DEHP would lead to preterm birth. In addition to tyramine, we also found that

phenethylamine was positively associated with DEHP metabolites. Phenethylamine is a phenylalanine derivate in brain. Mazumder's molecular docking model demonstrated that phenethylamine could increase the generation of reactive oxygen species [52]. And Capuron et al. suggested that inflammation was positively associated with phenylalanine at the expense of tyrosine [43]. All these observations supported that phthalate exposures may lead to adverse health outcome through inflammation and oxidative stress by inducing perturbations in metabolic pathways centering around tyrosine metabolism.

Interestingly, we found that the coefficient of hydrolyzed primary metabolite of DEHP (MEP) is higher than those of oxidative secondary metabolites of DEHP (MEHHP and MEOHP), which suggested that oxidative DEHP metabolites might be less toxic than hydrolyzed DEHP metabolites. This was in consistent with Hauser's study, where urinary MEHP levels were found to be significantly positively associated with sperm DNA damage in male, while the urinary MEHHP and MEOHP levels were significantly negatively associated with sperm DNA damage, indicating that DEHP oxidative metabolites may be less toxic [4]. Thus, individual vulnerability and sensitivity to DEHP exposure may depend on the individual functionality of the phase one enzymes to hydrolyze DEHP.

Besides confirming the features enriched in the tyrosine metabolism, we also confirmed bilirubin with level one evidence, where our data showed a positive association between serum bilirubin and increase in MEHP level. The biochemical function and toxicity of bilirubin have always been controversial. In previous study, the function of this endogenous compound was reported inconsistently. Some previous studies have demonstrated the neurotoxicity of unconjugated bilirubin (UCB) in preterm neonate suggesting that gestational age-specific free bilirubin level may increase the risk of bilirubin-induced neurotoxicity in premature infants [53-55], while many studies showed its antioxidant properties in vitro and in animal [56,57]. There are two potential explanations for the increasing level of bilirubin in our result. First, DEHP exposure may have adverse effect on neurodevelopment in newborn by increasing level of bilirubin. Second, bilirubin is not effective as an antioxidant in human being. Previous study has

corroborated this explanation. Dani et al observed the increase in plasma antioxidant capacity with the decrease plasma level of bilirubin in preterm birth [58]. Further studies are required to confirm our speculations.

In addition to the above metabolic features which were significantly associated with several phthalate metabolites, we observed perturbations in thiamine metabolism associated with MEP during late pregnancy. Correspondingly, we confirmed the identify of thiamine in HILIC ESI and that of pyruvate in C18 ESI with level one confidence, which were both negatively associated with MEP during late pregnancy. Thiamine is an essential vitamin which cannot be synthesized in human body and require exogenous source. Thiamin is absorbed into cells by specific transporters and is converted into active thiamine pyrophosphate (TPP) by thiamine pyrophosphokinase (TPK) in the cytoplasm, from which it can be transported to the mitochondria, and there TPP serves as a cofactor for three distinct ketoacid dehydrogenases including pyruvate dehydrogenase complex, α-ketoglutarate dehydrogenase and branched-chain α-keto acid dehydrogenase [59]. One of the key enzymes of glucose metabolism is pyruvate dehydrogenase [60]. Shaffer et al. found that the maternal urinary level of T1T3avg MEP (average of first and third trimester MEP concentrations) was positively associated with gestational diabetes mellitus (GDM) [61]. And Bartáková et al. observed that the plasma levels of BMI-adjusted thiamine in women with GDM in mid-trimester were significantly lower than control groups [62]. Our finding suggested that thiamine may serve as a mediator in the association between MEP and GDM, and provided a potential mechanism of MEP leading to GDM that Diethyl phthalate (DEP) exposure could alter glucose metabolism by disturbing thiamine metabolism and incease the risk of GDM during late pregnancy.

There are several limitations in this study. First, the cross-sectional nature of the current analysis precludes the ability to make any causal inferences about the association between maternal phthalates exposure and biological perturbations. Second, although we control for several important confounding factors, we were not able to adjust for the impact of diurnal pattern, which may have effect on intra-

individual variation in response to phthalate exposure and in phthalate metabolism. Third, using untargeted high-resolution metabolomics method, we can only confirm the metabolites in the limited reference standards. Thus, we may miss numerous significant metabolites related to both adverse health outcomes and phthalate exposure. Moreover, we used raw p-value instead of using FDR correction to select significant metabolic features to conduct pathway analysis using mummichog, there might be false discoveries on significant features due to multiple evaluation in a single experiment, Finally, our study had limited sample size focused on pregnant African American women. Therefore, our results may not be generalizable to other population.

Conclusion

With the help of highly sensitive platform, untargeted HRM, our results demonstrate that urinary phthalate levels are associated with perturbations in biological pathways closely connected with inflammation and oxidative stress. Besides, we also found that DEP exposure during late pregnancy is related to decreasing maternal level of thyroxine and thiamine. And DEHP exposure during late pregnancy is associated with increasing maternal level of tyramine and phenethylamine. The findings support future hypothesis-testing investigations on potential molecular mechanisms underlying the impact of maternal phthalates exposure on adverse health outcomes.

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Tables

Table 1. Characteristics of participants

Characteristics	Overall (N=73)	Grady Memorial Hospital (N=46)	Emory University Hospital Midtown (N=27)	p-value [a]
Age [years], mean (SD)	25.96 (4.86)	24.67 (4.24)	28.15 (5.13)	0.005**
BMI (kg/m^2), mean (SD)	28.24 (6.65)	28.09 (7.00)	28.50 (6.13)	0.728
Parity, n (%)				
Nulliparous	31 (42)	20 (43)	11 (41)	0.951
Nonnulliparous	41 (56)	25 (54)	16 (59)	
Education, n (%)				
High school or less	41 (56)	37 (80)	4 (15)	
Undergraduate	20 (27)	7 (15)	13 (48)	<0.001**
Graduate	12 (16)	2 (5)	10 (37)	
Insurance type, n (%)				
Medicaid	56 (77)	45 (98)	11 (41)	<0.001**
Private insurance	17 (23)	1 (2)	16 (59)	
Baby sex, n (%)				
Female	39 (53)	23 (50)	16 (59)	0.601
Male	34 (47)	23 (50)	11 (41)	
Tobacco use, n (%)				
Clinical visit 1 (Yes)	10 (14)	9 (20)	1 (4)	0.148
Clinical visit 1 (No)	60 (82)	37 (80)	23 (85)	
Clinical visit 2 (Yes)	3 (4)	3 (7)	0 (0)	0.549
Clinical visit 2 (No)	66 (90)	41 (89)	25 (93)	
Alcohol use, n (%)				
Clinical visit 1 (Yes)	5 (7)	4 (9)	1 (4)	0.652
Clinical visit 1 (No)	64 (88)	41 (89)	23 (85)	
Clinical visit 2 (Yes)	0 (0)	0 (0)	0 (0)	-
Clinical visit 2 (No)	69 (95)	44 (96)	25 (93)	
Marijuana use, n (%)				
Clinical visit 1 (Yes)	16 (22)	15 (33)	1 (4)	0.006**
Clinical visit 1 (No)	53 (73)	30 (65)	23 (85)	
Clinical visit 2 (Yes)	2 (3)	2 (4)	0 (0)	0.528
Clinical visit 2 (No)	66 (90)	41 (89)	25 (93)	
Gestational age (week), mean (SD)				
Clinical visit 1	11.59 (2.20)	11.30 (2.35)	12.07 (1.85)	0.094
Clinical visit 2	25.74 (1.58)	25.60 (1.54)	25.98 (1.64)	0.277
Post-delivery	38.65 (1.55)	38.45 (1.66)	38.99 (1.31)	0.14

[a] Mann-Whitney U Test, Pearson's Chi-squared test or Fisher's Exact Test
**P<0.01

Table 2. Creatinine-adjusted phthalate metabolite concentrations (μg/g) of study participants

Sample size (73)	First clinical visit			Second clinical visit			Fourth national report on human exposure to environmental chemicals[3]	
	% Detect	GM	IQR (25^{th}, 75^{th})	% Detect	GM	IQR (25^{th}, 75^{th})	Age≥20 (2015-2016) GM	Non-Hispanic blacks (2015-2016) GM
MEP	100	72.5	29.62, 161.27	100	62.45	28.6,91.54	35.9	54.9
MBP	83.6	5.98	3.39, 11.58	86.3	7.31	4.34, 11.95	9.36	10.9
MiBP	82.3	5.79	3.52, 11.97	86.3	5.58	3.58, 10.10	8.03	9.29
MBzP	100	4.05	2.12, 7.09	98.6	4.27	1.88, 7.56	3.87	5.57
MEHP	87.7	1.33	0.61, 3.62	91.8	1.46	0.74, 2.78	#	1.27
MEOHP	97.3	2.62	1.44, 5.24	95.9	2.74	1.71, 4.59	3.36	3.66
MEHHP	100	4.77	2.26, 8.14	95.9	3.55	2.15, 5.74	5.40	5.96
MECPP	75.7	6.68	3.29, 12.22	60.3	5.61	3.41, 8.73	8.28	8.51
∑DEHP [a]	-	0.03	0.02, 0.06	-	0.03	0.02, 0.05	-	-

Acronym: GM, geometric mean; IQR, interquartile range
[a]∑DEHP is the molar sum of MEHP, MEHHP, and MEOHP.
Not calculated: proportion of results below limit of detection was too high to provide a valid result.

Table 3. Number of significant metabolic features (p-value<0.005) associated with phthalate exposure in technical column

Clinical visit & technical column	Total number of features extracted	MEP	MBP	MiBP	MBzP	MEHP	MEOHP	MEHHP	∑DEHP	Number of unique features [a]
Visit 1-HILIC	15310	70	117	93	97	100	67	47	63	507
Visit 1-C18	12812	57	49	57	81	47	46	44	44	337
Visit 2-HILIC	15167	403	92	53	124	85	95	87	98	801
Visit 2-C18	12754	231	91	36	158	77	100	97	95	634

[a] Number of unique metabolic features that were statistically significantly associated with at least one or more phthalate metabolites.

Table 4. Chemical identity [a] of the metabolic features significantly associated with phthalate metabolites (p-value<0.005) in this study.

m/z	RT	Annotated metabolites	Adduct form	Pathways	Associated phthalate metabolites [c] & mean change [b] (95% confidence intervals)
122.0965	30.9	Phenethylamine	M+H	Tyrosine metabolism	MEHP β= 0.121 (0.092, 0.176) MEHHP β= 0.039 (0.032, 0.052) MEOHP β= 0.037 (0.030, 0.050) ∑DEHP β= 4.934 (4.016, 6.630)
130.0499	58	5-oxo-l-proline	M+H	Aspartate and asparagine metabolism	MEP β= -0.00006 (-0.00008, 0.00002)
138.0914	22.5	Tyramine	M+H	Tyrosine metabolism	MEHP β= 0.110 (0.076, 0.175) MEHHP β= 0.039 (0.030, 0.054) MEOHP β= 0.038 (0.030, 0.053) ∑DEHP β= 4.924 (3.847, 6.914)
138.0914	22.5	Phenylethanolamine	M+H	Urea cycle metabolism	MEHP β= 0.110 (0.076, 0.175) MEHHP β= 0.039 (0.030, 0.054) MEOHP β= 0.038 (0.030, 0.053) ∑DEHP β= 4.924 (3.847, 6.914)
168.1019	26.1	3-Methoxytyramine	M+H	Tyrosine metabolism	MBzP β= -0.014 (-0.019, -0.005)
252.1077*	25.5	5'-deoxyadenosine	M+H	Urea cycle metabolism	MEP β= 0.00019 (0.00015,0.00025)
265.1108	44.3	Thiamine (B1)	M+H	Thiamin metabolism	MEP β= -0.0004 (-0.0005, -0.0002)
288.2167	30.8	Octanoylcarnitine	M+H	Mitochondrial Beta-Oxidation of Short Chain Saturated Fatty Acids	MBP β= -0.061 (-0.080, -0.026)
585.2707*	49.1	Bilirubin	M+H	Porphyrin Metabolism	MEHP β= 0.079 (0.052, 0.131)
777.6931	30.7	Thyroxine	M+H	Tyrosine Metabolism	MEP β= -0.00008 (-0.00010, -0.00004)
87.0087	24.7	Pyruvate	M-H	Pyruvate Metabolism; Tyrosine metabolism; Thiamin metabolism; Methionine and cysteine metabolism; Pyrimidine metabolism; Purine metabolism; Glycine, serine, alanine and threonine metabolism; Arginine and Proline Metabolism; Butanoate metabolism; Glycolysis and Gluconeogenesis; Aminosugars metabolism; Glutamate metabolism; Carbon fixation; Alanine and Aspartate Metabolism	MEP β= -0.00010 (-0.00013, -0.00005)

Acronym: m/z, mass to charge ratio; RT, retention time.

[a] Chemical identification on the candidate metabolic features was conducted by matching peaks by accurate mass and retention time to authentic reference standards in an in-house library run under identical conditions using tandem mass spectrometry.

[b] β were adjusted for age (years), BMI at first clinical visit (kg/m2), Parity, insurance type, baby's gender, gestational age at each clinical, tobacco, alcohol and drug use withing 1 month before clinical visit

[c] All identified metabolites were during late pregnancy

*There were multiple pure peaks on its EIC plot.

Figures

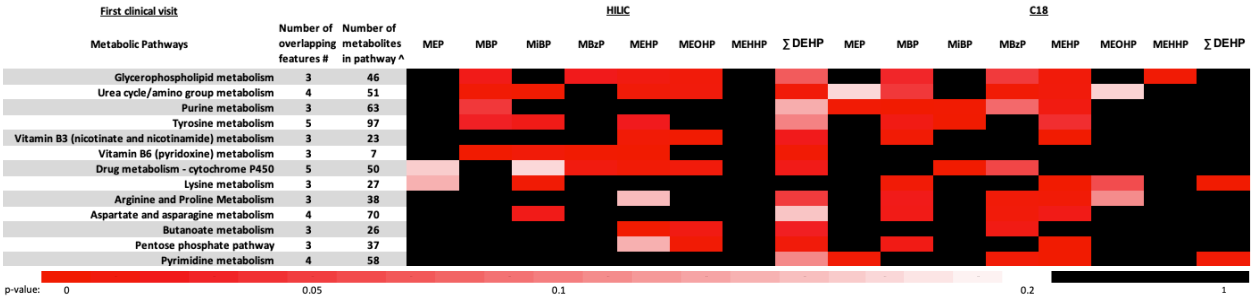

Fig1. Metabolic pathways associated with ≥4 phthalate metabolites models at the first clinical visit. Cells were colored according to the strength (i.e. p-value) of the association between each of metabolic pathways and significant features (p-value< 0.005) that were associated with phthalate metabolites. Pathways are ranked according to the total number of the significant pathway-phthalate metabolites associations ($p < 0.05$) in the HILIC column and the C18 column.
^Average number of metabolites within the specific metabolic pathway on significant models.
#Number of metabolic features in the samples with m/z matched to the metabolites within the specific metabolic pathway.

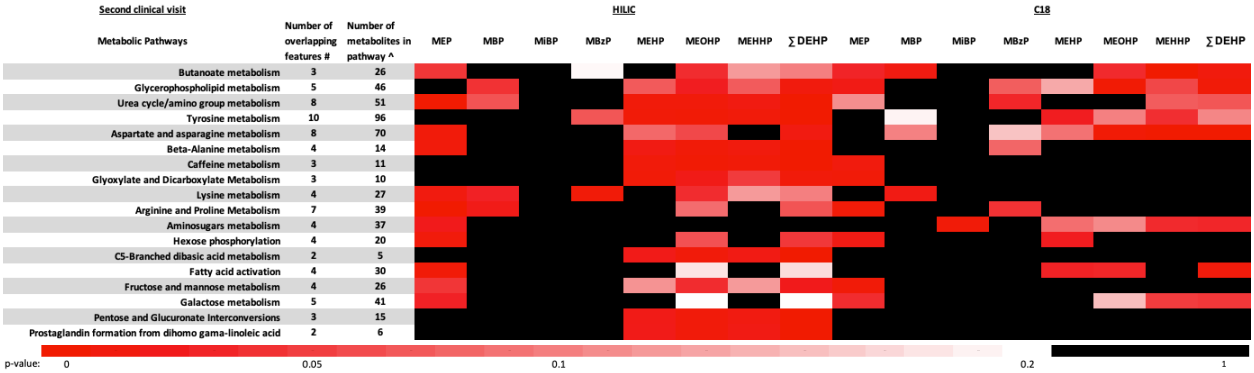

Fig2. Metabolic pathways associated with ≥4 phthalate metabolites models at the second clinical visit. Cells were colored according to the strength (i.e. p-value) of the association between each of metabolic pathways and significant features (p-value< 0.005) that were associated with phthalate metabolites. Pathways are ranked according to the total number of the significant pathway-phthalate metabolites associations ($p < 0.05$) in the HILIC column and the C18 column.
^ Average number of metabolites within the specific metabolic pathway on significant models.
#Number of metabolic features in the samples with m/z matched to the metabolites within the specific metabolic pathway.

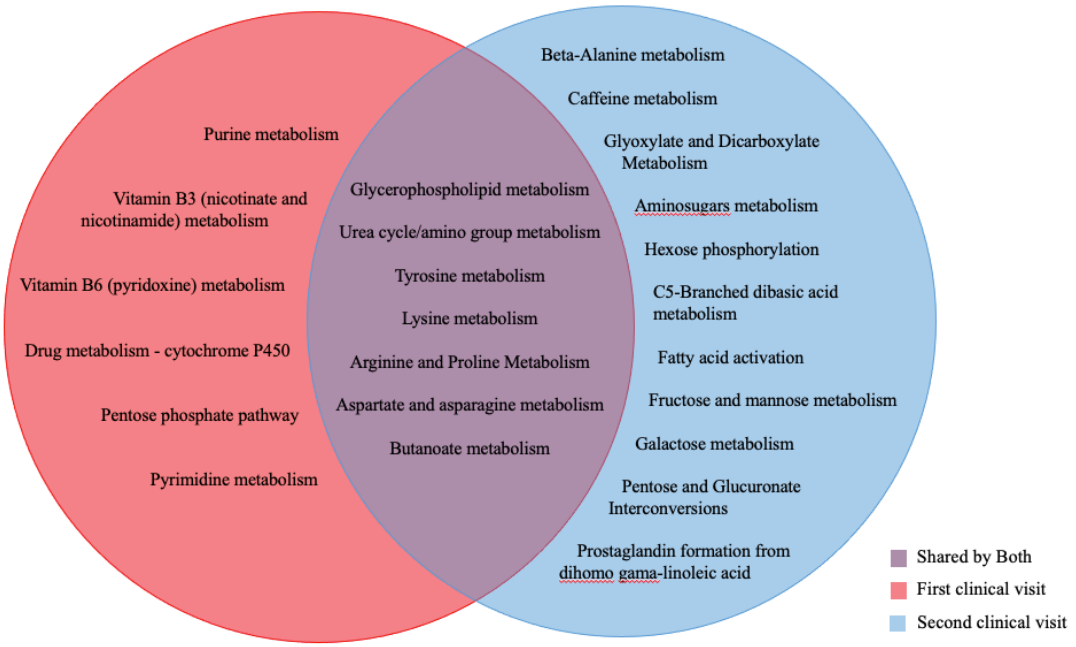

Fig3. Venn diagram of metabolic pathways related to phthalate exposure in pregnant African American women during early and late pregnancy.

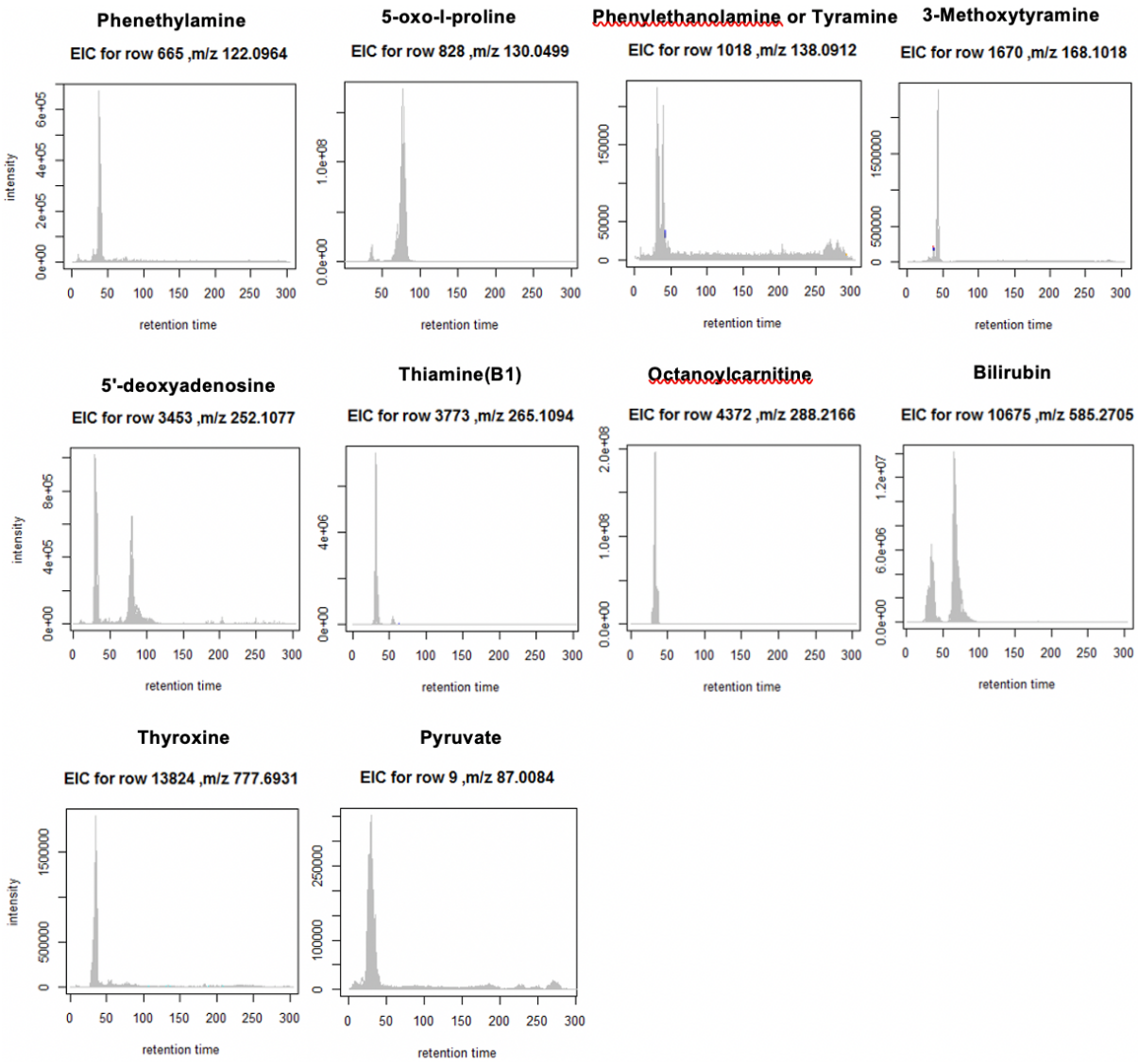

Fig 4. The extracted ion chromatograph of identified chemicals. The metabolites were considered to be acceptable for chemical identification that had one or multiple pure peaks.

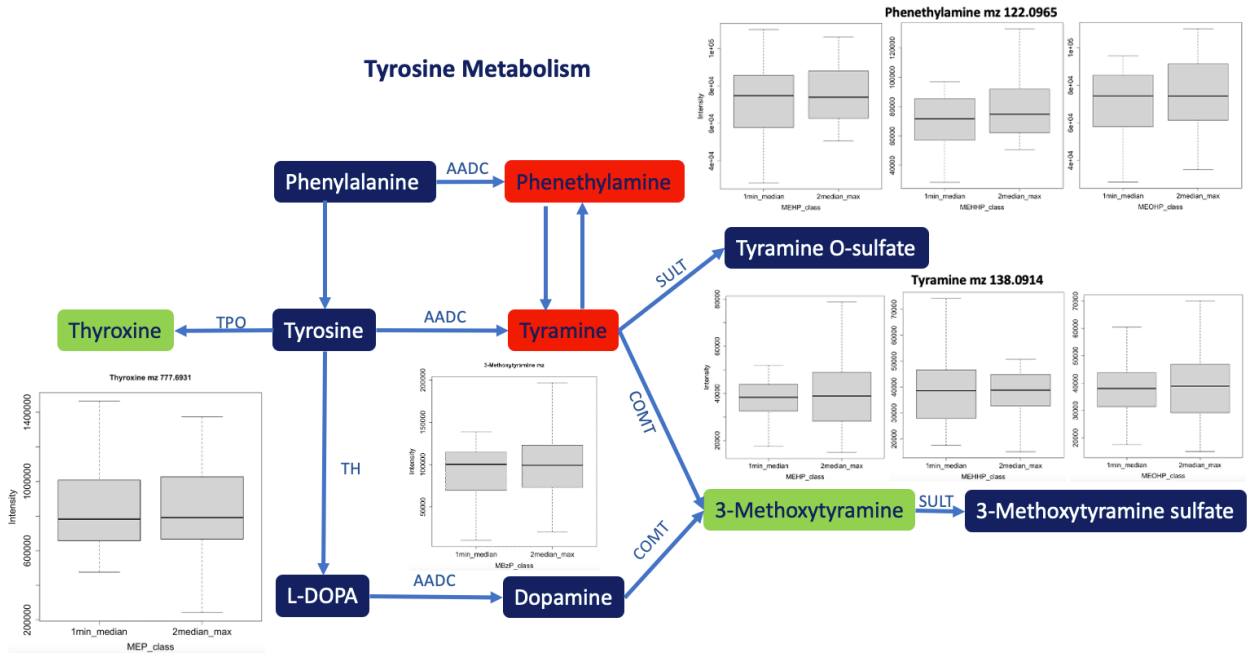

Fig5. Confirmed metabolites with level one confidence in the Tyrosine Metabolism pathway associated with maternal phthalate exposure: positive association (red) and negative association (green). Acronym: TPO, Thyroid peroxidase; AADC, aromatic l-amino acid decarboxylase; TH, tyrosine hydroxylase; SULT, Sulfotransferase; COMT, Catechol-O-methyltransferase