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The associations between pre-conception phthalate exposure and the serum metabolome in women undergoing infertility treatment

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2019

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An abstract of
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Abstract

The associations between pre-conception phthalate exposure and the serum metabolome in women undergoing infertility treatment

By Jillian E. Nelson

Phthalates, a class of synthetic chemicals with a wide spectrum of commercial uses, are ubiquitous endocrine disruptors, with demonstrated anti-androgenic properties. Past studies have shown an association between higher preconception exposure to phthalates and lower fertility in women; however, the biological mechanisms remain unclear. Our study aimed to utilize untargeted high-resolution metabolomics to identify serum metabolites and pathways associated with maternal preconception phthalate exposure. Our analysis included 184 women in the Environment and Reproductive Health (EARTH) study that underwent in vitro fertilization (IVF) at the Massachusetts General Hospital Fertility Center (2005-2016). On the same day during controlled ovarian stimulation, women provided a serum sample, which was analyzed for metabolomics using liquid chromatography coupled with high-resolution mass spectrometry and two chromatography columns, and a urine sample, which was analyzed for 11 phthalate metabolites. We used multivariable generalized linear models to identified metabolic features associated with urinary phthalate metabolite concentrations, followed by enriched pathway analysis. A total of 10,803 and 12,968 metabolic features were detected in the serum in the C18 negative and HILIC positive columns, respectively. Metabolic pathway enrichment analysis revealed 50 pathways in the C18 negative column and 50 pathways in the HILIC positive column that were significantly associated with at least one of the 11 urinary phthalate metabolites or molar sum of di-2-ethylhexyl phthalate metabolites ($\sum$DEHP). The classes of pathways that were most often associated with phthalate exposure were lipid metabolism, amino acid metabolism, and oxidative stress. Our study provides further insight into the biological pathways, including lipid and amino acid metabolism and oxidative stress, that may be mediating the observed associations between phthalate exposures and lower fertility in women.

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Abstract.

Background. Phthalates, a class of synthetic chemicals with a wide spectrum of commercial uses, are ubiquitous endocrine disruptors, with demonstrated anti-androgenic properties. Past studies have shown an association between higher preconception exposure to phthalates and lower fertility in women; however, the biological mechanisms remain unclear.

Objectives. Our study aimed to utilize untargeted high-resolution metabolomics to identify serum metabolites and pathways associated with maternal preconception phthalate exposure.

Methods. Our analysis included 184 women in the Environment and Reproductive Health (EARTH) study that underwent in vitro fertilization (IVF) at the Massachusetts General Hospital Fertility Center (2005-2016). On the same day during controlled ovarian stimulation, women provided a serum sample, which was analyzed for metabolomics using liquid chromatography coupled with high-resolution mass spectrometry and two chromatography columns, and a urine sample, which was analyzed for 11 phthalate metabolites. We used multivariable generalized linear models to identified metabolic features associated with urinary phthalate metabolite concentrations, followed by enriched pathway analysis.

Results. A total of 10,803 and 12,968 metabolic features were detected in the serum in the C18 negative and HILIC positive columns, respectively. Metabolic pathway enrichment analysis revealed 50 pathways in the C18 negative column and 50 pathways in the HILIC positive column that were significantly associated with at least one of the 11 urinary phthalate metabolites or molar sum of di-2-ethylhexyl phthalate metabolites (∑DEHP). The classes of pathways that were most often associated with phthalate exposure were lipid metabolism, amino acid metabolism, and oxidative stress.

Conclusion. Our study provides further insight into the biological pathways, including lipid and amino acid metabolism and oxidative stress, that may be mediating the observed associations between phthalate exposures and lower fertility in women.

Introduction

There is growing evidence that certain environmental exposures can negatively affect reproductive outcomes and fertility. The 2013 Joint Committee Opinion on 'Exposure to Toxic Environmental Agents' states that environmental exposures in preconception and prenatal periods have long-lasting effects on reproductive health, while a lack of regulatory processes within the government has resulted in their ubiquity in the marketplace and environment (ACOG Committee. 2013). These chemicals are present in the air, in food and water supplies, and in everyday personal care products, often with minimal or no testing conducted on their effects on reproductive and other health outcomes. These toxins disproportionately affect minority populations and people of lower socio-economic status who are more likely to be exposed through their occupation or home environment. Further research is needed in this area to identify which exposures are harmful and the mechanisms through which they may influence reproductive outcomes (CDC 2013; Hauser and Calafat, 2005).

Phthalates, a class of synthetic chemicals with a wide spectrum of commercial uses, are ubiquitous endocrine disruptors, with demonstrated anti-androgenic properties. Exposure can occur through dermal contact with phthalate containing shampoos, lotions, deodorants and other cosmetics, ingestion of medications with phthalate containing coatings, or inhalation of phthalates released into the air from vinyl surfaces or other similar products. Phthalates have short half-lives and are quickly metabolized in the body. However, despite this rapid excretion, several animal and human studies have implicated phthalates as female reproductive toxicants with specific effects on disrupting ovarian function (Gray et al., 2006).

Past studies have shown that there is an association between higher exposure to phthalates and lower fertility in women, including reduced ovarian reserve (Messerlian et al., 2016), lower oocyte yield (Hauser and Gaskins, 2015), lower probability of pregnancy (Heudorf et al., 2007), and increased risk of pregnancy loss (Toft et al., 2012). In a study among women undergoing assisted reproduction, adjusted models showed that women with the highest urinary levels of Di(2-ethylhexyl)phthalate (∑DEHP) metabolites (the predominant high molecular weight phthalate) had significantly reduced probability of pregnancy and live birth compared to women with lower levels. And while the other phthalate metabolites (i.e., monoethyl phthalate (MEP), monobutyl phthalate (MBP), mono-isobutyl phthalate (MiBP), monobenzyl phthalate (MBZP1), mono-(3-carboxypropyl) phthalate (MCPP), and monocarboxy-isononly phthalate (MCNP) were not significantly associated with decreased probability of clinical pregnancy or live birth, nearly all models showed small to moderate reduced likelihood of these outcomes when the highest quartile was compared with the lowest quartile (Hauser et al., 2015). While experimental studies have shed some insight into the biological mechanisms that may be mediating these associations, almost all have taken a targeted approach when examining potential biomarkers. Very few have applied a metabolomic approach, which may help further inform the research on the pathways linking phthalate exposure with detrimental health outcomes in humans.

Studies on phthalate exposures and the serum metabolome have most commonly found changes in lipid metabolism associated with increased phthalate exposure. In 2021, a case control study with 120 participants was published finding that urinary phthalate metabolites were associated with changes in metabolic features related to type 2 diabetes pathways (Duan et al., 2021). A group examining the effect of DEHP exposure on mice found that after 10 weeks

of exposure, there were significant changes to lipids and carnitines within the serum metabolome (Klöting et al., 2015). A study conducted on 115 pregnant women between weeks 20-36 of gestation, found through pathway analysis that as concentrations of 11 urinary phthalate metabolites increased, lipid biosynthesis, inflammation, sphingolipid signaling and nucleotide degradation increased. These metabolic changes can be markers of inflammation and have potential to negatively impact pregnancy (Zhou et al., 2018). In another study, which investigated the link between urinary phthalates and serum metabolomics during early and late pregnancy in African-American women, the authors found that phthalate metabolites were associated with perturbations in four inflammation- and oxidative-stress-related pathways including glycerophospholipid, urea cycle, arginine, and tyrosine metabolism (Zhang et al., 2021).

Building off this existing literature, the aim of our study was to utilize untargeted high-resolution metabolomics to identify metabolites and pathways associated with maternal preconception phthalate exposure.

Methods

Study Design and Participants

The project uses data from the Environment and Reproductive Health (EARTH) study, a prospective cohort study designed to evaluate environmental and nutritional determinants of fertility among couples presenting for infertility treatment and evaluation at the Massachusetts General Hospital Fertility Center (2005-2019). In brief, all women 18 to 45 years at enrollment were eligible to participate and approximately 60% of those contacted by the research nurses participated in the study. At enrollment, height and weight were measured by trained research study staff to calculate body mass index (BMI (kg/m^2)), and data on demographics, medical and

reproductive history, and lifestyle characteristics were collected via questionnaire. Women were then followed prospectively through their ART cycles until failure or live birth. The EARTH study was approved by the Human Studies Institutional Review Boards of the MGH and the Harvard T.H. Chan School of Public Health. All study participants signed an informed consent after the study procedures were explained by research study staff.

Since the metabolomics sub-study was originally designed to evaluate air pollution exposure, 345 women with complete air pollution data who underwent a fresh, autologous ART cycle between 2005 and 2015 were initially eligible (Gaskins et al. 2019a). We then randomly selected 200 women, due to budgetary constraints, to have their stored serum samples sent for metabolomics analysis. As described previously (Gaskins et al. 2021), there was little difference in demographic or ART cycle characteristics between the women who were and were not included in the sub-study. Of the 200 women with metabolomics data, 184 women (92%) had a urine sample (analyzed for phthalate metabolites) that was collected on the same day as their serum sample.

Phthalate Assessment

All the women in our study underwent a fresh assisted reproduction technology (ART) cycle where they provided up to two urine samples during controlled ovarian stimulation. Urine was collected in a sterile polypropylene cup. After measuring specific gravity (SG) using a handheld refractometer (National Instrument Company, Inc.), the urine was divided into aliquots and frozen at -80°C. These samples were then shipped, overnight on dry ice, to the Centers for Disease Control and Prevention for the measurement of urinary phthalate concentrations, including: MEHP, MEHHP, MEOHP, MECPP, MiBP, MBP, MBzP, MEP, MCOP, MCNP, and MCPP.

The analytical approach, based on solid phase extraction coupled with high performance liquid chromatography-isotope dilution tandem mass spectrometry, followed standard QA/QC procedures as previously described (Silva et al., 2007). The limits of detection (LOD) were 0.5-1.2 µg/L (MEHP), 0.2-0.7 µg/L (MEHHP and MEOHP), 0.2-0.6 µg/L (MECPP), 0.1-0.2 µg/L (MCPP), 0.2-0.7 µg/L (MCOP), 0.2-0.6 µg/L (MCNP), 0.2-0.3 µg/L (MBzP), 0.4-0.8 µg/L (MEP), and 0.4-0.6 µg/L (MBP). We calculated the molar sum of DEHP metabolites (∑DEHP) by dividing each metabolite concentration by its molecular weight and then summing: [(MEHP*(1/278.34)) + (MEHHP*(1/294.34)) + (MEOHP*(1/292.33)) + (MECPP*(1/308.33))].

High-Resolution Metabolomics

Women participating in EARTH also provided a non-fasting blood sample via venipuncture at a routine morning appointment (between 7AM to 10AM) during the monitoring phase of controlled ovarian stimulation. Serum was centrifuged, aliquoted, and stored at -20°C initially before being transferred to Harvard for storage at -80°C. These samples were shipped on dry ice to Emory University where metabolomics analyses were conducted using established protocols. Samples were treated with two volumes of acetonitrile for analysis in triplicate, conducted via liquid chromatography coupled with high-resolution mass spectrometry (Dionex Ultimate 3000 NANO; Thermo Orbitrap Fusion). Both HILIC positive and C18 negative chromatography columns were used to maximize the scope of this analysis. The inclusion of two reference plasma samples, NIST 1950 and pooled human plasma purchased from Equitech Bio ensured normalization, and was used for batch evaluation, retention time alignment, and post hoc quantification.

Conversion of raw data files to .mzML files using Proteowizard allowed for extraction of metabolic features using apLCMS and XMSanalyzer. This included peak detection, noise filtering, m/z and retention time alignment, feature quantification, and data quality filtering (Chambers et al., 2012; Uppal et al., 2013; Yu et al., 2009). Mass to charge ratio (m/z), retention time, and ion intensity were noted for each metabolic feature detected. Quality control restrictions for continuing the analysis included only features detected in >10% of samples and with a median coefficient of variation <30% and Pearson correlation >0.7. The median intensities for replicate features was log transformed for the next analysis steps.

Statistical Analysis

Urinary phthalate metabolite concentrations below the LOD were replaced with a value equal to the LOD/√2 (Hornung and Reed 1990). To adjust for urinary dilution, the following formula was used: Pc = P[(1.015 - 1)/SG - 1], where Pc is the SG-corrected phthalate metabolite concentration (µg/L), P is the measured phthalate metabolite concentration (µg/L), and 1.015 is the mean (and median) SG level in the study population (Smith et al., 2012). We used SG-corrected phthalate metabolite concentrations in all analyses.

To investigate the relationship between urinary phthalate metabolites and the composition of the serum metabolome, multivariable linear regression models were used, adjusted for age, BMI, smoking status, education, protocol, race, and infertility diagnosis. Separate models were conducted for each metabolic feature detected in each chromatography chrome (i.e., serum C18 column with negative ESI and HILIC column with positive ESI). Multiple comparison correction was conducted using the Benjamini-Hochberg false discovery rate (FDR_{B-H}) procedure, a widely used procedure in MWAS studies, at a 5% false positive threshold. A

pathway enrichment analysis was conducted utilizing mummichog (v. 1.0.10), a bioinformatics platform that infers and categorizes functional biological activity directly from mass spectrometry output, without prior metabolite validation. We selected metabolic features at raw p-values <-0.05 for pathway analysis. Pathways were classified using the Human Metabolome Database (Wishart et al., 2022).

Results

The women in our analysis were majority White (85%), highly educated (91% had at least a college degree), non-smokers (97%) (**Table 1**). The mean (standard deviation) age and BMI was 34.7 (3.8) years and 23.9 (4.7) kg/m^2, respectively. There was a similar distribution of male (28%), female (33%), and unexplained (39%) infertility diagnoses at entry into the cohort. Women in the highest quartile of urinary ∑DEHP concentrations were less likely to have a graduate degree and more likely to be White and have a female factor infertility diagnosis compared to women in the lowest quartile. Age, BMI, smoking status, and controlled ovarian stimulation treatment protocol were similar across urinary ∑DEHP quartiles. The detection rate and distribution of each urinary phthalate metabolite or molar sum concentration in our population is shown in **Table 2**. The median concentration of ∑DEHP metabolites was 0.14 µmol/L (IQR 0.08-0.26). The lowest median phthalate concentration was MEHP at 2.34 µg/L (IQR 1.22-5.11). The highest median phthalate concentration was MEP at 36.79 µg/L (IQR 16.51-115.19).

A total of 23,771 metabolic features were detected in serum- 10,803 features in the C18 negative column and 12,968 features in the HILIC positive column. While hundreds of serum metabolic features were associated with each urinary phthalate metabolite or molar sum

concentration at a raw $p<0.05$, only a handful of metabolic features remained statistically significant at a raw $p<0.005$ and after FDR correction (**Table 3**). Since these more stringent p-value thresholds did not result in enough metabolites to use as inputs into mummichog, we selected a significance threshold of raw $p<0.05$ for the pathway analysis. Across both columns, a range of 1000 (for MEHP) to 1579 (for MCOP) serum metabolic features were associated with the urinary phthalate concentrations at a significance level of $p<0.05$.

Metabolic pathway enrichment analysis revealed 50 pathways in the C18 negative column and 50 pathways in the HILIC positive column that were significantly associated with at least one of the 11 urinary phthalate metabolites or ∑DEHP concentrations (**Figure 1 and 2**). While there were a handful of overlapping pathways, some distinct patterns emerged. For example, in the C18 negative column, glycosylphosphatidylinositol(GPI)-anchor biosynthesis, nitrogen metabolism, and vitamin B3 metabolism were uniquely associated with urinary ∑DEHP and its metabolites (MECPP, MEHHP, MEHP, and MEOHP). In the HILIC positive column, prostaglandin formation from arachidonate, linoleate metabolism, and leukotriene metabolism were pathways associated with ∑DEHP and at least two of its metabolites. The ascorbate (vitamin C) and aldarate metabolism pathway was associated with ∑DEHP and its metabolites in both the C18 negative and HILIC positive columns.

In regards to the other non-DEHP phthalate metabolites, common pathways identified included aspartate and asparagine metabolism, which was associated with MCOP, MCPP, MEP, and MiBP exposures, and methionine and cysteine metabolism, which was associated with MBP, MBZP, MCPP, and MiBP exposures in the C18 negative column. Across all phthalate exposures,

the most common types of pathways involved lipid metabolism, amino acid metabolism, oxidative stress, or inflammation pathways.

Discussion

In our study of 184 women, we found that preconception urinary phthalate metabolite concentrations were associated with hundreds to thousands of metabolic features in the serum. Metabolic pathways associated with these features were most commonly lipid metabolism, amino acid metabolism, oxidative stress, and inflammation. Our findings provide insight into potential molecular mechanisms in women that may underlie the association between phthalate exposure and lower fertility in women.

Our main results are in line with three previous studies examining the association between urinary phthalate metabolites and the serum or plasma metabolome. For example, several studies showed that as phthalate exposure increased in their participants, common trends were upregulation of lipid biosynthesis, higher serum lipid concentrations, and evidence of inflammation and oxidative stress (Duan et al., 2021, Zhou et al., 2018, Klöting et al., 2015). Specifically, a case-control study that included 60 type 2 diabetes cases and 60 age-, sex-, and BMI-matched controls, Duan et. al found that common pathways associated with ∑DEHP exposure were galactose metabolism, B vitamin pathways, pyrimidine metabolism, and amino acid metabolism (Duan et al., 2021). They listed arginine and alanine metabolism and aspartate and glutamate metabolism as the most significant amino acid pathways. All of these pathways were found to be significantly associated with at least one or more phthalates in our study. The authors took these pathways together as signs of inflammation or oxidative stress in their participants. Similarly, a 2018 study, which utilized targeted metabolomics in plasma and urine

samples of 115 pregnant women at 26 weeks gestation, noted that increased urinary phthalate concentrations were associated with an increase in lipid, hormone, and nucleic acid metabolism. This study also classified such metabolic changes as signs of inflammation or oxidative stress (Zhou et al., 2018). Our results also largely coincide with a 2021 study, which focused on 73 pregnant women and collected samples in early and late pregnancy. Out of their list of significant pathways associated with at least four phthalate exposures, 10 out of the 13 early pregnancy pathways and 11 out of the 18 late pregnancy pathways matched to the pathways identified in our study. The majority of the overlapping pathways were amino acid, galactose, vitamin and pyrimidine metabolism pathways in early pregnancy and carbohydrate, lipid, or amino sugar metabolism pathways in later pregnancy (Zhang et al., 2021). In summary, the findings of our study are consistent with those of other groups. Despite key differences between studies in terms of study design, sample population, and metabolomics analysis, the past literature provides strong evidence for the association of phthalate exposures with metabolic changes that are indicative of oxidative stress and inflammation.

Most significant pathways identified in this study were related to amino acid metabolism, lipid metabolism, or vitamin pathways. Other notable pathways include TCA cycle pathway, pyrimidine metabolism, and activation of arachidonic acid. These metabolic changes taken together are indicators of oxidative stress. Per recent literature, oxidative stress and inflammation may negatively impact pregnancy or reproductive outcomes (Betteridge et al., 2000; Mullarky et al., 2015; Zhang et al., 2021). This connects our metabolomics results to potential mechanisms for adverse reproductive outcomes. To elaborate, a review of oxidative stress and pregnancy pathologies cites that oxidative stress has been implicated with fertility

issues and adverse maternal and fetal health outcomes at all stage of pregnancy. However, the role of oxidative stress in these outcomes is not yet fully understood and varies based on individual maternal health (Pereira et al., 2014). In vitro experiments conducted in 2013 showed an increase in markers of oxidative stress in human placental cells linked to increased exposure to MEHP, an active metabolite of DEHP (Tetz et al., 2013). A 2015 nested case-control study suggested an association between oxidative stress and increased preterm birth among 482 mothers in a prospective birth cohort (Ferguson et al., 2015a). This group then published a study using the same cohort of women which showed that an increase in urinary phthalate metabolites was associated with an increase in oxidative stress biomarkers. Considering these analyses together provides a potential link between phthalate exposure, oxidative stress biomarkers, and preterm birth (Ferguson et al., 2015B).

One interesting finding in our analysis was that the DEHP metabolites and their combined molar sum tended to have a unique metabolic fingerprint compared to the other phthalate metabolites. Key pathways associated with DEHP exposure were glycosylphosphatidylinositol (GPI)-anchor biosynthesis, nitrogen metabolism, and vitamin B3 (nicotinate and nicotinamide) metabolism in the C18 negative column as well as prostaglandin formation from arachidonate, linoleate metabolism, and leukotriene metabolism in the HILIC positive column and the ascorbate (vitamin C) and aldarate metabolism pathway across both columns. This was not unexpected, as animal and in vivo studies have demonstrated that phthalates likely have differential biological effects with some being more or less toxic. In past studies, DEHP has been one of the most common phthalate exposures linked to adverse reproductive, obstetric, and birth outcomes (Gray et al., 2006; Hauser et al., 2015). Further research on the unique pathways

associated with DEHP exposures in this and other studies could generate greater insight into the processes which link DEHP exposure to adverse reproductive outcomes.

While the metabolomics analyses of the women in the EARTH cohort provides valuable insight into the biochemical mechanisms that may mediate the downstream health effects of phthalate exposure, there are limitations to take into account. Conducting multiple regression analyses with 12 exposures and over 10,000 metabolic features poses a risk for false positives and type 1 error. To reduce this, multiple levels of significance were considered throughout the study. For pathway analysis, a raw level of $P<0.05$ was selected to ensure there were sufficient numbers of metabolites to obtain meaningful results. This was justified by the small and exploratory nature of this study; however, it does increase the risk of chance findings. Another limitation to note is that women undergoing infertility treatment may not be generalizable to all reproductive aged women. The women in our cohort were majority White and of high socioeconomic status, potentially limiting the applicability of our findings to other race/ethnicities and women of lower socioeconomic status. Our median phthalate concentrations were similar to those of the 2007-2012 NHANES study, which measured phthalate concentrations in urine samples from adults ages 18-80, which increases the generalizability of our findings. Their results for weighted geometric mean concentrations of MEHP, MEHHP, and MEOHP were 2.1, 12.5, and 7.5 μg/L, respectively. Our findings for these phthalates were 2.34, 12.28, and 8.1 μg/L. One concern we had is that phthalates have short half-lives in the body and the snapshot concentrations in the urine sample data may not be representative of overall or lifetime exposure to these chemicals or exposure during other critical time periods (Hoppin et al., 2002). We encourage future research with greater cohort

diversity and multiple phthalate assessments to continue to explore the association between phthalates and the serum metabolome. While we were able to account for a wide variety of lifestyle characteristics, since this was an observational study, there still remains the possibility of residual confounding. Finally, the use of non-fasting blood samples may have had an impact on the metabolomics results. To minimize this potential impact, we applied a comprehensive metabolomics workflow, which we and others have successfully applied to the analysis of non-fasting samples, using pooled standards and internal references.

In conclusion, we have demonstrated through untargeted high-resolution metabolomics and pathway analysis that phthalate exposures are associated with lipid metabolism, amino acid metabolism, vitamin pathways and oxidative stress pathways. Taken together with previous literature, our findings suggest that these biochemical mechanisms may be mediating the observed associations between phthalate exposures and lower fertility in women.

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Table 1. Demographic and reproductive characteristics of 184 women in the Environment and Reproductive Health Study by quartiles of urinary ∑ DEHP concentration.

			Quartile of ∑ DEHP			
		Total	Q1	Q2	Q3	Q4
	Number of Women	184	46	46	46	46
Age	Mean (SD)	34.7 (3.8)	34.2 (4.1)	34.8 (3.9)	34.3 (3.7)	35.5 (3.7)
	Min-Max	21-43	28-42	27-43	27-42	21-42
BMI	Mean (SD)	23.9 (4.7)	23.8 (4.6)	24.0 (5.0)	23.8 (4.3)	24.2 (4.9)
	Min-Max	16.1-45.8	16.7-45.8	16.5-38.9	16.1-35.5	17.5-37.3
Education	High school/some college (%)	8.7	8.7	13	6.5	6.67
	College graduate (%)	31.2	19.6	30.4	34.8	40
	Graduate degree (%)	60.1	71.7	56.5	58.7	53.3
Smoking Status	Never (%)	72.8	82.6	56.5	84.8	67.4
	Past (%)	24.5	17.4	39.1	15.2	26.1
	Current (%)	2.7	0	4.4	0	6.5
Protocol	Antagonist (%)	12.5	8.7	10.9	19.6	10.9
	Flare (%)	11.4	13	10.9	6.5	15.2
	Luteal phase agonist (%)	76.1	78.3	78.2	73.9	73.9
Race	Caucasian (%)	84.8	67.4	87	91.3	93.5
	Black/African American (%)	1.6	6.5	0	0	4.3
	Asian (%)	9.8	21.7	6.5	6.5	2.2
	Other (%)	3.8	4.4	6.5	2.2	0
Infertility Diagnosis	Male factor (%)	28.3	37	23.9	23.9	28.2
	Female Factor (%)	33.1	28.2	34.8	26.1	43.5
	Unexplained (%)	38.6	34.8	41.3	50	28.3

Table 2. Distribution of urinary phthalate metabolite concentrations (metabolite or molar sum) measured among 184 women in the Environment and Reproductive Health Study.

Phthalate	Units	LOD	% Detect	Min	SG-Adjusted Median (IQR)	Max
∑DEHP metabolites	µmol/L	--	--	0.02	0.14 (0.08-0.26)	15.98
MEHP	µg/L	0.5-1.2	77%	0.27	2.34 (1.22-5.11)	394.29
MEHHP	µg/L	0.2-0.7	99%	0.50	12.28 (6.05-23.08)	1264.29
MEOHP	µg/L	0.2-0.7	99%	0.65	8.21 (4.30-15.31)	1061.79
MECPP	µg/L	0.2-0.6	100%	3.00	18.51 (11.21-37.41)	2046.43
MEP	µg/L	0.4-0.8	100%	2.86	36.79 (16.51-115.19)	3623.40
MBP	µg/L	0.4-0.6	98%	0.71	13.72 (7.43-23.90)	169.00
MCPP	µg/L	0.1-0.2	95%	0.35	3.32 (1.85-9.81)	160.31
MiBP	µg/L	0.2-0.3	97%	0.53	8.87 (4.50-16.71)	95.53
MBzP	µg/L	0.2-0.3	94%	0.14	3.87 (1.79-7.51)	172.13
MCOP*	µg/L	0.2-0.7	100%	1.88	34.45 (10.56-72.96)	780.00
MCNP*	µg/L	0.2-0.6	97%	0.42	3.83 (2.19-7.58)	163.04

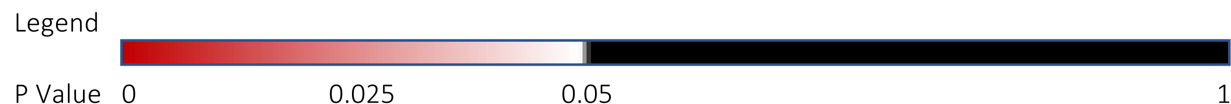
Abbreviations: IQR, interquartile range; LOD, limit of detection; min, minimum; max, maximum; SG, specific gravity.

*14 women did not have their urine samples analyzed for MCOP or MCNP

Table 3. Significant metabolic features associated with each urinary phthalate metabolite or molar sum concentration among 184 women in the Environment and Reproductive Health Study.

	C18 Negative (N = 10803)					HILIC Positive (N = 12968)				
	Raw P<0.05	Raw P <0.005	Raw P <0.0005	FDR P<0.20	FDR P<0.05	Raw P<0.05	Raw P<0.005	Raw P<0.0005	FDR P<0.20	FDR P<0.05
∑DEHP	454	82	28	34	15	596	142	67	84	56
MEHP	431	82	32	34	18	569	136	64	78	51
MEHHP	485	86	30	31	14	614	135	62	79	51
MEOHP	431	78	29	35	15	600	141	67	84	57
MECPP	453	82	28	30	15	602	145	64	81	59
MEP	496	102	27	30	12	887	116	27	26	13
MBP	690	112	16	16	1	761	131	27	27	14
MCPP	543	99	23	20	5	815	114	40	42	16
MIBP	410	48	8	2	1	595	71	14	5	3
MBZP	730	172	43	98	6	582	89	24	19	8
MCOP	772	108	23	20	7	807	121	22	19	3
MCNP	609	82	23	22	4	523	66	15	6	3

Figure 1. Metabolic pathways significantly associated with ∑DEHP and MECPP, MEHHP, MEHP, and MEOHP (**Panel A**), MBP (**Panel B**), MBZP (**Panel C**), MCNP (**Panel D**), MCOP (**Panel E**), MCPP (**Panel F**), MEP (**Panel G**), and MiBP (**Panel H**) concentrations.



A.

Classification	Pathway	C18 Negative Total Metabolites	DEHP	MECPP	MEHHP	MEHP	MEOHP	HILIC Positive Total Metabolites	DEHP	MECPP	MEHHP	MEHP	MEOHP
Oxidative stress	Nitrogen metabolism	6	67%	50%	67%	50%	67%	7					
Amino acid metabolism	Chondroitin sulfate degradation	8	38%	38%	63%		38%	7					
Oxidative stress	Heparan sulfate degradation	9	34%	34%	56%		34%	8					
Biosynthesis	Hyaluronan Metabolism	6	34%	34%	34%	34%	34%	3					
Carbohydrate metabolism	Pentose and Glucuronate Interconversions	14	29%	29%	36%		22%	12					
Amino acid metabolism	Aspartate and asparagine metabolism	63	26%	26%	24%	23%	24%	86					
Amino acid metabolism	Arginine and Proline Metabolism	36	25%	23%	23%	20%	23%	39					
Vitamin pathway	Vitamin B3 (nicotinate and nicotinamide) metabolism	21	24%	20%	24%	20%	24%	23					
Amino acid metabolism	Propanoate metabolism	13	24%	24%		24%	24%	5					
Amino acid metabolism	Alanine and Aspartate Metabolism	22	23%	23%	23%	19%	23%	18					
DNA metabolism	Pyrimidine metabolism	59	21%	19%	17%	14%	14%	5					
Vitamin pathway	Ascorbate (Vitamin C) and Aldarate Metabolism	35	20%	20%	20%	18%	18%	14	29%	29%			
Amino acid metabolism	Aminosugars metabolism	20	20%	20%	20%	20%	20%	16					
Inflammation	Arachidonic acid metabolism	29						36	23%	23%		20%	
Lipid metabolism	Prostaglandin formation from arachidonate	39						27	30%	30%	26%		
Lipid metabolism	Leukotriene metabolism	31						30	24%	24%			
Lipid metabolism	Bile acid biosynthesis	34						36	23%				25%
Lipid metabolism	Carnitine shuttle	21						33	22%	22%			
Lipid metabolism	Linoleate metabolism	37						46	20%	20%	22%	20%	

B.

Classification	Pathway	C18 Negative Total Metabolites	MBP	HILIC Positive Total Metabolites	MBP
Oxidative stress	Ascorbate (Vitamin C) and Aldarate Metabolism	35	23%	14	
Amino acid metabolism	Methionine and cysteine metabolism	30	24%	50	
Vitamin pathway	Vitamin B9 (folate) metabolism	12	34%	8	
Lipid metabolism	Fatty acid activation	23	27%	34	
Lipid metabolism	Fatty acid oxidation	2	100%	9	
Lipid metabolism	Mono-unsaturated fatty acid beta-oxidation	2	100%	2	
Lipid metabolism	Bile acid biosynthesis	34		36	31%
Lipid metabolism	Carnitine shuttle	21		33	31%
Endocrine disruption	Androgen and estrogen biosynthesis and metabolism	35		40	23%
Endocrine disruption	C21-steroid hormone biosynthesis and metabolism	47		78	21%
Lipid metabolism	Squalene and cholesterol biosynthesis	10		18	45%

C.

Classification	Pathway	C18 Negative Total Metabolites	MBZP	HILIC Positive Total Metabolites	MBZP
Amino acid metabolism	Methionine and cysteine metabolism	30	27%	50	20%
Inflammation	Histidine metabolism	31	20%	34	
Inflammation	Arachidonic acid metabolism	29		36	23%
Lipid metabolism	Linoleate metabolism	37		46	20%
Lipid metabolism	De novo fatty acid biosynthesis	30		34	21%

D.

Classification	Pathway	C18 Negative Total Metabolites	C18 Negative MCNP	HILIC Positive Total Metabolites	HILIC Positive MCNP
DNA metabolism	Pyrimidine metabolism	59	19%	53	
Lipid metabolism	Saturated fatty acids beta-oxidation	3	67%	7	
Vitamin pathway	Vitamin H (biotin) metabolism	4	50%	2	
Lipid metabolism	Carnitine shuttle	21		33	22%
Amino acid metabolism	Aminosugars metabolism	20		16	25%
Vitamin pathway	Vitamin B6 (pyridoxine) metabolism	12		17	24%
Drug metabolism	Drug metabolism - other enzymes	18		22	23%
Amino acid metabolism	N-Glycan Degradation	9		11	28%
Amino acid metabolism	Glutamate metabolism	14		17	24%
Lipid metabolism	Fatty acid oxidation, peroxisome	4		4	50%

E.

Classification	Pathway	C18 Negative Total Metabolites	MCOP	HILIC Positive Total Metabolites	MCOP
Carbohydrate metabolism	Pentose and Glucuronate Interconversions	14	43%	12	
Amino acid metabolism	Aspartate and asparagine metabolism	63	26%	86	
Amino acid metabolism	Arginine and Proline Metabolism	36	37%	39	
Lipid metabolism	Arachidonic acid metabolism	29	35%	36	
Lipid metabolism	Glycosphingolipid metabolism	21	34%	30	
Energy metabolism	TCA cycle	27	30%	14	
Amino acid metabolism	N-Glycan Degradation	9	45%	11	
Amino acid metabolism	Lysine metabolism	24	34%	38	
Amino acid metabolism	Linoleate metabolism	37	33%	46	
Heme biosynthesis	Porphyrin metabolism	22	37%	13	
Endocrine disruption	C21-steroid hormone biosynthesis and metabolism	47	26%	78	
Lipid metabolism	Bile acid biosynthesis	34	30%	36	
Lipid metabolism	Saturated fatty acids beta-oxidation	3	67%	7	
Lipid metabolism	Omega-3 fatty acid metabolism	6		13	39%
Lipid metabolism	De novo fatty acid biosynthesis	30		34	33%
Lipid metabolism	Fatty Acid Metabolism	18		23	40%
Lipid metabolism	Fatty acid activation	23		34	30%
Enzyme cofactor metabolism	Biopterin metabolism	21		15	34%
Carbohydrate metabolism	Fructose and mannose metabolism	16		20	30%
Lipid metabolism	Di-unsaturated fatty acid beta-oxidation	4		5	60%
Lipid metabolism	Polyunsaturated fatty acid biosynthesis	3		3	67%

F.

Classification	Pathway	C18 Negative Total Metabolites	C18 Negative MCPP	HILIC Positive Total Metabolites	HILIC Positive MCPP
Amino acid metabolism	Chondroitin sulfate degradation	8	38%	7	43%
Oxidative stress	Heparan sulfate degradation	9	34%	8	38%
Amino acid metabolism	Aspartate and asparagine metabolism	63	21%	86	
Amino acid metabolism	Arginine and Proline Metabolism	36	31%	39	
Amino acid metabolism	Alanine and Aspartate Metabolism	22	28%	18	
Oxidative stress	Ascorbate (Vitamin C) and Aldarate Metabolism	35	26%	14	36%
Amino acid metabolism	Methionine and cysteine metabolism	30	30%	50	
Amino acid metabolism	Beta-Alanine metabolism	13	31%	20	30%
Carbohydrate metabolism	Hexose phosphorylation	20	30%	16	
Amino acid metabolism	Glutamate metabolism	14	29%	17	
Carbohydrate metabolism	Glycolysis and Gluconeogenesis	24	25%	21	29%
Energy metabolism	TCA cycle	27	26%	14	
Lipid metabolism	Prostaglandin formation from arachidonate	39	21%	27	
Lipid metabolism	Porphyrin metabolism	22	32%	13	
Amino acid metabolism	Tryptophan metabolism	86	20%	107	
Vitamin pathway	Vitamin B1 (thiamin) metabolism	8	38%	7	
Biosynthesis	Hyaluronan Metabolism	6		3	67%
Vitamin pathway	Vitamin B5 - CoA biosynthesis from pantothenate	6		7	43%
Vitamin pathway	Vitamin B9 (folate) metabolism	12		8	38%
Carbohydrate metabolism	Galactose metabolism	21		28	25%
Lipid metabolism	Phosphatidylinositol phosphate metabolism	19		22	28%
Amino acid metabolism	Glycine, serine, alanine and threonine metabolism	43		61	23%
Oxidative stress	Pyruvate Metabolism	10		6	67%

G.

Classification	Pathway	C18 Negative Total Metabolites	C18 Negative MEP	HILIC Positive Total Metabolites	HILIC Positive MEP
Amino acid metabolism	Aspartate and asparagine metabolism	63	16%	86	
Amino acid metabolism	Arginine and Proline Metabolism	36	20%	39	
Oxidative stress	Pentose phosphate pathway	25	24%	28	
Lipid metabolism	Phosphatidylinositol phosphate metabolism	19	22%	22	
Drug metabolism	Drug metabolism - cytochrome P450	25	20%	39	
Lipid metabolism	Leukotriene metabolism	31		30	24%
Amino acid metabolism	Methionine and cysteine metabolism	30		50	22%
Lipid metabolism	Fatty acid oxidation	2		9	34%
Amino acid metabolism	Selenoamino acid metabolism	13		20	25%
Oxidative stress	Phytanic acid peroxisomal oxidation	6		6	50%
Lipid metabolism	Mono-unsaturated fatty acid beta-oxidation	2		2	100%
Inflammation	Putative anti-Inflammatory metabolites formation from EPA	7		7	43%

H.

Classification	Pathway	C18 Negative Total Metabolites	C18 Negative MiBP	HILIC Positive Total Metabolites	HILIC Positive MiBP
Amino acid metabolism	Aspartate and asparagine metabolism	63	18%	86	
DNA metabolism	Pyrimidine metabolism	59	17%	53	
Aminosugars metabolism	Aminosugars metabolism	20	25%	NA	
Oxidative stress	Glutathione Metabolism	10	30%	16	
Amino acid metabolism	Methionine and cysteine metabolism	30	24%	50	
Inflammation	Histidine metabolism	31	26%	34	
Amino acid metabolism	Tryptophan metabolism	86		107	15%
Amino acid metabolism	Butanoate metabolism	26		30	24%
Energy metabolism	Electron transport chain	2		4	50%
Amino acid metabolism	Valine, leucine and isoleucine degradation	25		27	26%
Lipid metabolism	Propanoate metabolism	13		5	40%
Energy metabolism	TCA cycle	27		14	36%
Lipid metabolism	Fatty acid oxidation, peroxisome	4		4	50%