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Urinary Paraben Concentrations and Associations with the Periconceptional Urinary Metabolome: Untargeted and Targeted Metabolomics Analyses of Participants from the Early Pregnancy Study

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Background: Parabens, found in everyday items from personal care products to foods, are chemicals with endocrine-disrupting activity, which has been shown to influence reproductive function.

Objectives: This study investigated whether urinary concentrations of methylparaben, propylparaben, or butylparaben were associated with the urinary metabolome during the periconceptional period, a critical window for female reproductive function. Changes to the periconceptional urinary metabolome could provide insights into the mechanisms by which parabens could impact fertility.

Methods: Urinary paraben concentrations were measured in paired pre- and postconception urine samples from 42 participants in the Early Pregnancy Study, a prospective cohort of 221 women attempting to conceive. We performed untargeted and targeted metabolomics analyses using ultrahigh-performance liquid chromatography quadrupole time-of-flight mass spectrometry. We used principal component analysis, orthogonal partial least-squares discriminant analysis, and permutation testing, coupled with univariate statistical analyses, to find metabolites associated with paraben concentration at the two time points. Potential confounders were identified with a directed acyclic graph and used to adjust results with multivariable linear regression. Metabolites were identified using fragmentation data.

Results: Seven metabolites were associated with paraben concentration (variable importance to projection score >1, false discovery rate–corrected q-value <0.1). We identified four diet-related metabolites to the Metabolomics Standards Initiative (MSI) certainty of identification level 2, including metabolites from smoke flavoring, grapes, and olive oil. One metabolite was identified to the class level only (MSI level 3). Two metabolites were unidentified (MSI level 4). After adjustment, three metabolites remained associated with methylparaben and propylparaben, two of which were diet-related. No metabolomic markers of endocrine disruption were associated with paraben concentrations.

Discussion: This study identified novel relationships between urinary paraben concentrations and diet-related metabolites but not with metabolites on endocrine-disrupting pathways, as hypothesized. It demonstrates the feasibility of integrating untargeted metabolomics data with environmental exposure information and epidemiological adjustment for confounders. The findings underscore a potentially important connection between diet and paraben exposure, with applications to nutritional epidemiology and dietary exposure assessment. https://doi.org/10.1289/EHP12125

Introduction

Parabens are common antimicrobial preservatives used in personal care products, cosmetics, foods, and medications since the 1930s.[1–4] Exposure typically occurs through ingestion of foods and medications and dermal application of personal care products, with detectable urinary concentrations present in populations worldwide.[5–14] *In vitro* and *in vivo* studies suggest parabens are endocrine-disrupting chemicals (EDCs) that can influence reproductive function through estrogenic mechanisms,[1] including via drug metabolizing enzymes (DMEs), which are critical to both detoxification processes and hormone metabolism (Figure S1).[15–17] Parabens can bind to peroxisome proliferator–activated receptors (PPARs), a group of nuclear receptors that play a role in mediating the expression of DMEs.[18–20] When DME function impacts hormone metabolism, shifts in the bioavailability of endogenous hormones and metabolite levels can be reflected in changes to the metabolome.[21] Although there are only limited epidemiological studies of paraben exposure and human female reproductive function, they suggest reduced fecundability associated with methylparaben exposure,[22] decreased menstrual cycle length with butylparaben exposure,[23] and diminished ovarian reserve in a fertility treatment population with propylparaben exposure.[24] Another study reported no association between paraben exposure and *in vitro* fertilization (IVF) treatment outcomes.[25] Because subfertility can have major physical, emotional, and financial consequences, the identification of reproductive toxicants is needed.[26]

Metabolomics is a sensitive tool for characterizing metabolite changes in response to environmental exposures, with the potential to illuminate mechanisms of toxicity and biological responses to exposures.[21] To our knowledge, three studies have examined associations between human paraben exposure and the urinary[27,28] and serum metabolomes.[29] These studies suggest that paraben exposure may influence nuclear receptor pathways, energy metabolism, and the urea pathway.[27–29] None of these studies focused on the periconceptional period, a critical window for female reproductive function.[26,30,31]

To better understand the potential reproductive effects of paraben exposure, we used untargeted and targeted metabolomics analyses to examine associations between periconceptional exposure to methylparaben, propylparaben, and butylparaben and the urinary metabolome in a cohort of women attempting natural conception. Because parabens could modulate currently unidentified metabolic pathways, we used a hypothesis-generating, untargeted approach to evaluate the effects of paraben exposure, paired with targeted analysis for metabolite identification. We also conducted

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a targeted assessment of phase II endogenous metabolites previously identified as sensitive to endocrine disruptor exposure, along with other metabolites specifically identified in relation to paraben exposure.[32]

Methods

Study Population

We used urine samples from 42 participants in the Early Pregnancy Study (EPS), a prospective cohort study of 221 women in North Carolina attempting pregnancy who had no known fertility problems.[33,34] In brief, from 1982 to 1986, participants were enrolled when they stopped using contraception. They collected daily first morning urine samples for 6 months if they did not conceive or for 8 wk after the last menstrual period if they became pregnant. Participants were interviewed on enrollment, and they kept daily logs of menstrual bleeding and sexual intercourse.[33] For this study, we selected 42 of the 221 participants who were 27–31 y old, had a body mass index (BMI; kg/m^2) <25, were White, and whose pregnancies ended with a singleton live birth (22 male, 20 female). These inclusion criteria aimed to create a homogeneous population to minimize the interindividual variability of metabolomic profiles. Sample selection was carried out by first identifying the subset of all EPS participants meeting our inclusion criteria with sufficient sample volume available for analysis (>0.5 mL). Then, in order of study enrollment, we selected samples from the first 22 pregnancies resulting in a male baby and the first 20 pregnancies resulting in a female baby. The sample size was chosen to enable metabolomic analysis on a single 96-well plate to minimize potential interbatch variability. The Yale University institutional review board determined the analyses for this study were not human subjects research (ID 2000022845).

Urine Specimen Collection, Storage, and Pooling

Daily urine specimens were collected in preservative-free polypropylene jars, stored in home freezers for up to 2 wk, and transported on ice to a central freezer at the National Institute of Environmental Health Sciences (NIEHS). Specimens were stored at −20°C until initial study analyses were complete and kept at −80°C for long-term storage.[35] As part of a previous research study, urine specimens from each menstrual cycle were pooled within individual women and analyzed for multiple environmental biomarkers of exposures, including parabens.[35] The pooling strategy combined equal aliquots from three specimens collected ∼1 wk apart on consecutive Mondays, if available, across a menstrual cycle beginning after the end of menstrual bleeding. Most daily specimens used for pooling were collected on a Monday ($n = 173/252$, 69%), but if a Monday sample was unavailable, the nearest available day was used (Table S1).[35] Because parabens are rapidly metabolized, with a half-life of 20–90 min and high intraindividual variability, pooled rather than single urine samples were analyzed to better represent exposure across a menstrual cycle.[5,36–41]

Conception Cycle and Early Pregnancy Samples

We analyzed a total of 84 paired conception cycle and early pregnancy pooled samples from 42 participants. "Conception cycle" samples contain three specimens from the menstrual cycle in which conception occurred, including two collected after the end of menstrual bleeding and before ovulation, and a third collected after ovulation/conception but before implantation (typically about a week after ovulation). The exact date of ovulation (and by inference, conception) was previously determined by an algorithm using the ratio of estrone-3-glucuronide and pregnanediol-3-glucuronide measured in unpooled, daily urine specimens from across each participant's conception cycle.[42,43] Pregnancy was defined as a human chorionic gonadotropin (hCG) level of >0.025 ng/mL for ≥3 consecutive days, also using daily urine specimens.[34] After identification of pregnancy, the day of implantation was defined as the first day of the pregnancy where hCG was ≥0.01 ng/mL.[44] "Early pregnancy" pooled samples include three postimplantation specimens collected ∼1 wk apart, and covering the time period of 4–6 wk after the last menstrual period, or the first 3 wk of *in utero* development.

Paraben Exposure Assessment

Quantification of parabens in urine. Methylparaben, propylparaben, and butylparaben concentrations (ng/mL) were measured at the U.S. Centers for Disease Control and Prevention (U.S. CDC) in 2010 in pooled urine samples using the high-performance liquid chromatography–isotope-dilution tandem mass spectrometry method reported in Ye et al. (2005).[45] The specific gravity of each sample was measured with an Atago PAL-10S refractometer and used to adjust paraben concentrations to account for varying urinary dilution across samples, as previously described.[46] Paraben measurements for this study were approved by the NIEHS institutional review board, and the analysis of de-identified specimens at the U.S. CDC laboratory was determined not to constitute human subjects research.

Quality control. Ten random individual (nonpooled) quality control (QC) samples were assessed for evidence of contamination by measuring the free and total concentrations of each paraben. Because parabens are generally excreted as urinary conjugates, relatively high percentages of unconjugated or free parabens may indicate contamination during collection, handling, or storage.[38] Contamination is unlikely when the free paraben concentration is <20% of the total amount detected.[38] In all samples, the percentage of free biomarkers was <20% or below the limit of detection (LOD), suggesting systematic contamination was not evident. The methylparaben concentration of one pooled sample was missing due to other quality control issues during quantification and was excluded from analyses.

Concentrations below the LOD. Many butylparaben concentrations were nondetectable [$LOD_{butylparaben} = 0.2$ ng/mL, $n = 46/84$ (55%)], but we did not impute values <LOD.[47] Instead, we *a priori* planned to use a categorical structure to model butylparaben, with the lowest concentration category comprising concentrations <LOD. Methylparaben and propylparaben concentrations were treated continuously in statistical models used to adjust for confounding. For these models, we imputed the concentration of one propylparaben sample that was <LOD [$LOD_{propylparaben} = 0.2$ ng/mL, $n = 1/84$ (1.2%)], with a single imputation using a maximum likelihood procedure assuming a log-normal distribution based on the distribution of phenol measurements >LOD.[47] No methylparaben samples were <LOD ($LOD_{methylparaben} = 1.0$ ng/mL), and because no metabolites were associated with butylparaben concentrations, no adjusted models including butylparaben were constructed.

Paraben exposure categories. The multivariate statistical techniques we used required treating paraben concentrations categorically. Because EDCs can exhibit nonmonotonic dose–response relationships, we included three concentration categories with a stable reference group and maximum contrast between categories.[48,49] For methylparaben and propylparaben, the low concentration category was defined as below the median concentration; the medium concentration category as median to 75th percentile concentration; and the high concentration category as >75th percentile concentration (Table 1). For butylparaben, concentrations <LOD were classified as the low category; LOD to the median as the medium category; and >median concentration

Table 1. Paraben concentration (ng/mL, specific gravity–adjusted) categories stratified by conception cycle or early pregnancy status for 42 participants in the Early Pregnancy Study.

	Conception cycle			Early pregnancy		
Concentration category[a]	Methylparaben[b]	Propylparaben	Butylparaben	Methylparaben	Propylparaben	Butylparaben
Low						
Samples (n)	21	21	22	22	20	22
Concentration	3.9–116.9	0.7–47.9	<LOD	4.9–57.9	0.1–13.9	<LOD
Medium						
Samples (n)	10	10	10	10	12	10
Concentration	117.0–241.9	48.0–124.9	0.3–1.6	58.0–200.9	14.0–89.9	0.1–1.5
High						
Samples (n)	10	11	10	10	10	10
Concentration	242.0–924.0	125.0–691.9	1.7–31.1	201.0–723.1	90.0–640.4	1.6–76.8

Note: LOD, limit of detection.
[a]For methylparaben and propylparaben, the low concentration category includes concentrations <median; the medium category includes concentrations from the median to 75th percentile; and the high category includes concentrations >75th percentile. For butylparaben, the low concentration category is <LOD ($LOD_{butylparaben} = 0.2$ ng/mL); the medium category is below the median concentration of detectable samples; and the high category is above the median concentration of detectable samples.
[b]One conception cycle methylparaben sample is missing due to quality control issues.

as the high category, where the median refers to the median among concentrations >LOD.

Untargeted Metabolomics Analysis

Details of sample preparation and metabolomics analysis for this study have been previously published.[46] In brief, samples were prepared using a water extraction process for hydrophilic interaction chromatography–mass spectrometry (HILIC-MS, negative mode, polar metabolites) and a methanol extraction for reversed-phased liquid chromatography-mass spectrometry (RPLC-MS, positive mode, nonpolar metabolites). A QC based on pooling together equal aliquots from each of the 84 samples was also prepared according to both methods. All samples were analyzed by untargeted ultrahigh-performance liquid chromatography (UHPLC) quadrupole time-of-flight (QToF) mass spectrometry (Xevo-G2-XS-QToFMS; Waters Corporation), with run order randomized.

Untargeted Metabolomics Data Processing

Initial untargeted data processing used ProteoWizard (version 3.0.10158) to convert .raw files to .mzML files (see Supplemental Material, ProteoWizard settings).[50] XCMS (version 3.8)[51] running on R (version 3.4; R Development Core Team) was used for deconvolution and peak alignment, with a K-nearest neighbor imputation of missing values. A total of 18,822 HILIC mode features and 12,794 RPLC mode features were detected. Adjustment for analytical variability was carried out using the MetCleaning R package (version 1.0.0; X. Shen and Z. Zhu) with support vector regression, and metabolite features were specific gravity–adjusted to account for differences in urinary dilution.[52] Data were assessed for normality using the Shapiro-Wilk test, along with the performance of multiple data transformations ($\log_2$, $\log_{10}$, Glog); as all transformations applied still left 20%–30% of features with a nonnormal distribution, we decided to use nonparametric statistical techniques. All features with a relative standard deviation (RSD; standard deviation of the peak area divided by the mean peak area across QC samples) >0.3 were excluded to maximize feature reproducibility, resulting in the retention of 81% (15,158/18,822) of HILIC mode features and 94% (12,023/12,794) of RPLC mode features.[53–56] Finally, all metabolite features were mean-centered and Pareto-scaled (divided by the square root of the feature standard deviation).[57,58]

Targeted Metabolomics Analysis

Metabolite identification. We gathered fragmentation data from the QC sample ~2.5 y after the initial untargeted analysis. Where available, we used commercial standards to check putative metabolite identifications. Standards purchased included 3-(4-hydroxy-3-methoxyphenyl)propionic acid (Sigma-Aldrich, CAS 1135-23-5; putative identification for metabolite 1), ethyl 2,4-dihydroxy-6-methylbenzoate (Sigma-Aldrich, CAS 2524-37-0; putative identification for metabolite 1), argininosuccinic acid disodium salt hydrate (Sigma-Aldrich, CAS 918149-29-8 anhydrous; putative identification for metabolite 2), bergenin (Chromadex, CAS 477-90-7; putative identification for metabolite 2), and lignoceric acid (Sigma-Aldrich, CAS 557-59-5; putative identification for metabolite 3). Fragmentation data were gathered for each of the seven metabolites associated with paraben exposure and for a standard if it was detected using the same liquid chromatography–mass spectrometry (LC-MS) methods and instrument as in the untargeted analysis, with ramping from 10 to 40 eV (see Supplemental Excel File for fragmentation data). Fragmentation patterns were compared with those of commercial standards and data in public libraries using the SIRIUS and CFM-ID (Competitive Fragmentation Modeling-Identification) software.[59–61]

Drug metabolizing enzyme metabolites. We also ran additional standards based on our hypothesis that paraben exposure could alter DME function via nuclear receptor pathways. Because phase II metabolism increases the production of sulfated metabolites in the urine, we examined whether select compounds previously found to be associated with methylparaben exposure in Sprague-Dawley rats were altered in our cohort, including aromatic sulfate conjugates (o-aminophenol sulfate; Santa Cruz Biotechnology, CAS 67845-79-8; catechol sulfate, CAS 4918-96-1; quinol sulfate, CAS 17438-29-8) and sulfonated steroids (pregnenolone sulfate; Sigma-Aldrich, CAS 1247-64-9; 17-hydroxypregnenolone sulfate, Cayman Chemicals, CAS 28901-70-4).[32] Standards were unavailable for catechol sulfate and quinol sulfate, so these compounds were assessed by evaluating MSe data from the QC sample using SIRIUS software.[59]

Statistical Analyses

All statistical analyses were carried out in R (version 4.04; R Development Core Team). (Please contact the authors with inquiries regarding data availability.)

Paraben distribution. We calculated medians and interquartile ranges (IQRs) of urinary paraben concentrations (ng/mL, specific gravity–adjusted) across the cohort (Table 2), stratified by conception cycle or early pregnancy time point and participant characteristics, and compared the EPS cohort paraben concentrations with those of other cohorts of women of reproductive age. We also calculated Spearman correlations between the parabens.

Multivariate analysis. We used principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis

Table 2. Urinary paraben concentrations (ng/mL, specific gravity–adjusted) stratified by participant characteristics for 42 participants in the Early Pregnancy Study.

		Methylparaben		Propylparaben		Butylparaben	
Characteristic	Participants (*n*)	Conception cycle median (IQR)	Early pregnancy median (IQR)	Conception cycle median (IQR)	Early pregnancy median (IQR)	Conception cycle median (IQR)	Early pregnancy median (IQR)
All participants	42	129.1 (25.0, 388.6)	53.2 (25.9, 198.3)	50.8 (9.3, 126.0)	18.5 (6.6, 88.2)	<LOD (<LOD, 1.5)	<LOD (<LOD, 1.0)
Acetaminophen							
No	31	84.0 (25.0, 203.2)	50.8 (23.7, 128.1)	46.3 (8.9, 125.4)	13.9 (5.8, 87.1)	<LOD (<LOD, 1.5)	<LOD (<LOD, 1.5)
Yes	11	178.2 (42.0, 641.1)	108.2 (49.3, 301.6)	89.9 (10.9, 130.5)	22.9 (10.0, 84.9)	0.4 (<LOD, 1.5)	0.4 (<LOD, 0.8)
Aspirin							
No	19	73.6 (20.2, 209.6)	41.2 (18.3, 196.7)	45.5 (7.6, 110.6)	11.0 (4.9, 80.5)	<LOD (<LOD, 0.9)	<LOD (<LOD, 0.6)
Yes	23	129.1 (49.7, 382.3)	86.3 (33.3, 201.5)	62.9 (25.4, 137.8)	35.9 (10.3, 88.1)	0.8 (<LOD, 3.4)	<LOD (<LOD, 2.7)
Antibiotics							
No	35	129.1 (22.7, 388.6)	52.6 (23.7, 202.4)	62.1 (9.6, 132.8)	20.7 (7.8, 94.7)	<LOD (<LOD, 1.4)	<LOD (<LOD, 0.9)
Yes	7	73.6 (63.9, 154.3)	53.9 (29.7, 106.3)	42.7 (17.3, 58.7)	8.2 (5.1, 57.7)	<LOD (<LOD, 3.4)	<LOD (<LOD, 7.1)
Vitamins							
No	21	94.4 (47.8, 216.0)	53.9 (33.0, 250.0)	46.3 (11.9, 115.1)	20.7 (7.9, 89.4)	0.4 (<LOD, 3.3)	0.4 (<LOD, 3.2)
Yes	21	135.5 (21.1, 488.7)	50.8 (21.4, 129.8)	62.1 (9.1, 134.6)	16.3 (6.4, 75.2)	<LOD (<LOD, 1.2)	<LOD (<LOD, 0.9)
Age (y)							
<27	9	327.0 (49.9, 639.1)	108.2 (33.5, 298.7)	114.0 (10.0, 146.0)	98.0 (16.3, 123.2)	<LOD (<LOD, 1.6)	<LOD (<LOD, 0.3)
27–31	22	105.6 (23.6, 177.2)	52.2 (20.3, 220.0)	44.9 (7.6, 95.3)	13.3 (6.8, 68.7)	0.4 (<LOD, 2.1)	0.3 (<LOD, 2.8)
>31	11	67.3 (32.4, 226.9)	46.1 (25.9, 90.3)	46.3 (18.4, 132.8)	20.7 (6.4, 56.5)	<LOD (<LOD, 0.9)	<LOD (<LOD, 1.4)
Body mass index (kg/m^2)							
Low, <18.5	5	73.6 (24.1, 174.4)	41.2 (27.0, 53.9)	42.7 (8.0, 124.0)	13.9 (5.2, 266.3)	<LOD (<LOD, 1.6)	0.3 (<LOD, 4.7)
Normal, 18.5–24.9	37	129.1 (26.4, 290.3)	58.1 (25.9, 200.0)	54.5 (10.0, 131.0)	20.7 (7.7, 84.8)	<LOD (<LOD, 1.5)	<LOD (<LOD, 0.9)
Marijuana							
No	39	105.6 (25.0, 388.6)	52.6 (23.7, 196.7)	54.5 (8.9, 128.8)	16.3 (7.1, 87.1)	<LOD (<LOD, 1.5)	<LOD (<LOD, 1.0)
Yes	3	160.6 (89.1, 167.5)	78.6 (52.3, 400.8)	45.5 (27.8, 84.8)	22.9 (13.9, 331.6)	<LOD (<LOD, 3.9)	<LOD (<LOD, 2.5)
Alcohol (drinks/month)							
0 drinks	7	58.5 (23.2, 266.1)	33.0 (21.1, 163.7)	9.1 (8.0, 83.5)	12.9 (5.7, 123.5)	0.4 (<LOD, 1.3)	<LOD (<LOD, 0.5)
1–16 drinks	22	157.5 (49.7, 476.5)	53.2 (26.2, 186.0)	67.4 (43.4, 123.8)	15.1 (5.0, 53.7)	<LOD (<LOD, 1.2)	<LOD (<LOD, 0.8)
≥17 drinks	13	69.3 (17.5, 169.8)	89.6 (33.5, 193.4)	31.0 (5.5, 124.0)	55.8 (8.8, 121.5)	0.8 (<LOD, 3.9)	0.9 (<LOD, 2.2)
Caffeine (mg/month)							
≤1,920 mg	13	103.2 (23.6, 240.3)	33.5 (20.8, 293.8)	86.5 (8.1, 141.0)	35.9 (3.6, 109.7)	0.4 (<LOD, 1.6)	<LOD (<LOD, 0.7)
1,921–5,999 mg	15	94.4 (42.2, 563.0)	50.8 (29.5, 98.9)	46.3 (18.4, 105.6)	13.8 (7.1, 29.2)	0.8 (<LOD, 2.6)	0.9 (<LOD, 3.3)
≥6,000 mg	14	129.1 (49.7, 174.9)	82.4 (38.3, 203.5)	50.0 (14.1, 108.8)	41.2 (9.6, 85.8)	<LOD (<LOD, 0.3)	<LOD (<LOD, <LOD)
Smoker							
No	40	141.5 (26.0, 339.4)	56.0 (25.9, 201.2)	54.6 (9.8, 127.8)	21.8 (7.4, 89.9)	0.3 (<LOD, 1.6)	<LOD (<LOD, 1.3)
Yes	2	66.9 (41.9, 91.8)	23.7 (18.8, 28.6)	27.6 (14.2, 41.1)	6.8 (5.8, 7.8)	all <LOD	all <LOD
Recent OC use[a]							
No	25	141.5 (27.8, 212.8)	58.1 (25.9, 200.0)	62.1 (10.0, 131.0)	20.7 (5.3, 71.7)	<LOD (<LOD, 1.5)	<LOD (<LOD, 2.0)
Yes	17	105.6 (23.6, 451.7)	50.8 (21.4, 193.4)	47.1 (9.1, 114.0)	13.9 (7.7, 109.7)	<LOD (<LOD, 1.6)	<LOD (<LOD, 0.9)
Education (y)							
Some college, <16	8	141.0 (25.3, 187.6)	39.4 (15.3, 109.0)	50.8 (9.3, 69.0)	11.0 (4.4, 35.1)	<LOD (<LOD, 0.5)	<LOD (<LOD, 0.4)
College, 16	14	69.3 (47.8, 148.0)	56.0 (42.4, 117.2)	34.6 (8.2, 129.8)	32.1 (9.6, 89.7)	0.6 (<LOD, 1.5)	0.2 (<LOD, 1.0)
Graduate school, >16	20	157.5 (20.6, 451.7)	70.9 (20.6, 221.9)	67.4 (25.7, 134.7)	18.5 (7.4, 91.5)	0.3 (<LOD, 2.3)	<LOD (<LOD, 2.3)
Income (USD)							
<$20,000	16	176.3 (50.8, 487.6)	88.0 (25.6, 212.5)	76.4 (22.1, 136.2)	46.8 (6.7, 107.4)	1.2 (<LOD, 3.3)	0.4 (<LOD, 2.0)
$20,000 – $29,999	16	66.3 (17.3, 135.5)	56.0 (30.0, 196.2)	28.5 (6.9, 140.6)	17.9 (5.1, 91.5)	<LOD (<LOD, 0.2)	<LOD (<LOD, 0.2)
≥$30,000	10	144.8 (30.4, 369.6)	46.0 (25.9, 107.9)	59.1 (30.0, 94.5)	12.8 (8.6, 49.9)	0.8 (<LOD, 14.0)	0.5 (<LOD, 2.7)
Health care job							
No	30	151.0 (32.8, 476.5)	56.0 (25.9, 201.9)	63.2 (9.3, 129.9)	14.6 (6.8, 74.4)	0.4 (<LOD, 1.8)	0.3 (<LOD, 1.7)
Yes	12	58.5 (21.1, 151.2)	46.5 (30.1, 147.3)	46.7 (21.5, 68.8)	28.3 (11.3, 124.9)	<LOD (<LOD, 1.3)	<LOD (<LOD, 0.5)
Year of collection[b]							
1983	CC 39 EP 38	117.9 (22.7, 388.6)	68.3 (26.2, 203.5)	62.1 (8.9, 128.8)	24.7 (8.4, 90.8)	<LOD (<LOD, 1.7)	<LOD (<LOD, 1.8)
1984	CC 3 EP 4	116.7 (91.5, 138.7)	29.7 (22.9, 36.7)	45.5 (28.7, 50.0)	4.8 (4.5, 4.9)	<LOD (<LOD, 0.6)	<LOD (<LOD, 0.2)
Season[b]							
Winter (Jan–Mar)	CC 11 EP 7	116.7 (37.8, 324.8)	46.1 (29.7, 93.9)	45.5 (8.3, 142.1)	4.9 (4.8, 31.2)	0.4 (<LOD, 1.3)	<LOD (<LOD, 0.3)
Spring (Apr–Jun)	CC 13 EP 11	174.4 (55.6, 216.0)	91.1 (16.3, 271.9)	89.9 (46.3, 126.7)	75.2 (16.8, 126.9)	0.4 (<LOD, 2.4)	0.4 (<LOD, 0.9)
Summer (Jul–Sept)	CC 12 EP 15	94.4 (40.8, 438.5)	52.6 (37.1, 196.7)	62.5 (25.7, 108.1)	13.8 (6.5, 74.5)	<LOD (<LOD, 3.0)	0.9 (<LOD, 3.3)
Fall (Oct–Dec)	CC 6 EP 9	38.1 (17.2, 66.6)	78.6 (20.1, 108.2)	9.1 (6.1, 22.4)	16.3 (8.2, 55.8)	<LOD (<LOD, 1.5)	<LOD (<LOD, <LOD)

Table 2. (*Continued.*)

Characteristic	Participants (*n*)	Methylparaben		Propylparaben		Butylparaben	
		Conception cycle median (IQR)	Early pregnancy median (IQR)	Conception cycle median (IQR)	Early pregnancy median (IQR)	Conception cycle median (IQR)	Early pregnancy median (IQR)
Weekend specimens[b,c]							
No	CC 34 EP 27	141.5 (47.8, 437.8)	51.9 (21.1, 117.3)	50.8 (10.5, 126.0)	13.9 (5.8, 73.5)	0.3 (<LOD, 1.8)	0.3 (<LOD, 1.5)
Yes	CC 8 EP 15	59.3 (17.5, 149.6)	89.6 (30.3, 236.6)	44.4 (6.9, 200.1)	37.7 (8.3, 115.6)	<LOD (<LOD, 0.5)	<LOD (<LOD, 0.9)

Note: Participant characteristics, including medication, vitamin, and other substance use, were reported at the study intake interview. Apr, April; CC, conception cycle; Dec, December; EP, early pregnancy; IQR, interquartile range; Jan, January; Jul, July; Jun, June; LOD, limit of detection; Mar, March; OC, oral contraceptive; Oct, October; Sept, September; USD, U.S. dollars.

[a]Recent oral contraceptive use was reported as occurring in the 90 d prior to study enrollment.

[b]The number of participants with this characteristic differs between the conception cycle and early pregnancy. This is indicated with CC or EP next to the number of participants.

[c]Weekend specimens indicates whether there is ≥1 weekend day urine specimen included in the pooled sample. Most specimens were collected on a Monday, but if a Monday specimen was not available, the nearest day was included (see Table S1 for the days of the week represented in pooled samples).

(OPLS-DA) to evaluate associations between paraben concentration categories and the high dimensional metabolomics data. First, we used PCA to assess whether paraben concentration category was associated with any of the principal components of the metabolomics data.[58] Conception cycle and early pregnancy data were analyzed separately, given the major hormonal and physiological changes accompanying pregnancy, as were data from different mass spectrometry analysis modes (HILIC-MS for polar metabolites, and RPLC-MS for nonpolar metabolites), for a total of four comparisons. Concentration categories for each paraben were then overlayed onto PCA plots to determine whether they corresponded to data clustering. PCA results were assessed using scree plots (the number of principal components explaining $\sim 50\%$ of the variance in the data), R^2 (how much variance in the data was explained by the model), and Q^2 (the predictive ability of the model).

Next, we used OPLS-DA to determine the variation associated with concentration categories using R^2X, R^2Y, Q^2, and the number of features where the variable importance to projection or VIP score was >1.[62] A less conservative VIP score was chosen to ensure we did not overlook metabolites potentially associated with paraben concentration. Comparisons were stratified by conception cycle or early pregnancy status, analysis mode, and concentration category (low vs. medium, low vs. high, medium vs. high), for a total of 18 comparisons. Because multigroup OPLS-DA can yield ambiguous results, we chose to compare only two paraben concentration categories at a time.[63] Results of OPLS-DA were cross-validated with a permutation test that randomly assigned concentration category, correlating the Q^2 and R^2Y of the original data with the distribution of Q^2 and R^2Y after 200 iterations of random permutation by concentration category.[64] Permutation models with a Q^2 intercept close to or below 0 indicate poorly fitting models when concentration category is randomly assigned; in turn, this suggests that the original OPLS-DA model accurately assigns concentration category.

Univariate analysis. A Wilcoxon rank-sum test was used to compare changes in peak area between concentration categories using the same 18 comparison groups of the OPLS-DA models. We used a Benjamini-Hochberg false discovery rate (FDR)-corrected p-value (q-value) of $q < 0.1$ to set the level of statistical significance; as with the VIP score, a less conservative q-value was chosen, given the discovery objectives of this analysis, to ensure no true associations were excluded from results.[65] We then combined OPLS-DA and Wilcoxon rank-sum test results to identify those features associated with paraben concentration category, using the criteria of both VIP >1 and $q < 0.1$, common criteria applied to identify potential biomarkers.[62]

Adjustment for confounding. Potential confounding factors in the relationship between paraben concentration and the urinary metabolome were mapped with a directed acyclic graph (DAG; Figure 1). We used the method outlined in Shrier and Platt (2008) to select covariates for model inclusion and evaluate remaining sources of bias using a DAG.[66] Then, we used multivariable linear regression models to adjust paraben–metabolite relationships.[67,68]

Metabolite intensities (dependent variable) and paraben concentrations (ng/mL, specific gravity–adjusted, independent variable) were natural log-transformed and treated continuously. To determine how to represent other covariates, we looked at unadjusted associations with metabolite intensity using clinically meaningful cut points and percentiles (quartiles, tertiles). Final forms were determined using visual inspection and the lowest value of the Akaike information criterion (AIC). Information on participant characteristics was gathered during an intake interview at study enrollment. We adjusted for age and alcohol use (reported as 12-oz beers, 4-oz. glasses of wine, or shots of hard liquor per day, week, and month, and combined into drinks per month) in three categories based on the 25th and 75th percentiles; BMI (based on self-reported height and weight) using clinical cut points of low ($<18.5\,kg/m^2$) or normal ($18.5–24.9\,kg/m^2$); caffeine (reported as cups of caffeinated beverage per day, week, and month, and converted to mg caffeine per month) in three categories using tertiles; years of education (highest level and years reported) in three categories (high school or some college, college graduate, and graduate/professional school); and income (reported as total pretax family income per year) in three categories using tertiles. Season was defined by the median specimen aliquot date for pooled samples and was included because of seasonal trends in personal care product use.[69] Year of sample collection was included to account for the fact that exposures may change over time, though most samples ($n = 77/84$, 92%) were from the same year. Although no dietary information was collected from participants, dietary patterns can differ between weekends and weekdays.[70] We therefore used pooled samples with weekend collections (Table S1) as a diet proxy variable to adjust for potential differences in diet between weekends and weekdays. There were 8 ($n = 8/42$, 19%) conception cycle pooled samples and 15 ($n = 15/42$, 36%) early pregnancy pooled samples that contained weekend days. The diet proxy variable had two categories: pooled samples containing weekdays only ($n = 61/84$, 73%) and pooled samples including at least 1 weekend day ($n = 23/84$, 27%). The remaining covariates were treated as dichotomous categorical variables (yes/no): health care occupation (occupation on enrollment reported and categorized by industry), smoker (current smoking status reported on enrollment), marijuana use (reported as any use in the 3 months prior to enrollment), vitamin use, aspirin use, acetaminophen use, antibiotic use, and oral contraceptive use in the 90 d prior to study enrollment. All medications and supplements used in the 3 months prior to study enrollment were reported by name, along with dose and frequency; these

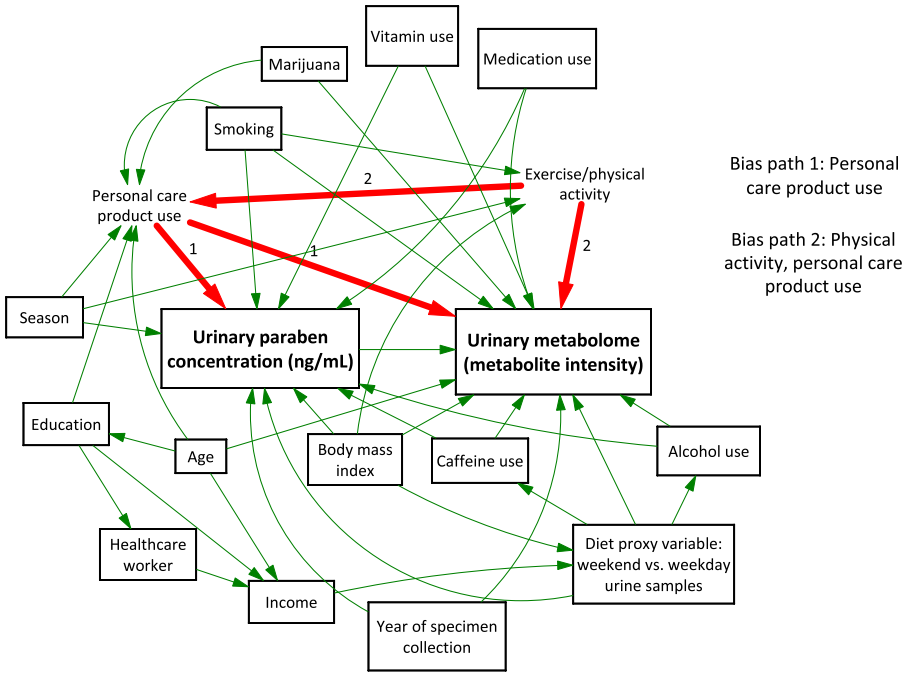

Figure 1. Directed acyclic graph showing potential confounders in the relationship between urinary paraben concentration and the urinary metabolome and bias paths remaining after adjustment. Thin green lines connecting covariates indicate that associations on those paths are blocked, and potential confounding is accounted for in regression models. Any remaining paths from urinary paraben concentration to the urinary metabolome—regardless of arrow direction—indicate possible sources of bias and are represented by thick red lines with numbers corresponding to bias paths. Bias path 1 is via personal care product use, and bias path 2 is via physical activity and personal care product use. Boxed variables are measured for study participants and included in regression models, whereas unboxed variables are neither measured for study participants nor included in regression models.

were then categorized into classes of medications for covariate adjustment.

Regression models. We constructed 13 multivariable linear regression models to adjust each paraben–metabolite relationship where VIP was >1 and $q<0.1$. Models were labeled by metabolite number (1–7) and letter (a–d) when multiple models were run for a given metabolite (conception cycle, early pregnancy, methylparaben, propylparaben). In addition to 11 single-pollutant models with just one paraben, we also constructed two bipollutant models including both methylparaben and propylparaben because for two metabolites, both methylparaben and propylparaben independently were associated with metabolite intensity. Both parabens were included in these models to determine whether associations remained after mutual adjustment. Paraben regression coefficients or estimates are the percent change in metabolite intensity for every 1% change in paraben concentration. For interpretation, we also calculated the estimated percent change in metabolite intensity when the paraben concentration doubled. Residual plots were visually examined for normality for all models.

Drug metabolizing enzyme metabolites. We calculated median intensity and IQR across paraben concentration category groups for those sulfated DME metabolites identified in our data, catechol sulfate and pregnenolone sulfate. We also visually compared intensities across concentration categories, stratified by conception cycle or early pregnancy status, using box plots.

Metabolite Annotation

Metabolites associated with paraben concentration category (VIP >1 and $q<0.1$) before adjustment were selected for identification. Initial screening of potential matches for metabolite identification was carried out on the METLIN database,[71] with criteria including a mass error [parts per million (ppm)] of Δppm <25, retention time within 2 min to account for instrument differences, and a metabolite match that was biologically plausible (endogenous human metabolites or metabolites from potential external exposures) if information was available (84 candidate matches were considered). Putative matches with medications not reported in study intake interviews, used only in experimental settings after the years of EPS specimen collection, or receiving U.S. Food and Drug Administration (U.S. FDA) approval after specimen collection were excluded ($n=6$), leaving 78 potential matches. Based on these criteria, available standards ($n=5$) were purchased (see "Targeted Metabolomics Analysis"). Fragmentation data were used for metabolite identification, first compared with fragmentation data from commercial standards and public libraries and then analyzed using the SIRIUS and CFM-ID tools.[59,61] The certainty of a metabolite identification was classified using the scoring system of the Metabolomics Standards Initiative (MSI; see Table 4 notes for scoring system description).[72,73]

Results

Paraben Urinary Concentration Distribution

For all parabens, conception cycle urinary concentrations were higher than early pregnancy concentrations (Table 2). Methylparaben and propylparaben concentrations were higher for participants who reported taking acetaminophen and aspirin, were younger, and were nonsmokers, and concentrations were highest in the spring and lowest in the fall (Table 2). Methylparaben and propylparaben concentrations were correlated during conception cycles (Spearman's $r=0.8$) and early pregnancy ($r=0.8$), whereas correlations between butylparaben concentrations and the other parabens were <0.5. Comparison of EPS paraben concentrations with those from other cohorts of reproductive-age women (sample collection years 1997–2019) found paraben concentrations were variable across all studies, without any trend over time.[2,37,39,74–78] EPS concentrations

Table 3. Results of OPLS-DA showing variation associated with urinary paraben concentration category for 42 participants in the Early Pregnancy Study.

		HILIC data				RPLC data			
Sample time point	Paraben concentration comparison (n)	R^2X	R^2Y	Q^2	Discriminant features (n)[a]	R^2X	R^2Y	Q^2	Q^2
Conception cycle	Methylparaben	—	—	—	—	—	—	—	—
	L (21)–M (10)	0.23	0.98	−0.026	0	0.42	0.95	0.32	1
	L (21)–H (10)	0.24	0.98	0.14	2	0.38	0.96	−0.084	0
	M (10)–H (10)	0.19	0.99	−0.16	0	0.44	0.96	−0.16	0
	Propylparaben	—	—	—	—	—	—	—	—
	L (21)–M (10)	0.24	0.97	−0.27	0	0.41	0.96	−0.053	0
	L (21)–H (11)	0.25	0.98	0.24	5	0.41	0.95	0.21	0
	M (10)–H (11)	0.27	0.99	−0.21	0	0.43	0.96	−0.36	0
	Butylparaben	—	—	—	—	—	—	—	—
	L (22)–M (10)	0.22	0.97	−0.072	0	0.42	0.95	−0.023	0
	L (22)–H (10)	0.22	0.99	−0.16	0	0.41	0.93	−0.39	0
	M (10)–H (10)	0.25	0.99	−0.50	0	0.43	0.99	−0.35	0
Early pregnancy	Methylparaben	—	—	—	—	—	—	—	—
	L (22)–M (10)	0.23	0.97	−0.087	0	0.40	0.95	−0.16	0
	L (22)–H (10)	0.26	0.95	−0.24	2	0.42	0.92	−0.43	0
	M (10)–H (10)	0.26	0.99	−0.052	0	0.43	0.99	−0.017	0
	Propylparaben	—	—	—	—	—	—	—	—
	L (20)–M (12)	0.20	0.98	−0.073	0	0.39	0.96	−0.39	0
	L (20)–H (10)	0.27	0.95	−0.087	0	0.42	0.95	−0.22	0
	M (12)–H (10)	0.25	0.99	0.22	0	0.44	0.99	0.32	1
	Butylparaben	—	—	—	—	—	—	—	—
	L (22)–M (10)	0.22	0.96	0.12	0	0.41	0.95	−0.028	0
	L (22)–H (10)	0.21	0.99	−0.016	0	0.42	0.94	−0.092	0
	M (10)–H (10)	0.27	0.99	0.061	0	0.42	0.97	−0.0080	0

Note: Only features with a relative standard deviation <0.3 were included in this analysis: 15,158/18,822 features from HILIC data, and 12,023/12,794 features from RPLC data. R^2X represents the variation in the data related to concentration category; R^2Y indicates group separation by concentration category; and Q^2 indicates predictive performance of the model. If the difference between R^2Y and Q^2 is >0.3, this suggests overfitting of the model. Paraben concentration categories of low, medium, and high are defined in Table 1. —, no data; H, high concentration category; HILIC, hydrophilic interaction chromatography; L, low concentration category; M, medium concentration category; OPLS-DA, orthogonal partial least-squares discriminant analysis; RPLC, reversed-phase liquid chromatography; VIP, variable importance to projection score.

[a]Discriminant features are those with VIP > 1 based on OPLS-DA analysis, and $q < 0.1$ based on the Wilcoxon rank-sum test comparing changes in peak area between paraben concentration categories. The q-value is a Benjamini-Hochberg false discovery rate–corrected p-value.

were within range of other cohorts, including more recent studies with samples collected during the period 2010–2019.

Although metabolomic analysis of samples after long-term storage can raise concerns about degradation, we did not observe a systematic pattern of increasing or decreasing paraben concentrations within the EPS cohort across the years of sample collection (Table 2). Together, the careful storage and specimen handling procedures, absence of sample contamination, and similar urinary concentrations in comparison with present-day cohorts support the viability of these specimens for analysis even after long-term storage.

Multivariate and Univariate Analyses

Results of PCA showed no separation of groups based on paraben concentration categories (Figure S2). Although the R^2 values of the PCA models indicate that 25%–47% of the variance is explained by the first two principal components, the predictive ability (Q^2) of the models is low: ≤0.3 in all cases (Table S2). For RPLC data, the first three principal components explain 50% of the variance of the data, whereas for HILIC data, more than five principal components are needed to explain 50% of the variance.

Results of the OPLS-DA models (Table 3) indicated that the models did not accurately assign concentration categories. Differences >0.3 between R^2Y and Q^2 indicate overfitting of a model, and in all models, the difference was >0.3. The findings of this analysis were validated using a permutation test (Table S3), where Q^2 intercepts were all >0.

Using the results of the Wilcoxon rank-sum test, we found 11 discriminant features with $q < 0.1$ (Tables 3 and 4). Although all of these features also had a VIP > 1, because of OPLS-DA model overfitting, we consider the VIP score to be of lesser importance in this instance. Two metabolites were associated with paraben concentration in both conception cycle and early pregnancy samples and independently associated with methylparaben and propylparaben concentrations, for a total of 7 unique metabolites across the 11 discriminant features. No metabolites were associated with butylparaben. Five metabolites were detected in HILIC mode, which focuses on polar metabolites, including hydrophilic conjugated and glucuronidated metabolites associated with detoxification, and two were detected in RPLC mode, which focuses on nonpolar metabolites.

Multivariable Linear Regression Models

After adjustment for potential confounding factors, three metabolites remained associated with paraben concentrations (Table 5 and Table S4; Figure 2): metabolites 2 and 5 detected in HILIC mode and metabolite 3 detected in RPLC mode. Metabolites 2 and 5 were associated with methylparaben in both conception cycles and early pregnancy (models 2a, 2d, 5a, and 5d). Across these four models, a doubling of methylparaben concentration was associated with a 59%–75% [95% confidence interval (CI): 33%, 111%; $p \le 0.00005$ in all models] increase in metabolite intensity. After adjustment, metabolites 2 and 5 were also associated with propylparaben, and a doubling of propylparaben concentration was associated with a 32% (95% CI: 0%, 73%; 2b, $p = 0.05$; 5b, $p = 0.02$) increase in metabolite intensity for both. Metabolite 3 was associated with methylparaben in the conception cycle, with a doubling of methylparaben concentration associated with a −14% (95% CI: −26%, −1%, $p = 0.03$) decline in metabolite intensity. Across the 13 models, methylparaben remained associated with metabolite intensity after adjustment in all seven models that included methylparaben (Figure 2). In contrast, propylparaben remained associated with metabolite intensity in only two of the eight models including propylparaben. Of

Table 4. Identification of metabolites associated with urinary paraben concentration for 42 participants in the Early Pregnancy Study.

Metabolite number	Mode, sample time point	Paraben concentration comparison[a]	*m/z* and RT (sec)	VIP[b]	*q*-Value[c]	Raw fold change	Putative identification	MSI level of certainty and rationale	Additional information
1	HILIC, conception cycle	Propylparaben low-high	*m/z*: 195.0669 RT: 37.71	3.77	0.039	14.9	Acetosyringone $C_{10}H_{12}O_4$ CAS: 2478-38-8 PubChem CID: 17198 Theoretical mass: 196.074107 Class: acetophenone	MSI Level 2: MS/MS fragmentation data matched with public library, CFM-ID score = 0.93 (top match)	Signature of smoke from hardwoods detected in $PM_{2.5}$ analyses; found in smoked foods and in smoke flavoring added to foods
2	HILIC, conception cycle	Methylparaben low-high	*m/z*: 327.0735 RT: 347.30	4.24	0.0070	14.2	Fertaric acid $C_{14}H_{14}O_9$ PubChem CID: 22298372 Theoretical mass: 326.063782 Class: hydroxy-cinnamic acid	MSI Level 2: MS/MS fragmentation data matched with public library (HMDB), SIRIUS score = 100%	Antioxidant found in grapes, grape seeds, grape juice, wine, and wheat
2	HILIC, conception cycle	Propylparaben low-high		3.88	0.039	14.2			
2	HILIC, early pregnancy	Methylparaben low-high		3.07	0.0076	4.3			
3	RPLC, conception cycle	Methylparaben low-medium	*m/z*: 386.3972 RT: 498.75	2.74	0.075	0.5	Methyl tricosanoate CAS: 2433-97-8 PubChem CID: 75519 $C_{24}H_{48}O_2$ Theoretical mass: 368.365979 Class: fatty acid methyl ester	MSI Level 2: MS/MS fragmentation data matched with public library (Biodatabase), SIRIUS score = 100%	In-source ammonium (NH_4) adduct of methyl tricosanoate, which is found in olives/olive oil
4	HILIC, conception cycle	Propylparaben low-high	*m/z*: 522.1288 RT: 346.53	3.73	0.072	5.9	Unidentified	MSI Level 4: unknown spectral signal can be reproducibly detected and quantified	No information available
5	HILIC, conception cycle	Methylparaben low-high	*m/z*: 591.1843 RT: 349.96	3.76	0.023	7.1	$C_{21}H_{36}O_{19}$ Theoretical mass: 592.185628 Class: glycerolipid	MSI Level 3: putative identification of compound class; MS/MS fragmentation data does not match public libraries	No information available
5	HILIC, conception cycle	Propylparaben low-high		3.65	0.00024	6.1			
5	HILIC, early pregnancy	Methylparaben low-high		4.29	0.0047	5.5			
6	HILIC, conception cycle	Propylparaben low-high	*m/z*: 613.1669 RT: 349.10	3.67	0.039	3.0	Catechin 3′,5-diglucoside $C_{27}H_{34}O_{16}$ CAS: 105330-54-9 PubChem CID: 73813318 Theoretical mass: 614.185234 Class: flavonoid o-glycoside	MSI Level 2: MS/MS fragmentation data matched with public library (HMDB), SIRIUS score = 99.8%	Found in apples, green vegetables, green/black tea, grapes/wine, cocoa, barley, lentils
7	RPLC, early pregnancy	Propylparaben med-high	*m/z*: 634.1677 RT: 652.65	3.48	0.037	0.6	Unidentified	MSI Level 4: unknown spectral signal can be reproducibly detected and quantified	No information available

Note: MSI levels of certainty[72,73] are defined as: Level 1/identified metabolites—two orthogonal analytical techniques applied to the analysis of both the metabolite of interest and to a chemical reference standard of suspected structural equivalence, with all analyses performed under identical analytical conditions in the same laboratory; Level 2/putatively annotated compounds—Levels 3 and 4 plus spectral similarity with public or commercial libraries; Level 3/putatively characterized compound classes—Level 4 plus spectral and/or physiochemical properties consistent with a particular class of organic compounds; Level 4/unknown—discernible spectral signal that can be reproducibly detected and quantified. CAS, Chemical Abstracts Service Registry Number; CFM-ID, Competitive Fragmentation Modeling-Identification; HILIC, hydrophilic interaction chromatography; HMDB, Human Metabolome Database; MSI, Metabolomics Standards Initiative; MS/MS, tandem mass spectrometry; MS/MS data, fragmentation data; *m/z*, mass-to-charge ratio; OPLS-DA, orthogonal partial least-squares discriminant analysis; $PM_{2.5}$, air particulate matter $\leq$2.5 micrometers in aerodynamic diameter; PubChem CID, unique identification number for each substance in the PubChem database; RPLC, reversed-phase liquid chromatography; RT, retention time; sec, seconds; VIP, variable importance to projection from OPLS-DA.

[a]Paraben concentration categories are defined in Table 1.

[b]The VIP score is from the OPLS-DA analysis.

[c]The *q*-value is a Benjamini-Hochberg false discovery rate-corrected *p*-value based on the Wilcoxon rank-sum test comparing changes in peak area between paraben concentration categories.

Table 5. Change in metabolite intensity associated with urinary paraben concentration (ng/mL, specific gravity–adjusted) after adjusting for confounding using multivariable linear regression in 42 participants in the Early Pregnancy Study.

Metabolite number (model)[a]	Sample time point	Paraben	Intensity change when paraben concentration doubles (95% CI)	Adjusted R^2
1 (a)	Conception cycle	Propylparaben	28% (−4%, 71%)	−0.02
2 (a)	Conception cycle	Methylparaben	75% (45%, 111%)	0.69
2 (b)	Conception cycle	Propylparaben	32% (0%, 73%)	0.19
2 (c)[b]	Conception cycle	Methylparaben	103% (49%, 175%)	0.70
		Propylparaben	−15% (−35%, 12%)	
2 (d)	Early pregnancy	Methylparaben	62% (38%, 90%)	0.60
3 (a)	Conception cycle	Methylparaben	−14% (−26%, −1%)	0.27
4 (a)	Conception cycle	Propylparaben	15% (−7%, 41%)	0.16
5 (a)	Conception cycle	Methylparaben	59% (33%, 89%)	0.59
5 (b)	Conception cycle	Propylparaben	32% (5%, 66%)	0.21
5 (c)[b]	Conception cycle	Methylparaben	72% (27%, 133%)	0.58
		Propylparaben	−8% (−30%, 20%)	
5 (d)	Early pregnancy	Methylparaben	69% (49%, 93%)	0.70
6 (a)	Conception cycle	Propylparaben	15% (−5%, 39%)	−0.09
7 (a)	Early pregnancy	Propylparaben	2% (−9%, 13%)	0.01

Note: The adjusted R^2 is for the full model, and full regression results are included in supplementary materials (Table S4). The full model includes paraben concentration; acetaminophen, aspirin, antibiotic, and vitamin use; age; BMI; marijuana use; alcohol consumption; caffeine intake; smoking status; recent oral contraceptive use; education; income; health care occupation; year of specimen collection; season of specimen collection; and whether a pooled sample includes weekend days. BMI, body mass index; CI, confidence interval.
[a]Metabolite numbers (1–7) correspond to the metabolites described in Table 4, and model letters (a–d) differentiate models run for the same metabolite.
[b]Bipollutant model, including both methylparaben and propylparaben.

the 11 models including just a single paraben, 10 were associated with increased metabolite intensities.

We also constructed two bipollutant models (models 2c and 5c) with both methylparaben and propylparaben (Figure 2). In these models, after adjustment methylparaben remained associated with metabolite intensity [2c, 1.02 (95% CI: 0.58, 1.46), $p = 0.0002$; 5c, 0.78 (95% CI: 0.34, 1.22), $p = 0.002$], whereas the association between metabolite intensity and propylparaben

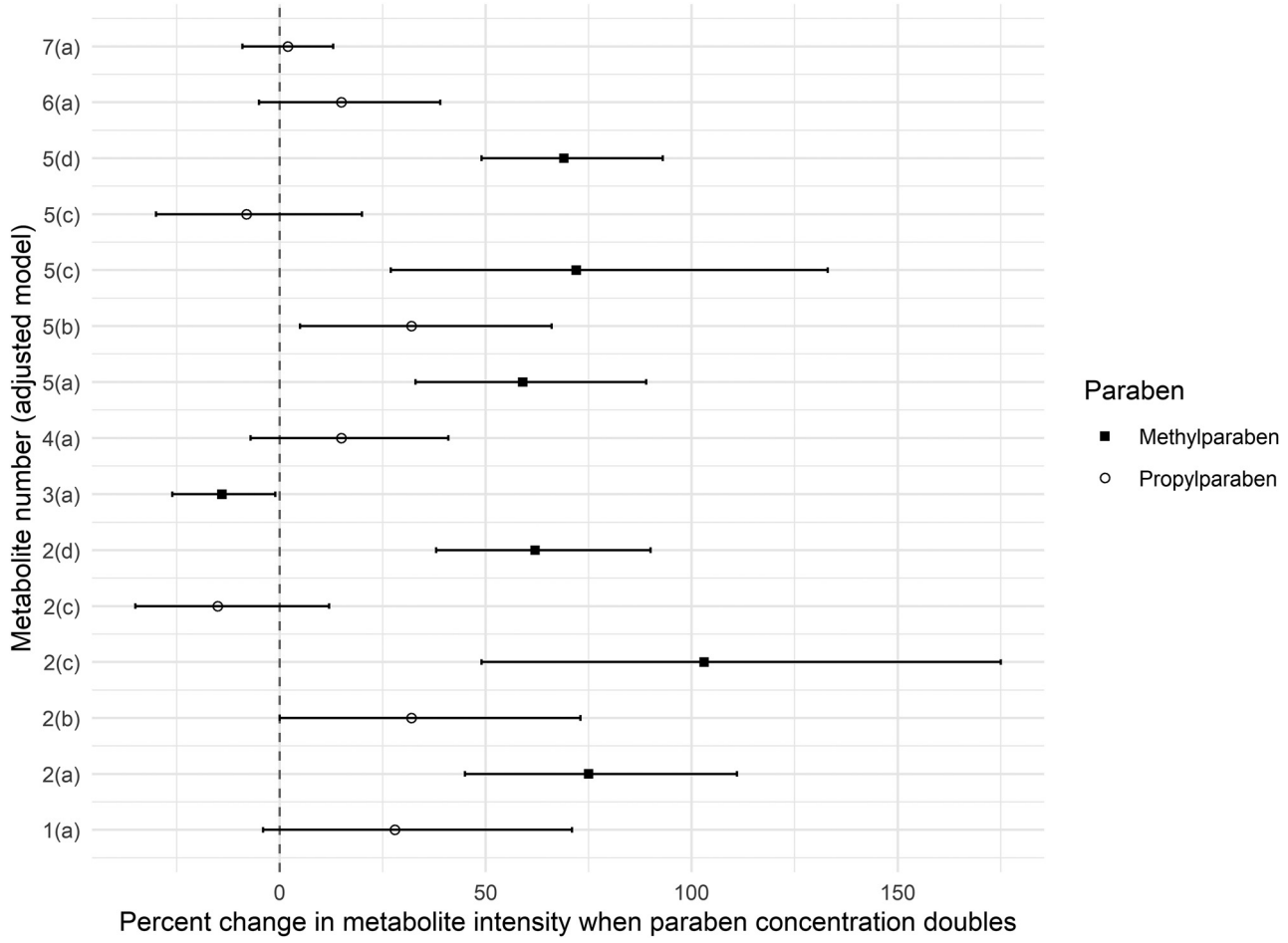

Figure 2. Percent change in metabolite intensity associated with a doubling of urinary paraben concentration (ng/mL, specific gravity–adjusted) after adjustment for confounding using multivariable linear regression in 42 participants in the Early Pregnancy Study. Error bars represent 95% confidence intervals. Metabolite numbers (1–7) correspond to the metabolites described in Table 4, and model letters (a–d) correspond to adjusted models summarized in Table 5, with full model results found in Table S4. Covariates used for adjustment can be visualized in Figure 1 and are also listed in Table 2. Data for this figure are from Table 5.

was attenuated [2c, −0.23 (95% CI: −0.62, 0.16), $p = 0.22$; 5c, −0.12 (95% CI: −0.51, 0.26), $p = 0.51$].

Metabolite Annotation

We putatively identified 5 of the seven metabolites associated with paraben concentration, at varying levels of certainty (Table 4). Fragmentation data are available in the Supplemental Excel File. Four of the metabolites were identified at MSI level 2 as food or flavoring compounds: acetosyringone (metabolite 1), an acetophenone found in hardwood smoke, smoked foods, and smoke flavorings; fertaric acid (metabolite 2), a hydroxycinnamic acid found in grapes and wheat; methyl tricosanoate (metabolite 3), a fatty acid methyl ester found in olives and olive oil; and catechin 3′,5-diglucoside (metabolite 6), a flavonoid o-glycoside found in certain plant-based foods. For each of these compounds, we matched our fragmentation data with that available in SIRIUS and CFM-ID because no standards were available, which would have allowed confirmation of identification to MSI level 1. In addition, resolving flavonoids (metabolite 6) and similar structures is limited in untargeted metabolomics methods; thus, although there is high confidence in the substructure of the metabolite, there is more ambiguity in the substitutions and stereochemistry of the metabolite. Identifications at MSI level 3 included a glycerolipid without any information about source or function (metabolite 5). Finally, two compounds (metabolites 4 and 7) remained unidentified at MSI level 4, without any matching fragmentation data in public tandem mass spectrometry (MS/MS) spectral libraries. After adjustment for confounding, fertaric acid, methyl tricosanoate, and the glycerolipid remained strongly associated with paraben concentrations, whereas associations with acetosyringone, catechin 3′,5-diglucoside, and the unidentified metabolites were attenuated.

Drug Metabolizing Enzyme Metabolites

We detected pregnenolone sulfate (MSI level 1) and catechol sulfate (MSI level 2) using the LC-MS methods but did not observe systematic differences in peak area across paraben concentration categories based on visual assessment of boxplots (Table S5; Figure S3).

Discussion

In this study, we investigated associations between urinary concentrations of three widely used parabens and the urinary metabolome during the periconceptional period, a critical window for fertility. This study provides the first results related to paraben exposure and the metabolome during this critical window.[22–25] We found seven metabolites were associated with urinary paraben concentrations, four of which we identified as diet-related metabolites or flavoring compounds, and none of which were associated with endocrine-disrupting pathways.

Three studies have investigated associations between paraben exposure and the human metabolome. Zhao et al. examined associations between methylparaben, propylparaben, and ethylparaben and the urinary metabolome in 88 pregnant Chinese women at 10–15 wk gestation.[27] Using UHPLC coupled with triple quadrupole mass spectrometry (positive mode), they identified 60 differentially expressed metabolites in spot urine samples using Mann-Whitney *U* tests and Spearman correlations, without adjusting for multiple comparisons or confounders. Differential metabolites were from energy metabolism pathways, including the purine metabolism pathway and the urea cycle.[27] Lee et al. assessed associations between paraben concentrations in spot urine samples and aeroallergen sensitization in South Korean children.[28] Using gas chromatography time-of-flight mass spectrometry methods, they found propylparaben was associated with 15 differential metabolites, including from serine and glycine metabolism, branched-chain amino acid metabolism, and ammonia recycling.[28] Bessonneau et al. used nontargeted liquid chromatography coupled to high-resolution mass spectrometry (negative mode) to examine spot serum samples from California women firefighters and office workers.[29] In office workers only, butylparaben was associated with inflammation, potentially through bile acids, which are signaling molecules with hormonal actions that can regulate energy metabolism through nuclear receptor activation, including PPARα.[29]

Our study, which used pooled samples for more representative estimates of urinary paraben concentration and detailed control of potential confounding factors, was unable to confirm any of the differential metabolites associated with paraben concentrations in previous studies. In contrast to these other studies, we identified novel relationships between urinary paraben concentration and diet-related metabolites. Diet is a known route of exposure to parabens, which have been added as antimicrobial and antifungal agents to prevent spoilage since the 1930s.[4,40,79] Parabens are heat-resistant, odorless and tasteless, often used in combination, and can be combined with other preservatives, making them useful in food processing contexts.[79] Recent studies of parabens in foods in the United States and China found detectable levels in 90%–99% of samples, with methylparaben and propylparaben more common than butylparaben.[80,81] Although there are no studies of the prevalence of parabens in foods in the 1980s during the EPS sampling years, parabens are classified as "generally recognized as safe" ingredients by the FDA, meaning they have an extensive history of use in food prior to 1958, and are limited to a maximum concentration of 0.1% in food products.[4,79]

We found four diet-related metabolites to be associated with paraben concentration before adjustment. Acetosyringone (metabolite 1) is a plant metabolite and component of wood smoke[82] that contributes to the smoky aroma of naturally smoked foods and is a component of smoke flavorings added to foods.[83] Foods that are smoked or smoke-flavored could also contain paraben preservatives. Fertaric acid (metabolite 2) is an antioxidant found in grapes, grape juice and wine, and bread wheat.[84–87] A study of parabens in foods from the United States found methylparaben in 85% and propylparaben in 45% of fruit samples, which included raisins and grapes among the fruits sampled.[80] The same study detected methylparaben in 67% and propylparaben in 21% of beverages sampled including wine, and methylparaben in 98% and propylparaben in 82% of grains sampled. Fruits had the lowest mean concentration of parabens and grains had the highest.[80] Methyl tricosanoate (metabolite 3) is a metabolite of the olive plant, *Olea europaea*, and we detected an in-source ammonium adduct of the compound. A common source of methyl tricosanoate is olive oil.[88] Studies of parabens in food cooking oils and fats found methylparaben in 40% of samples from the United States, and 100% of samples from China.[80,81] Catechin 3′5-diglucoside (metabolite 6) is found in many plant-based foods, especially green and black teas, grapes and wines, fruits, cocoa, barley, and lentils.[89]

After adjustment, two of the three metabolites strongly associated with paraben concentration were diet-related (fertaric acid and methyl tricosanoate), along with a third unidentified glycerolipid (metabolite 5). Associations between paraben concentrations and acetosyringone and catechin 3′5-diglucoside were somewhat attenuated, with wider CIs including but centered well above 1 (Figure 2). Our observation of a negative association between methylparaben concentration and methyl tricosanoate metabolite intensity could help explain why the metabolite discriminated between low-medium but not low-high concentration categories: reduced methyl tricosanoate intensity at high concentrations could make it difficult to discriminate between the two categories. Although these results underscore the general importance of diet to

paraben concentrations, the metabolites we identified should not be interpreted as particularly paraben-rich exposure sources; future research could examine the relative source contributions of different food products to paraben exposure and how parabens enter the food supply.

Our findings emphasize the importance of including dietary data in metabolomic studies of paraben exposure. Because no dietary information was available for the EPS cohort, we developed a proxy variable for regression models that indicated whether a pooled urine sample included any weekend days, since diet tends to differ on weekends in comparison with diets on weekdays.[70] In adjusted models, this variable was strongly associated with fertaric acid intensity, and for all four dietary metabolites the direction of association was consistent, with higher metabolite intensities in samples containing weekend days (Table S4). Results of the full regression models (Table S4) also showed consistent directional trends in changes in metabolite intensity associated with other covariates, including medication use, age, and BMI; the presence of these trends is reassuring, given that we expected these factors to impact the metabolome. In these models, the covariates most strongly associated with metabolite intensity were acetaminophen use, vitamin use, age, marijuana use, health care job, season, and weekend days in sample. The participant characteristics showing the largest differences in paraben concentrations were acetaminophen use, aspirin use, age, smoking status, and season (see Table 2). Taken together, these findings suggest that medication use, age, and season may be some of the most important confounding factors, information that could benefit the design of future studies of paraben exposure and the metabolome.

Studies of parabens in foods have estimated the daily dietary intake of methylparaben to be 4–5.5 times that of propylparaben,[80,81] and in the EPS cohort, median methylparaben concentrations were $\sim$2.5 times higher than propylparaben concentrations (Table 2). We also observed more consistent and stronger associations between methylparaben and metabolite intensity (Table 5, Figure 2): in single-pollutant adjusted models, a doubling of methylparaben concentration increased metabolite intensities 59%–75% (95% CI: 33%, 111%), whereas a doubling of propylparaben concentration increased metabolite intensity 32% (95% CI: 0%, 73%). In the bipollutant models, after mutual adjustment methylparaben remained associated with metabolite intensity whereas propylparaben did not, likely reflecting the cohort's higher methylparaben concentrations. Although it is also possible that methylparaben may confound propylparaben exposure; because they are found in similar foods and products, are often used in combination, and have correlated concentrations within the cohort, it is difficult to hypothesize what specific paths of exposure might underlie these results. Together, our findings may reflect that the higher concentrations of methylparaben in the EPS cohort more strongly influence the metabolome in comparison with the lower concentrations of propylparaben.

Previous research has shown that parabens—especially those with longer linear alkyl side-chains like propylparaben and butylparaben—can activate PPARα and PPARγ nuclear receptors.[18–20] PPAR nuclear receptors, among others, mediate the expression of DMEs such as cytochrome P450s (CYPs), UDP-glucuronosyltransferases (UGTs), and sulfotransferases (SULTs), which play key roles in detoxification processes and hormone metabolism and bioavailability underlying normal reproductive function.[18] Although it is possible that further research could link this study's unidentified metabolites to hormone metabolism, we did not observe metabolites in steroid hormone classes to be impacted. The two sulfonated steroids previously associated with methylparaben exposure in rats[32] that we were able to identify using targeted analysis in our samples, catechol sulfate and pregnenolone sulfate, were not associated with paraben concentrations (Table S5; Figure S3). Instead, our findings emphasize the contribution of diet to urinary paraben concentrations, reflecting markers of paraben exposure rather than the biological effects of paraben exposure.

We detected more changes in the metabolome associated with paraben concentration during conception cycles (six metabolites across eight comparisons) than in the early pregnancy period (three metabolites). This could reflect normal physiologic changes of pregnancy, including pregnancy-associated changes to metabolism. Although the mechanisms are not fully understood, hormonal changes in pregnancy influence DME expression, including CYP and UGT enzymes.[16,90,91] During pregnancy, the UGT enzymes that metabolize parabens increase in activity; this can result in subtherapeutic levels of some medications and increased renal clearance.[90,91] Although it is unclear how early these shifts occur, previous research in the EPS cohort reported a decline in creatinine concentrations from before to after implantation, indicating that pregnancy-related changes in kidney function may begin as early as 3–6 wk gestation.[52] We observed early pregnancy paraben concentrations were lower than conception cycle concentrations, even after adjusting for urinary dilution (Table 2). This contrasts with expectations based on increased renal clearance of medications during pregnancy but could help explain why we observed fewer metabolomic changes in the early pregnancy samples. It is unlikely study participants made behavioral changes in response to pregnancy that modified their urinary paraben concentration profiles, because early pregnancy samples were often collected before a pregnancy was recognized and before the onset of nausea and vomiting of pregnancy, which typically begins at 6 wk after the last menstrual period.

The strengths of our study include the use of pooled rather than single spot urine samples to estimate paraben exposure. Spot samples, as used in the other metabolomics studies of paraben exposure, may inaccurately estimate exposure given the short half-life and high intraindividual variability of parabens.[5,36–39] Within-subject sample pooling helps address the potential measurement error associated with chemicals like parabens, which can lead to attenuation bias in estimates of exposure–outcome relationships.[92–94] The differences between the differential metabolites identified in our study and in previous studies could relate to different study populations (China, South Korea, United States; pregnant women at 10–15 wk gestation, children, office workers, preconception and pregnant women at <6 wk gestation) and biofluids analyzed, their use of spot samples, and the different metabolomics methods and analytical platforms employed in each study, leading to coverage of different chemical spaces.[27–29] Different paraben exposure profiles across the studies may also contribute to differences in findings. Concentrations of methylparaben and propylparaben were higher in EPS (median methylparaben conception cycle 129.1 ng/mL, early pregnancy 53.2 ng/mL; median propylparaben conception cycle 50.8 ng/mL, early pregnancy 18.5 ng/mL) in comparison with those reported in Zhao et al. [geometric mean (GM) methylparaben 19.45 ng/mL, propylparaben 1.18 ng/mL] or Lee et al. (median of third quintile methylparaben 13.8 ng/mL).[27,28] However, EPS butylparaben concentrations (median conception cycle and early pregnancy <0.2 ng/mL) were similar to those in Lee et al. (median of third quintile butylparaben 0.8 ng/mL).[28] Bessonneau et al. did not report study participants' paraben concentrations.[29] It is also possible that the metabolites we found to be associated with paraben concentrations are actually associated with another chemical or exposure that is co-occurring or highly correlated with parabens—including exposures unique to the 1980s—which could contribute to differences between study findings. In addition, these previous studies either did not control or minimally

controlled for confounding factors. In contrast, we controlled for numerous potential confounding factors, giving further confidence in the associations we observed that persisted after adjustment. The methods used in this study to combine untargeted and targeted metabolomics analyses with epidemiological assessment of and adjustment for confounding could be applied to larger cohorts. In the context of metabolomics, a highly sensitive tool for assessing both exposures and their effects, such adjustment for confounding is essential. Our use of an agnostic, untargeted metabolomics approach also revealed novel associations that would have been overlooked had we focused solely on our hypothesis that parabens are endocrine-disruptors influencing DMEs, underscoring the value of including untargeted analyses in environmental health research.

Our study has some weaknesses. Nearly half of butylparaben concentrations were <LOD, which may have limited our capacity to detect metabolomic changes associated with butylparaben. Our study is limited by small sample size, with just 42 participants. Although the participants in this study were not broadly representative at a population level, we leveraged selection criteria to amplify the subcohort's homogeneity so as to minimize interindividual metabolomic variability, increasing the potential to detect paraben–metabolome associations despite the smaller sample size. This approach, coupled with our use of pooled samples for exposure assessment to decrease potential measurement errors, increased the power of our study.[92] Because the paraben concentrations within our cohort were similar to those in other cohorts, our findings may have broader relevance.

We were unable to control for two important sources of bias in our adjusted models: personal care product use and exercise/physical activity (Figure 1). It is possible that including information about personal care product use and exercise/physical activity could alter adjusted results. Because this study used first morning urine samples, paraben concentrations likely reflect exposures occurring on the evening prior to specimen collection. Despite having a short half-life of <2 h, studies have found parabens to be detectable in urine for at least 48 h after dermal exposure, and 24 h after ingestion, suggesting that first morning urine samples could robustly reflect exposures from the previous evening and may even reflect exposures from the past 24–48 h, depending on exposure route.[40,41,95] Because personal care products are often used in the morning, the samples in this study might be less likely to reflect parabens from personal care product use and more likely to reflect dietary exposures, as evidenced by the metabolites we were able to identify. Sampling time thus could have influenced our findings and might help explain why we did not find metabolites associated with personal care product use despite this being a major route of paraben exposure. Results of adjusted models also showed a seasonal trend in metabolite intensities, with lower intensities in the winter rising to a peak in the summer and fall (Table S4). Previous studies have observed similar seasonal fluctuations in paraben concentrations, possibly related to trends in personal care product use.[69,96] These fluctuations underscore the importance of gathering data on personal care product use and exercise/physical activity.

We attempted to control for a third source of bias, diet, using a proxy variable of whether weekend days were present in pooled samples, because previous research has found weekend and weekday dietary patterns differ.[70] Although the collection of dietary information would improve accuracy and possibly influence the results of adjusted models, the precision with which the metabolomics data identified diet-related sources of paraben exposure (smoke flavoring, grapes, olive oil) is unlikely to be replicated using common dietary data collection tools like food frequency questionnaires. However, more specific dietary data could help explain the direction of specific metabolite–exposure relationships, such as the negative association between methylparaben and methyl tricosanoate. Although dietary patterns can change with the onset of pregnancy, the early pregnancy samples in this study are from prior to the typical onset of first trimester nausea and vomiting and often from before a pregnancy was recognized. Integrating metabolomics data and nutritional biomarkers with existing dietary data is an area of emerging research in nutritional epidemiology, with the potential to inform survey questions seeking to assess paraben and other environmental exposures.[97] Together, these sources of bias highlight the importance of collecting a broad array of information from study participants when trying to understand the metabolomic implications of environmental exposures.

Conclusions

We observed associations between urinary paraben concentrations and the periconceptional urinary metabolome, with detailed control for confounding. Four of the seven differential metabolites were consistent with dietary sources of paraben exposure, and three metabolites remained unidentified. We did not document associations between paraben concentrations and endocrine-disrupting pathways and were unable to confirm the differential metabolites associated with paraben concentrations in previous studies. This study demonstrated the feasibility and value of integrating untargeted metabolomics data with environmental exposure information and adjusting for confounding using epidemiological approaches; our methods can be scaled up for larger cohorts. Our findings support further investigation of dietary exposure pathways in environmental health and exposure studies, including the relative source contributions of different food products to paraben exposure and integration of metabolomics data into dietary exposure assessment, and underscore the need for further research on the biological and health implications of paraben exposure.

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