

Urinary Concentrations of Phthalate Metabolite Mixtures in Relation to Serum Biomarkers of Thyroid Function and Autoimmunity among Women from a Fertility Center

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BACKGROUND: Although previous epidemiological studies have explored associations of phthalate metabolites with thyroid function, no studies to date have assessed associations of mixtures with thyroid function and autoimmunity among potentially susceptible subgroups such as subfertile women.

OBJECTIVE: We aimed to explore associations of mixtures of urinary phthalate metabolites with serum markers of thyroid function and autoimmunity.

METHODS: This cross-sectional study included 558 women attending a fertility center who provided one spot urine and one blood sample at enrollment (2005–2015). We quantified urinary concentrations of eight phthalate metabolites using mass spectrometry, and biomarkers of thyroid function [thyroid-stimulating hormone (TSH), free and total thyroxine (fT₄, TT₄) and triiodothyronine (fT₃, TT₃), and autoimmunity [thyroid peroxidase and thyroglobulin antibodies (TPOAb and TgAb, respectively)] in serum using electrochemiluminescence assays. We applied principal component analysis (PCA) and Bayesian kernel machine regression (BKMR) to identify the main patterns of urinary phthalate metabolites. We used linear mixed models to assess the association between PCA-derived factor scores in quintiles and serum thyroid function and autoimmunity, adjusting for age, body mass index (BMI), specific gravity (SG), and, for the PCA, other factor scores.

RESULTS: We observed two factors using PCA, one representing the di(2-ethylhexyl) (DEHP) and another non-DEHP metabolites. Compared to women in the lowest quintile of the DEHP factor scores, women in the highest quintile had significantly lower serum concentrations of fT₄, TT₄, fT₃, and TT₃ [absolute difference: –0.62; 95% confidence interval (CI): –0.12, –0.01; *p* = 0.04; absolute difference: –8.31; 95% CI: –13.8, –2.85; *p* = 0.003; absolute difference: –0.37; 95% CI: 0.54, –0.19; *p* < 0.0001; and absolute difference: –0.21; 95% CI: –0.32, –0.10; *p* = 0.003, respectively]. Using BKMR, we observed that mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) was the primary contributor to these negative associations. DEHP and non-DEHP factor scores were not associated with serum TSH, TgAb, or TPOAb.

CONCLUSIONS: Mixtures of urinary DEHP metabolites were inversely associated with serum biomarkers of thyroid function but not with autoimmunity, which were within normal ranges for healthy adult women. <https://doi.org/10.1289/EHP6740>

Introduction

Phthalates are a class of endocrine-disrupting chemicals (EDCs) used in consumer and personal care products, leading to widespread general population exposure through ingestion, inhalation, and dermal absorption (CDC 2019; Hauser and Calafat 2005; Silva et al. 2004; Zota et al. 2014). Despite their relatively short elimination half-life, exposures are repeated, episodic, and chronic (Kao et al. 2012; Koch et al. 2006, 2012). Urine has been

shown to be the optimal matrix for exposure biomonitoring (Calafat et al. 2015). Phthalate metabolites have been detected in urine from representative samples of the U.S. general population from the National Health and Nutrition Examination Survey (NHANES) (CDC 2019), confirming their ubiquitous exposure.

Human and animal studies suggest that urinary phthalate metabolite concentrations negatively impact human health and adversely affect the endocrine system, metabolism, neurodevelopment, and behavior (Braun 2017; Casals-Casas et al. 2008; Desvergne et al. 2009; Grün and Blumberg 2007; James-Todd et al. 2012; Radke et al. 2018). Specifically, animal studies have documented altered thyroid function, including reduced thyroid hormone concentrations, biosynthesis, biotransformation, transport, and metabolism, after phthalate exposure (Dong et al. 2017; Liu et al. 2015; Zhai et al. 2014). Human studies have also reported alterations in thyroid function and on the hypothalamic–pituitary–thyroid (HPT) axis with phthalate metabolites, assessed both individually (Boas et al. 2012; Johns et al. 2015, 2016; Kim et al. 2019; Kuo et al. 2015; Meeker et al. 2007; Meeker and Ferguson 2011; Villanger et al. 2020) and also as a mixture (Romano et al. 2018; Villanger et al. 2020). However, thyroid autoimmunity, an important risk factor for thyroid dysfunction, has not been assessed in relation to phthalate metabolites despite evidence suggesting a possible association with other

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endocrine disruptors (Chailurkit et al. 2016; Duntas 2011; Skarha et al. 2019). Autoimmune antibodies can interfere with enzymes in the thyroid gland involved in the production of thyroid hormones, such as thyroperoxidase (TPO) and thyroglobulin (Tg), potentially resulting in inflammation that may ultimately affect the thyroid gland (Fröhlich and Wahl 2017).

In an effort to better understand the possible influence of phthalates on human thyroid function and thyroid autoimmunity, which may translate to human reproductive health effects, we investigated whether urinary phthalate metabolite concentrations were associated with serum markers of thyroid function and autoimmunity among women attempting conception and seeking fertility treatments. Urinary phthalate metabolite concentrations were evaluated as a mixture [applying principal component analysis (PCA) and Bayesian kernel machine regression (BKMR)] because humans are concurrently exposed to multiple manmade chemicals that may be associated with each other (Birnbau 2012; Bobb et al. 2015). We have previously found among women from the same study cohort that certain biomarkers of thyroid function and autoimmunity were associated with decreased ovarian reserve, which highlights a possible mechanism through which urinary phthalate biomarkers can adversely impact human reproduction (Korevaar et al. 2018). Although women in our cohort had mean serum thyroid function and autoimmunity biomarker concentrations mostly within normal ranges for healthy adult women, it is important to note that thyroid system is stringently regulated, as thyroid hormones are powerful stimulators; thus, any effects on the serum biomarker concentrations may have relevant physiological consequences.

Materials and Methods

Study Participants

We evaluated women enrolled in the Environment and Reproductive Health (EARTH) Study, an ongoing prospective cohort established to assess environmental and dietary determinants of fertility at the Massachusetts General Hospital (MGH) Fertility Center (Mínguez-Alarcón et al. 2016). Women between 18 and 45 y old were eligible to participate, and ~60% of those contacted by the research staff enrolled. This cross-sectional analysis includes 558 women who provided both a spot urine sample and a blood sample on the same day (at enrollment) between 2005 and 2015. A total of 398 women were excluded because of lack of serum thyroid and autoimmunity biomarker data, including 133 women who were using thyroid-interfering medication at study entry (predominantly levothyroxine, methimazole, propylthiouracil, amiodarone, antipsychotics, anticonvulsants, or high-dose steroids) as previously described (Korevaar et al. 2018).

The participant's date of birth was collected at entry, and weight and height were measured by trained study staff. Body mass index (BMI) was calculated as weight (in kilograms) divided by height (in meters squared). Research staff administered sociodemographic, lifestyle, and medical history questionnaires to participants at enrollment after they signed an informed consent. Also, study participants completed a comprehensive take-home questionnaire on family, medical, reproductive and occupational history, consumer products use, smoking history, and physical activity. Infertility was diagnosed using the Society of Assisted Reproductive Technology definitions (CDC 2015). The study was approved by the Human Subject Committees of the Harvard T.H. Chan School of Public Health, MGH, and the Centers for Disease Control and Prevention (CDC).

Exposure Assessment

At enrollment, each woman collected one spot urine sample in a sterile polypropylene specimen cup. Specific gravity (SG) was measured

at room temperature using a handheld refractometer (National Instrument Company, Inc.) calibrated with deionized water before each measurement. Because of the potential for bias, rather than correcting by SG, we instead used the unadjusted urinary phthalate metabolite concentrations and adjusted for SG by including this as a covariate in the statistical models (Barr et al. 2005; Schisterman et al. 2005). The urine was stored at -80°C , and samples were shipped on dry ice overnight to the CDC for analysis. As previously described (Silva et al. 2007), we used online solid-phase extraction coupled with isotope dilution–high-performance liquid chromatography–tandem mass spectrometry to quantify the urinary concentrations of phthalate metabolites, including monoethyl phthalate (MEP), mono-*n*-butyl phthalate (MBP), monoisobutyl phthalate (MiBP), monobenzyl phthalate (MBzP), mono(2-ethylhexyl) phthalate (MEHP), mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), and mono(2-ethyl-5-carboxypentyl) phthalate (MECPP). Limits of detection (LOD) are shown in Table S1 and ranged from 0.1 to 1.2 ng/mL. Concentrations below the LOD were assigned a value equal to the LOD divided by the square root of 2.

Outcome Assessment

A single nonfasting blood sample from each woman was collected via venipuncture on the same day of urine sample collection. Serum was then centrifuged, stored at -80°C , and eventually shipped on dry ice to the Department of Clinical Chemistry, Máxima Medical Center, to measure thyroid function and autoimmunity biomarkers. Concentrations of thyroid-stimulating hormone (TSH), free and total thyroxine (fT₄, TT₄) and triiodothyronine (fT₃, TT₃), and TPO and Tg antibodies (TPOAb and TgAb, respectively) were quantified using electrochemiluminescence assays (Cobas[®] e601 platform; Roche Diagnostics). Between-run coefficients of variation were 2.1%, 3.5%, 3.8%, 3.8%, and 7.7% for TSH, fT₄, TT₄, fT₃, and TT₃, respectively. Coefficients of variation were 12.4% and 7.1% for TPOAb at 33 or 100 international units (IU)/L, respectively, and 10.9% and 8.6% for TgAb at 76 and 218 IU/L, respectively. The clinical reference values were 0.4–4.0 mIU/L for TSH, 0.9–2.8 nmol/L for T₃, 3.5–6.5 pmol/L for fT₃, 58–161 nmol/L for T₄, and 10–24 pmol/L for fT₄ (Table 1). TPOAb and TgAb concentrations were considered positive if they were >35 IU/mL or >115 IU/L (manufacturer cutoffs), respectively.

Statistical Analysis

We presented the distribution of urinary concentrations of phthalate metabolites using percentiles and geometric means \pm standard deviations. Due to right skewness, we log_e-transformed urinary concentrations of phthalate metabolites and used Spearman's rank order correlation coefficient (*r*_s) to assess correlations between urinary biomarker concentrations. Then, we standardized to create *z*-scores, and variables were included in a PCA with varimax rotation. We selected PCA components with eigenvalues >1 in our analysis (O'Rourke and Hatcher 2013) and derived factor scores as a weighted linear combination of phthalate metabolites using the loading factors. To investigate potential nonlinearity in the exposure–response associations, factor scores derived from the PCA were both evaluated as continuous as well as categorized into quintiles, with the lowest quintiles considered as the reference group. We presented participants' demographic and baseline reproductive characteristics using median \pm interquartile ranges (IQRs) or counts (%) for all women included in this analysis and by quintiles of PCA-derived factor scores, which were evaluated using Kruskal-Wallis tests for continuous variables and chi-squared/Fisher's exact tests for categorical variables.

Table 1. Distribution of serum biomarkers of thyroid function and autoimmunity among 558 women in the Environment and Reproductive Health (EARTH) Study.

	Mean	SD	25th percentile	50th percentile	75th percentile	Normal range
TSH (mU/L)	2.05	1.02	1.35	1.80	2.53	0.4–4.0
Free T ₄ (pmol/L)	15.3	2.08	13.9	15.2	16.5	10–24
Total T ₄ (nmol/L)	98.0	18.7	84.9	95.8	108	58–161
Free T ₃ (pmol/L)	4.80	0.61	4.40	4.71	5.14	3.5–6.5
Total T ₃ (nmol/L)	1.83	0.69	1.57	1.78	2.02	0.9–2.8
TgAb (IU/mL)	61.3	218	10.8	14.7	23.3	<35
TPOAb (IU/mL)	25.9	53.9	9.17	11.7	16.0	<115

Note: IU, international units; SD, standard deviation; TgAb, thyroglobulin antibodies; TPOAb, thyroperoxidase antibodies; TSH, thyroid-stimulating hormone; T₃, triiodothyronine; T₄, thyroxine.

We used *r*s values to assess correlations between serum concentrations of thyroid function and autoimmunity biomarkers; serum concentrations of TgAb and TPOAb were right skewed, and we natural log transformed them before analysis to more closely approximate a normal distribution. Multivariable general linear models were used to evaluate the associations between factor scores derived from the PCA, evaluated both as continuous and categorical exposures, and thyroid and autoimmunity biomarkers. When modeling PCA scores by quintiles of their distribution, we present population marginal means (Searle et al. 1980) over groups, adjusted for all the covariates in the model (for which continuous variables were fixed at the mean level and categorical ones were weighted according to their relative frequencies). For models with continuous exposures, beta coefficients and 95% confidence intervals (95% CIs) are presented. To assess the robustness of the autoimmunity findings, we conducted sensitivity analyses including TgAb and TPOAb positivity and also further adjusted for TgAb and TPOAb positivity in the PCA mixture approach. In addition, we performed a sensitivity analysis of urinary phthalate metabolites concentrations, assessed individually, with serum TgAb and TPOAb concentrations due to the scarce epidemiologic literature between urinary phthalates and autoimmunity.

We next used BKMR, a nonparametric approach for chemical mixtures that flexibly models the joint effect of chemicals with a kernel function (Bobb et al. 2015; Valeri et al. 2017). BKMR allows evaluation of the relative importance of individual chemicals in the mixture–outcome association and visualization of the exposure–response association for each component of the mixture while accounting for the correlation between the mixture components. We presented results from the BKMR analysis by displaying the

difference in serum thyroid biomarker concentrations for a change in urinary concentration of phthalate metabolites between the 25th and 75th percentiles, along with corresponding credible intervals for this difference. Each measurement of loge-transformed phthalate metabolite concentrations was treated as a continuous predictor, and the other components of the mixture were set at their median values while adjusting for confounders.

We assessed confounding using prior knowledge on biological relevance and descriptive statistics from our study participants, which included factors associated with urinary phthalate biomarker concentrations and thyroid biomarkers that were not in the causal pathway between exposure and outcome. We initially consider potential confounders those variables showed in Table 2 and scores of the other factors. Models were finally adjusted for age (years), BMI (kilogram per meter squared), race (white vs. other), and scores of the other factors (continuous, not included in the BKMR models). We also included urinary SG as a covariate in the models to account for dilution. Statistical analyses were performed with SAS (version 9.4; SAS Institute Inc.), with the exception of the BKMR models, which were conducted using the R package BKMR (R 3.6.2; R Development Core Team) (Bobb et al. 2018). Statistical tests were two-tailed, and all *p*-values < 0.05 were conventionally regarded as statistically significant, although emphasis was placed on consistency of findings across analyses.

Results

Detection frequencies for the eight urinary phthalate metabolite concentrations exceeded 90%, with the exception of MEHP (68%)

Table 2. Demographics and reproductive characteristics {median [interquartile range (IQR)] or *n* (%)} by quintiles of principal component analysis (PCA)–derived factor scores for the di(2-ethylhexyl) (DEHP) and non-DEHP factors among 558 women in the present analysis from the Environment and Reproductive Health (EARTH) Study.

	Total cohort	DEHP factor scores			Non-DEHP factor scores		
		Q1 (<i>n</i> = 111)	Q5 (<i>n</i> = 111)	<i>p</i> -Value ^a	Q1 (<i>n</i> = 111)	Q5 (<i>n</i> = 111)	<i>p</i> -Value ^a
Demographics							
Age (y)	34.0 (32.0, 38.0)	34.0 (31.0, 38.0)	36.0 (32.0, 38.0)	0.30	35.0 (32.0, 38.0)	33.0 (31.0, 37.0)	0.009
White (race) [<i>n</i> (%)]	460 (83)	89 (81)	92 (83)	0.78	98 (88)	83 (75)	0.07
Body mass index (kg/m ²)	23.5 (21.3, 26.7)	23.3 (21.3, 25.7)	23.5 (21.4, 26.4)	0.41	22.7 (21.2, 25.2)	23.8 (21.4, 27.6)	0.01
Ever smoked [<i>n</i> (%)]	154 (28)	35 (32)	26 (23)	0.55	29 (26)	30 (28)	0.38
Education [<i>n</i> (%)]	—	—	—	0.98	—	—	0.32
High school/some college	111 (20)	19 (17)	24 (22)	—	19 (17)	29 (26)	—
College graduate	170 (30)	35 (32)	36 (32)	—	33 (30)	42 (38)	—
Graduate degree	277 (50)	57 (51)	51 (46)	—	59 (53)	40 (36)	—
Reproductive history							
Initial infertility diagnosis [<i>n</i> (%)]	—	—	—	0.64	—	—	0.52
Male factor	134 (24)	22 (20)	32 (29)	—	26 (23)	34 (31)	—
Female factor	195 (35)	40 (36)	40 (36)	—	38 (34)	37 (33)	—
Unexplained	229 (41)	48 (44)	39 (35)	—	47 (43)	40 (36)	—
History of ever being pregnant [<i>n</i> (%)]	234 (42)	47 (42)	39 (35)	0.59	47 (42)	45 (41)	0.95
History of infertility treatment [<i>n</i> (%)]	253 (45)	51 (46)	49 (44)	0.92	54 (49)	54 (49)	0.99

Note: —, no valid data available.

^aFrom Kruskal–Wallis tests for continuous variables and chi-squared tests for categorical variables. Some variables had missing data, including history of being of ever being pregnant (*n* = 2) and history of infertility treatment (*n* = 95).

(Table S1), and were similar to those reported in U.S. females from the general population (CDC 2018). Urinary concentrations of phthalate metabolites declined over the study period (2005–2015), with the exception of MBzP and MiBP, which remained stable. Urinary concentrations of MEHHP, MEOHP, and MECPP were highly correlated with each other ($r_s = 0.80–0.95$), as were MBP and MiBP concentrations ($r_s = 0.82$) (Table S1). Urinary concentrations of other phthalate metabolites were moderately correlated with each other ($r_s = 0.43–0.78$). We included the urinary phthalate metabolites in the PCA analysis and we identified two components (eigenvalue > 1), accounting for 69% and 14%, respectively, of the total variance in urinary phthalate metabolites concentrations (Table S2). Factor 1, or the di(2-ethylhexyl) (DEHP) factor, had high loading scores of urinary concentrations of the four DEHP metabolites measured (MEHP, MEHHP, MEOHP, and MECPP) (Table S3). Factor 2, which we refer to as the non-DEHP factor, was characterized by high loading scores of urinary MBP, MiBP, MEP, and MBzP concentrations. Median serum concentrations of TSH, fT₄, TT₄, fT₃, TT₃, TgAb, and TPOAb were 1.80 (IQR: 1.35, 2.53) mU/L, 15.2 (IQR: 13.9, 16.5) pmol/L, 95.8 (IQR: 84.9, 108) nmol/L, 4.71 (IQR: 4.40, 5.14) pmol/L, 1.78 (IQR: 1.57, 2.02) nmol/L, 14.7 (IQR: 10.8, 23.3) IU/mL, and 11.7 (IQR: 9.17, 16.0) IU/mL, respectively (Table 1). TPOAb positivity and TgAb positivity were detected in 100 (18%) and 58 (10%) women, respectively. Serum concentrations of thyroid function biomarkers were moderately to highly correlated (r_s range = 0.20–0.68), except for TSH (r_s range = 0.06–0.08). Serum autoimmunity biomarker concentrations were moderately correlated to each other ($r_s = 0.50$).

The 558 women included in this analysis had a median age of 34.0 (IQR: 32.0, 38.0) y and BMI of 23.5 (IQR: 21.3, 26.7) kg/m² and were predominantly white (83%), and 28% ever smoked (Table 2). Women in the highest quintile of the non-DEHP factor scores were more likely to be younger [median age of 33 (IQR: 31, 37) y vs. 35 (IQR: 32, 38) y] and heavier [median BMI of 23.8 (IQR: 21.4, 27.6) kg/m² vs. 22.7 (IQR: 21.2, 25.2) kg/m²] and less likely to be white (75% vs. 88%) compared to women in the lowest quintile. No other demographics, reproductive characteristics, or thyroid biomarkers significantly differed across quintiles of the DEHP and non-DEHP factor scores (Table 2). Women included in this analysis were similar in their demographic and reproductive characteristics compared to women who were excluded from the analysis because of a lack of measured serum concentrations of thyroid and autoimmunity biomarkers (Table S4).

In adjusted models, the DEHP factor was inversely related to serum concentrations of fT₄, TT₄, fT₃, and TT₃ (Table 3). Specifically, compared to women in the lowest quintile of the DEHP factor scores, women in the highest quintile had significantly lower serum concentrations of fT₄, TT₄, fT₃, and TT₃ [absolute difference: -0.62; 95% CI: -0.123, -0.01]; $p = 0.04$; absolute difference: -8.31; 95% CI: -13.8, -2.85; $p = 0.003$; absolute difference: -0.37; 95% CI: -0.54, -0.19; $p < 0.0001$; and absolute difference: -0.21; 95% CI: -0.32, -0.10; $p = 0.003$, respectively]. Similar associations were observed when models were further adjusted for TgAb and TPOAb positivity (data not shown). Nevertheless, the DEHP factor was unrelated to serum concentrations of TSH, TgAb, or TPOAb (as continuous or binary outcomes). In contrast, the non-DEHP factor was not related to any thyroid function or autoimmunity biomarkers we examined (Table 3). Consistently, no associations were also found between the DEHP and non-DEHP factors with TgAb or TPOAb positivity (data not shown). Additionally, we found, overall, no associations between urinary phthalate metabolite concentrations, assessed individually, and autoimmunity biomarkers, although we observed lower serum

Table 3. Serum biomarkers of thyroid function and autoimmunity by principal component analysis (PCA)-derived factor scores for the di(2-ethylhexyl) (DEHP) and non-DEHP metabolites among 558 women in the Environment and Reproductive Health (EARTH) Study.

	TSH (mU/L)	p	Thyroid function			Autoimmunity									
			Free T ₄ (pmol/L)	p-Value	Total T ₄ (nmol/L)	Free T ₃ (pmol/L)	p-Value	Total T ₃ (nmol/L)	p-Value	TgAb (IU/mL)	p-Value	TPOAb (IU/mL)	p-Value		
DEHP factor	Q1	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference		
	Q2	2.13 (1.93, 2.32)	0.30	15.4 (15.0, 15.8)	0.87	103 (98.9, 106)	0.07	4.84 (4.73, 4.95)	0.004	1.82 (1.74, 1.89)	0.004	22.8 (18.7, 27.7)	0.61	14.4 (12.4, 16.8)	0.90
	Q3	1.99 (1.79, 2.17)	0.98	15.3 (14.9, 15.6)	0.39	97.4 (94.0, 101)	0.04	4.81 (4.70, 4.92)	0.09	1.83 (1.76, 1.90)	0.01	22.7 (18.7, 27.6)	0.61	15.8 (13.7, 18.4)	0.34
	Q4	2.12 (1.93, 2.31)	0.62	15.4 (15.0, 15.8)	0.80	97.5 (94.1, 101)	0.06	4.84 (4.73, 4.96)	0.34	1.80 (1.73, 1.87)	0.003	20.4 (16.7, 24.8)	0.81	15.1 (13.0, 17.6)	0.61
	Q5	2.05 (1.85, 2.25)	0.37	14.9 (14.5, 15.3)	0.04	94.3 (90.7, 98.0)	0.003	4.56 (4.44, 4.68)	< 0.001	1.76 (1.68, 1.83)	0.0003	19.9 (16.2, 24.5)	0.72	13.1 (11.2, 15.4)	0.50
	β (95% CI)	0.01 (-0.08, 0.11)	0.78	-0.21 (-0.41, -0.01)	0.04	-2.66 (-4.44, -0.88)	0.004	-0.12 (-0.18, -0.06)	< 0.001	-0.07 (-0.11, -0.03)	0.0003	-0.07 (-0.17, 0.03)	0.17	-0.06 (-0.14, 0.02)	0.13
Non-DEHP factor	Q1	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference		
	Q2	2.00 (1.79, 2.19)	0.71	15.6 (15.2, 16.0)	0.43	101 (96.9, 105)	0.07	4.96 (4.83, 5.10)	0.005	1.87 (1.79, 1.96)	0.001	21.1 (17.1, 26.1)	0.44	16.3 (13.6, 19.4)	Reference
	Q3	1.87 (1.68, 2.05)	0.22	15.0 (14.6, 15.3)	0.005	96.4 (92.7, 100)	0.07	4.74 (4.62, 4.86)	0.005	1.73 (1.65, 1.80)	0.01	18.6 (13.1, 23.0)	0.44	13.5 (11.5, 15.8)	0.09
	Q4	2.14 (1.94, 2.34)	0.58	14.7 (14.3, 15.1)	0.006	94.6 (91.0, 98.2)	0.03	4.71 (4.60, 4.83)	0.009	1.83 (1.75, 1.90)	0.49	20.7 (16.9, 25.4)	0.98	13.2 (11.3, 15.4)	0.10
	Q5	2.22 (1.98, 2.45)	0.38	15.5 (15.0, 16.0)	0.43	101 (96.4, 105)	0.97	4.84 (4.71, 4.98)	0.28	1.92 (1.83, 2.01)	0.52	26.0 (20.4, 33.1)	0.25	16.9 (14.0, 20.3)	0.80
	β (95% CI)	0.09 (-0.04, 0.22)	0.18	-0.24 (-0.50, 0.02)	0.07	-0.72 (-3.08, 1.63)	0.55	-0.06 (-0.13, 0.02)	0.15	0.01 (-0.04, 0.06)	0.63	0.04 (-0.09, 0.18)	0.52	-0.01 (-0.11, 0.09)	0.87

Note: Data are presented as predicted marginal means (95% CI) unless otherwise noted, adjusted for age, BMI, race, specific gravity, and non-DEHP factor scores for DEHP factor analyses and DEHP factor scores for non-DEHP factor analyses. CI, confidence interval; IU, international units; TgAb, thyroglobulin antibodies; TPOAb, thyroperoxidase antibody; TSH, thyroid-stimulating hormone; T₃, triiodothyronine; T₄, thyroxine.

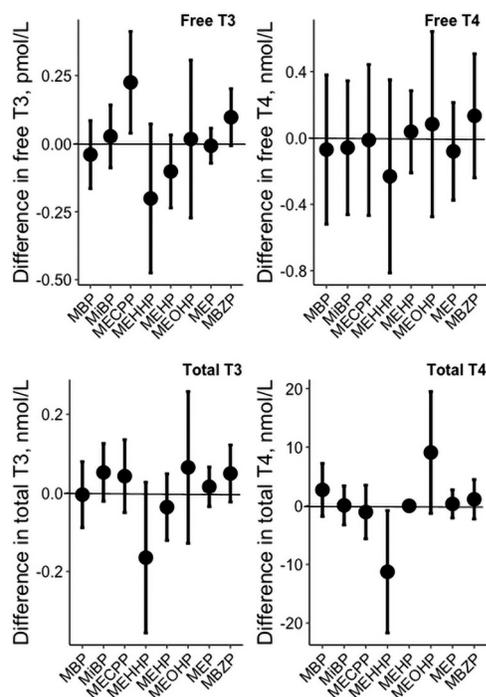


Figure 1. Differences with 95% credible intervals in thyroid biomarker concentration for an interquartile range change in each log-transformed phthalate metabolite, fixing other phthalate metabolite concentrations at median values. We used Bayesian kernel machine regression (BKMR) models adjusting for age, body mass index (BMI), race, and specific gravity. Numerical values are reported in Table S5. Note: MBP, mono-*n*-butyl phthalate; MBzP, monobenzyl phthalate; MECPP, mono(2-ethyl-5-carboxypentyl) phthalate; MEHHP, mono(2-ethyl-5-hydroxyhexyl) phthalate; MEHP, mono(2-ethylhexyl) phthalate; MEOHP, mono(2-ethyl-5-oxohexyl) phthalate; MEP, monoethyl phthalate; MiBP, monoisobutyl phthalate; T₃, triiodothyronine; T₄, thyroxine.

concentrations of TgAb or TPOAb with urinary concentrations of DEHP metabolites (Table S5).

We then conducted BKMR analyses focusing on thyroid biomarkers associated with the DEHP factor (i.e., fT₄, TT₄, fT₃, and TT₃) (Figure 1). For all four outcomes, MEHHP was found to be the most important contributor to the negative association between DEHP metabolites and thyroid biomarkers. For example, an IQR increase in MEHHP was associated with a reduction of -0.17 nmol/L in TT₄ (95% credible interval: $-0.36, 0.02$). For TT₃ and TT₄, a positive association was also observed with MEOHP. For serum fT₃, positive associations were instead observed with MECPP and MBzP. The interpretation of association detected with MEOHP and MEHHP requires caution, as these can still be influenced by the high correlation between these two metabolites, as also suggested by the wider credible intervals reported for DEHP metabolites.

Discussion

We evaluated whether urinary concentrations of eight phthalate metabolites, as a mixture, were associated with markers of thyroid function or thyroid autoimmunity among women seeking fertility treatment at a fertility center in Boston. Two main factors were identified using PCA: the DEHP factor, characterized by relatively high urinary concentrations of four DEHP metabolites, MEHP, MEHHP, MEOHP, and MECPP, and the non-DEHP factor, with relatively high urinary concentrations of MBP, MiBP, MEP, and MBzP. We found that DEHP factor scores were associated with lower serum concentrations of fT₄, TT₄, fT₃, and TT₃; however,

we found no associations for serum TSH, TgAb, or TPOAb concentrations. BKMR results identified urinary concentrations of MEHHP as the primary contributor to the lower serum fT₄, TT₄, fT₃, and TT₃ observed. Women in our cohort had serum thyroid function and autoimmunity biomarker concentrations within normal ranges. Nevertheless, the thyroid system is stringently regulated, and any effects on serum biomarker concentrations may have relevant physiological consequences, especially among this group of subfertile women. Since thyroid hormone regulates metabolism in practically all tissues in the human body, including those in the female reproductive tract, not only overt thyroid disease but also mild forms of thyroid dysfunction or/and autoimmunity have been associated with reduced overall fertility, which can be reversed by reestablishing a euthyroid state (Krassas et al. 2010). We observed decreased ovarian reserve with some biomarkers of thyroid function and autoimmunity highlighting a possible mechanism for adverse human reproduction by urinary phthalates and (Korevaar et al. 2018). Furthermore, alterations in maternal thyroid hormones have been associated with pregnancy complications (Allan et al. 2000), fetal development (Koibuchi 2008), and offspring health (Idris et al. 2005; Thompson et al. 2018). Thus, results from this analysis are noteworthy, as women included in this study are attempting pregnancy.

Using the same study cohort, we have previously identified similar PCA factors (DEHP and the non-DEHP) examining phthalate metabolite mixtures and birth weight (Chiu et al. 2018), as well as *in vitro* fertilization outcomes (Minguez-Alarcón et al. 2019), which have also been shown in another pregnancy cohort (Maresca et al. 2016). DEHP is commonly added to vinyl plastics to make them flexible and thus can be found in numerous consumer products, flooring and wall coverings, food contact applications, and medical devices. Precursors of MBP and MiBP are used as solvents and plasticizers for coatings, varnishes, and cellulose acetate (some are also found in personal care products); thus, these two phthalate metabolites may result from shared exposure sources as well (Braun et al. 2014; Hauser and Calafat 2005). By using PCA, we are converting a set of measured variables into principal components based on their correlations rather than the underlying biological effects of a mixture on the outcomes. Thus, the identified principal components reflect exposure patterns.

To our knowledge, only two epidemiological studies to date have investigated mixtures of phthalate metabolites in relation to thyroid hormones among European (Villanger et al. 2020) and U.S. pregnant women (Romano et al. 2018), and none of them applied the sophisticated BKMR approach to analyze chemical mixtures. Villanger and colleagues recently explored mixtures of urinary phthalate metabolites and stratification by iodine intake (measured using a food frequency questionnaire) in relation to thyroid hormones and TPOAb positivity measured in plasma samples of pregnant women in Norway with urinary phthalate metabolite concentrations between twice and three times higher than women in our study (Villanger et al. 2020). In this Norwegian cross-sectional study using PCA, and consistent with our results, the authors observed negative associations between one of the two factors, which had high loading scores of urinary DEHP/diisononyl phthalate (DiNP) metabolite concentrations and plasma fT₃ and TT₃, which were consistent across different levels of iodine intake. However, and contrary to our results, no associations between the DEHP/DiNP factor and plasma fT₄ and TT₄ were observed. Additionally, the authors reported higher plasma concentrations of TSH and lower fT₄ and TT₄, with high loading scores of the other factor, characterized by high urinary concentrations of MiBP, MnBP, and MBzP, only among women with sufficient iodine intake (≥ 150 μ g/d). Similar to our results, none of the two derived PCA factors were associated with TPOAb positivity regardless of

iodine consumption. Romano et al. (2018) reported negative associations between mixtures of urinary phthalate metabolites, calculated using weighted quantile sum regression, and serum concentrations of TT₄ that remained in secondary analyses, further adjusting for urinary iodine. This negative association was mainly driven by urinary MEP, MiBP, and MBzP, which had the largest weights within the mixture. Contrary to our results, the authors did not find any associations between the phthalate metabolite mixture and other measured thyroid biomarkers (TSH, TT₃, fT₄, and fT₃) among women in their study, despite having, overall, higher urinary phthalate metabolite concentrations compared to women included in our study. For example, urinary MEP among these pregnant women was about 5-fold higher compared to those among women in our study. Other differences between both studies included design (prospective vs. cross-sectional) and population included (pregnant vs. nonpregnant). Related to chemical mixtures and thyroid function and including a very different study population compared to ours, a cross-sectional study among NHANES participants 12 to 85 years of age and exploring a mixture of nine EDCs, including six phthalates, using structural equation models in relation to thyroid hormones, found no associations in females (Przybyla et al. 2018). Other epidemiological studies of pregnant women evaluated urinary phthalate metabolites individually in relation to thyroid function, and results have been inconsistent in terms of the direction of the associations, the timing of exposure during gestation, and the specific associated phthalate metabolite (Huang et al. 2007, 2016; Johns et al. 2015, 2016; Kuo et al. 2015; Yao et al. 2016). A recent meta-analysis that included data from adults, as well as children, found negative associations between DEHP metabolites and HPT axis markers, specifically serum TT₄ concentrations (Kim et al. 2019). Our study population includes women attempting to conceive through fertility treatments. Given the increasing prevalence of infertility (10%–15%) in modern societies (Inhorn and Patrizio 2015; Mascarenhas et al. 2012) and the fact that successful folliculogenesis, oocyte maturation, implantation, and early pregnancy maintenance all depend on the delicate interaction of the thyroid with the reproductive system, the identification of environmental factors that may adversely impact this balance is of public health concern.

Although it is important to identify mechanisms through which phthalate metabolites may alter thyroid homeostasis, our study was not designed to address this question. However, some *in vitro* and *in vivo* studies have elucidated potential mechanisms underlying phthalate metabolites' actions on the thyroid system. The HPT axis is a highly conserved and tightly controlled system, and phthalates have been shown to potentially disrupt this system by interfering at multiple sites. For example, phthalates have been shown to directly affect thyroid gland histology, interfere with TSH and thyrotropin-releasing hormone (TRH) receptor action, alter thyroid hormone biosynthesis and clearance, and also affect the production of binding proteins, as these are regulated by estrogens and competitively bind to thyroid-binding proteins based on their structural similarity (Dong et al. 2017; Liu et al. 2015; Sun et al. 2018; Zhai et al. 2014). At the level of the thyroid gland, DEHP exposure in rats caused histological changes in the thyroid that included follicular epithelial cell hypertrophy (characterized by an increase in the size of cells due to distended cytoplasm, which appeared otherwise foamy and vacuolated) and follicular epithelial cell hyperplasia (contributing to the narrowing of the thyroid follicular lumen) (Liu et al. 2015). Down-regulation of the TSH and up-regulation of the TRH receptor were also noted. Similarly, adverse effects on the protein levels of TRH in the hypothalamus (down-regulation), the protein and mRNA levels of TRH receptor in the pituitary (up-regulation), and on the TSH-receptor in the thyroid (down-regulation) after DEHP exposure in rats were reported (Sun et al. 2018). Altered thyroid

hormone biosynthesis, biotransformation, transport, and metabolism have been also demonstrated after exposure to DEHP, resulting in significant reductions in the serum concentrations of TT₃, TT₄, fT₃, and fT₄ (Liu et al. 2015; Sun et al. 2018). Moreover, Liu et al. reported that exposure to DEHP was associated with altered membranes and enzymes involved in iodization processes. More specifically, a study in rats demonstrated that exposure to dibutyl phthalate can amplify a thyroid autoimmune response mediated through changes in oxidative stress and proinflammatory factors such as interleukin 17 (Duan et al. 2018). Further epidemiological studies are needed to clarify the associations between investigate urinary phthalate metabolites and thyroid autoimmunity.

Our study has some distinct strengths. The most highlighted strength of the current manuscript is the use of the sophisticated BKMR approach to analyze chemical mixtures that, to our knowledge, has not been applied in the previous epidemiologic studies investigating mixtures of urinary phthalate metabolites in relation to thyroid function among women. Participants were derived from one center, enrolled in a well-established cohort study, and had homogeneous demographics, reducing the potential for confounding. Also, all serum and urine samples were collected and processed under one protocol prior to the determination of the thyroid function and thyroid autoimmunity biomarkers, and urinary phthalate metabolite concentrations were quantified at the CDC using the analytical approach used in other studies, including NHANES.

The current study also has several limitations. First, the cross-sectional design prevents establishing that exposures occur before the outcomes. Second, it may not be possible to generalize our findings to men or to women in the general population. Third, results should be interpreted with caution because coexposures to other chemicals were not accounted for, and exposure to phthalates may be reflective of other unknown lifestyle factors that might be affecting thyroid biomarkers. Fourth, residual confounding by unmeasured factors related to both phthalate and thyroid hormone absorption, distribution, metabolism, and excretion (e.g., urinary iodine) may be possible. Finally, there may be some concern regarding exposure misclassification because of the episodic exposure of these nonpersistent chemicals and their short biological half-lives (Perrier et al. 2016; Vernet et al. 2019).

Conclusion

We observed that mixtures of urinary DEHP metabolites were inversely associated with serum biomarkers of thyroid function and/or regulation of the HPT axis but not with autoimmunity among women attending a fertility center. Further epidemiologic research to confirm these findings in other study populations, especially older women who are at higher risk to develop thyroid autoimmunity, and experimental studies to investigate potential molecular mechanisms that explain the observed findings, are warranted given the potential public health impact of these results.

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The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the CDC, the US Government, the Department of Health and Human Services (DHHS), or the National Institutes of Health (NIH). The use of trade names is for identification only and does not imply endorsement by the CDC, the Public Health Service, or DHHS.

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