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Research Article

Associations Between Repeated Measures of Urinary Phthalate Metabolites With Hormones and Timing of Natural Menopause

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Abstract

Phthalates, ubiquitous endocrine-disrupting chemicals, may affect ovarian folliculogenesis and steroidogenesis. We examined the associations of urinary phthalate metabolites with hormones including estradiol, testosterone, follicle-stimulating hormone (FSH), sex hormone–binding globulin (SHBG), and anti-Müllerian hormone (AMH), and timing of natural menopause in midlife women. Data were from 1189 multiracial/multiethnic women aged 45 to 56 years without hormone therapy from the Study of Women's Health Across the Nation (SWAN). Urinary concentrations of 12 phthalate metabolites and hormones were repeatedly measured in 1999 to 2000 and 2002 to 2003, resulting in a total of 2111 observations. Linear mixed-effect models were used to calculate percentage differences (%D) and 95% CIs in serum concentrations of estradiol, testosterone, FSH, SHBG, and AMH. Cox proportional-hazards models were used to calculate hazard ratios (HRs) and 95% CIs of natural menopause. We observed statistically significant associations of phthalate metabolites with lower testosterone concentrations: MCOP with testosterone (%D: −2.08%; 95% CI, −3.66 to −0.47) and MnBP with testosterone (%D: −1.99%; 95% CI, −3.82 to −0.13), after adjusting for multiple comparisons with false discovery rates less than 5%. Lower AMH concentrations were also found with higher MECPP (%D: −14.26%; 95% CI, −24.10 to −3.14), MEHHP (%D: −15.58%; 95% CI, −24.59 to −5.50), and MEOHP (%D: −13.50%; 95% CI, −22.93 to −2.90). No associations were observed for other hormones or timing of natural menopause. These results suggest that exposure to phthalates may affect circulating levels of testosterone and diminish the ovarian reserve in midlife women. Given the widespread exposure, reduced exposure to phthalates may be a key step to prevent reproductive effects of phthalates.

Key Words: phthalates, endocrine-disrupting chemicals, hormones, anti-Müllerian hormone (AMH), menopause, midlife women

Abbreviations: %D, percentage difference; AMH, anti-Müllerian hormone; BMI, body mass index; CV, coefficient of variation; DEHP, di-(2-ethylhexyl) phthalate; FDR, false discovery rate; FMP, final menstrual period; FSH, follicle-stimulating hormone; HMW, high-molecular weight; HR, hazard ratio; HT, hormone therapy; IQR, interquartile range; LMW, low-molecular weight; LOD, limit of detection; MBzP, mono-benzyl phthalate; MCNP, mono-carboxyisononyl phthalate; MCOP, mono-carboxyoctyl phthalate; MCPP, mono-(3-carboxypropyl) phthalate; MECPP, mono-2-ethyl-5-carboxypentyl phthalate; MEHHP, mono-(2-ethyl-5-hydroxyhexyl) phthalate; MEHP, mono-(2-ethyl)-hexyl phthalate; MEOHP, mono-(2-ethyl-5-oxohexyl) phthalate; MEP, mono-ethyl phthalate; MiBP, mono-isobutyl phthalate; MnBP, mono-n-butyl phthalate; MPS, SWAN Multi-Pollutant Study; NHANES, National Health and Nutrition Examination Survey; PPARs, peroxisome proliferator-activated receptors; SHBG, sex hormone–binding globulin; SWAN, Study of Women's Health Across the Nation.

Phthalates have been widely used in the manufacturing of consumer products. Low-molecular-weight (LMW) phthalates, such as diethyl phthalate (DEP) and di-n-butyl phthalate (DnBP), are used as scent stabilizers in personal care products and cosmetics [1, 2]. High-molecular-weight (HMW) phthalates such as di(2-ethylhexyl) phthalate (DEHP) are employed as plasticizers in plastic tubing, food packaging materials, and medical devices [3]. Phthalates are released into the environment and enter the human body through oral ingestion, inhalation, and/or dermal contact [4]. On exposure, phthalates are rapidly metabolized and excreted in urine [1-3]. Despite the short half-lives of phthalates, most people in the United States have detectable levels of phthalate metabolites in urine, suggesting that phthalate exposure is widespread in the general population [5]. Women are often exposed to higher levels of phthalates than men, likely attributable to extensive use of personal care products and cosmetics [6, 7].

Recently, several phthalates have been identified as reproductive toxicants in animals and suspected endocrine-disrupting chemicals in humans [8]. Experimental studies suggest that phthalates can interfere with steroid hormone synthesis, possibly through suppressing aromatase transcripts and activating peroxisome proliferator-activated receptors (PPARs) in the granulosa cells [9, 10]. While several human

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studies have documented the associations between phthalates and sex hormones, most studies are cross-sectional, and the results are inconsistent [11-17].

To our knowledge, no previous epidemiologic studies have evaluated the associations of phthalates with hormones assessed longitudinally in midlife women. Since midlife women experience fluctuations in hormones, it is crucial to understand the potential effects of phthalates on hormone levels during the menopausal transition. Evaluating the effects of phthalate metabolites on anti-Müllerian hormone (AMH) in the naturally aging population is important to determine risks for phthalates-related accelerated ovarian aging. Owing to short excretion half-lives of phthalates in humans, repeated measures are critical to provide robust estimates of exposure over time.

We examined the associations between urinary phthalate metabolites and sex hormone levels including estradiol, testosterone, follicle-stimulating hormone (FSH), AMH, and sex hormone–binding globulin (SHBG) during the menopausal transition using data from the Study of Women's Health Across the Nation (SWAN). We also explored the associations between phthalate metabolites and timing of natural menopause. Furthermore, since whether alterations in sex hormone levels vary by menopausal status remains largely undetermined, we assessed whether the associations differ by menopausal stages.

Materials and Methods

Study Population

SWAN is a prospective cohort study of 3302 women of 5 racial/ethnic groups (White, Black, Hispanic, Chinese, and Japanese) aged 42 to 52 years and premenopausal at the baseline visit (1996-1997). Participants were recruited from 7 centers across the United States (Boston, Massachusetts; Chicago, Illinois; Southeast Michigan; Los Angeles, California; Newark, New Jersey; Oakland, California; Pittsburgh, Pennsylvania). The present analysis included participants from the SWAN Multi-Pollutant Study (MPS). The study design of the SWAN MPS is described elsewhere [18-20]. Briefly, the SWAN MPS was initiated in 2016 to examine the potential health effects of multiple environmental chemicals among midlife women. We used repository serum and urine samples from SWAN visit 3 (1999-2000) and visit 6 (2002-2003) for environmental exposure assessment. A total of 2694 participants remained in the study at SWAN visit 3 (1999-2000). We excluded 368 participants from Chicago and 278 from Newark since repository urine samples were not collected at these 2 sites. Additionally, 648 women with insufficient volumes of serum or urine samples were excluded, resulting in 1400 women who constitute the SWAN MPS. Women excluded because of insufficient biospecimens were different from those included in the analysis in that they were more likely to be obese, former or current smokers, and to have lower education attainments and lower physical activity levels [21]. The study protocols were approved by the institutional review board of each participating institution, and all study participants provided written informed consent.

The study design is illustrated in Supplementary Fig. S1 [22]. For the analysis of phthalate metabolites and sex hormones including estradiol, testosterone, FSH, and SHBG, we included women with repeated measures of phthalates and hormones at SWAN visit 3 (1999-2000) and visit 6 (2002-2003) while not receiving hormone therapy (HT). The final sample size was 1189 women with 2111 observations. AMH became undetectable among women reaching natural menopause, and thus SWAN used an ultrasensitive assay to detect low levels of AMH [23]. For the analysis of phthalate metabolites and AMH, we included a subset of 549 women with complete information on AMH, resulting in a total of 862 observations. Finally, for the analysis of phthalate metabolites and timing of natural menopause, we included 1082 women who were premenopausal at baseline, resulting in a total of 5017 person-years of follow-up.

Urinary Phthalate Metabolite Assessment

Spot urine samples were repeatedly collected during the menopausal transition, at SWAN visit 3 (1999-2000) and visit 6 (2002-2003) for phthalate measurements. Urinary concentrations of phthalate metabolites were assessed at NSF International (Ann Arbor, Michigan), a part of the Michigan Children's Health Exposure Analysis Resource (M-CHEAR) Laboratory Hub, using a method developed by the Centers for Disease Control and Prevention [24]. The analytical technique involved enzymatic deconjugation of glucuronidated phthalate monoester metabolites followed by online solid-phase extraction coupled to high-performance liquid chromatography–isotope dilution tandem mass spectrometry. The following 12 phthalate metabolites were measured in urine samples, including mono-ethyl phthalate (MEP), mono-isobutyl phthalate (MiBP), mono-n-butyl phthalate (MnBP), mono-benzyl phthalate (MBzP), mono-(2-ethyl)-hexyl phthalate (MEHP), mono-(2-ethyl-5-carboxylpentyl) phthalate (MECPP), mono-(2-ethyl-5-hydroxylhexyl) phthalate (MEHHP), mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP), mono-(3-carboxypropyl) phthalate (MCPP), mono-isononyl phthalate (MiNP), mono-carboxyoctyl phthalate (MCOP), and mono-carboxyisononyl phthalate (MCNP). The limits of detection (LODs) for individual metabolites ranged from 0.1 to 1.0 ng/mL. Phthalate metabolite concentrations below the LOD were assigned LOD/$\sqrt{2}$. MiNP was not included in data analysis because of its very low detection rates at SWAN visit 3 (1.1%) and visit 6 (0.7%). Comprehensive quality assessment/quality control procedures were conducted. The intra-assay coefficient of variation (CV) of low-, medium- and high-quality controls ranged from 3% to 20%, depending on the analyte. Urine creatinine was determined by the Cobas Mira analyzer (Horiba ABX) at visits 3 and 6 as markers of urine dilution.

Serum Hormone Assessment

Sex hormones (including estradiol, testosterone, FSH, and SHBG) were measured from fasting serum samples at each visit. As with the phthalates, hormone levels assessed at SWAN visit 3 (1999-2000) and visit 6 (2002-2003) were used for the analysis because of the short half-lives of these chemicals. These hormones were measured using the Automated Chemiluminescence System (ACS)-180 automated analyzer (Bayer Diagnostics Corp). Serum FSH concentrations were measured using 2 monoclonal antibodies directed to different regions on the beta subunit (Siemens catalog No. 04912924, RRID: AB_2895593), with an LOD of 1.05 mIU/mL. Serum estradiol concentrations were assessed using the rabbit anti-estradiol-6 ACS-180 immunoassay to increase sensitivity (Aviva Systems Biology catalog No. OAMA04038, RRID: AB_10877469), with an LOD of 1.0 pg/mL. Serum

testosterone concentrations were determined with the modified rabbit polyclonal anti-T ACS-180 immunoassay (Siemens catalog No. 05476206, RRID:AB_2783804), with an LOD of 2.19 ng/dL. SHBG assay was developed on-site using rabbit anti-SHBG antibodies (Siemens catalog No. 06520781, RRID: AB_2783801), with an LOD of 1.95 nM. Interassay and intra-assay CVs were 11% and 6% for estradiol, 12% and 6% for FSH, 10% and 8% for testosterone, and 10% and 6% for SHBG.

Anti-Müllerian Hormone Assessment

Fasting blood samples were collected between 8 and 10 AM on menstrual cycle days 2 through 5 to assess AMH. If 60 days had passed without drawing blood during this window because of irregular menstrual cycles, blood samples were collected at any time over the next 30 days. Serum was prepared promptly, frozen, and deposited at −80 °C until it was thawed for measurement. AMH was measured using the picoAMH enzyme-linked immunosorbent assay (ELISA) from Ansh Labs (Ansh Labs catalog No. AL-124, RRID: AB_2783675) with intraassay CVs ranging from 2.5% to 5.1% and interassay CVs ranging from 3.4% to 4.9%. The LOD was 1.85 pg/mL [23].

Natural Menopause Ascertainment

Natural menopause was assessed prospectively based on information collected at approximately annual or biannual interviews. The final menstrual period (FMP) or natural menopause was defined as 12 months of amenorrhea since the last menstrual period that was not due to other causes including hysterectomy, bilateral oophorectomy, or HT. At each visit, women were asked whether they had any menstrual bleeding in the last year, whether they had any menstrual bleeding in the last 3 months, and the date that she started her most recent menstrual bleeding. The age at the FMP was ascertained if a participant was reliably observed to have had a menstrual period followed by at least 12 consecutive months that were both HT free and bleed free.

Covariates

Covariates were selected based on a priori hypothesis [25]. Demographics, socioeconomic status, and health-related variables were collected through in-person interviews and self-administered questionnaires at annual visits. Only women without HT were included in this analysis. Demographic variables included age (time-varying), race/ethnicity, and study site. Race/ethnicity was categorized into White, Black, Chinese, and Japanese. Educational attainment was defined as high school or less, some college, college degree, or post-college degree. Health-related variables included smoking status (time-varying, never/former/current), physical activity (time-varying), and parity. Physical activity was estimated using the Kaiser Physical Activity Survey, which included 3 domains (sports/exercise, household/caregiving, and daily routine), with a minimum possible score of 3 and a maximum of 15 [26]. Parity was categorized into parous and nulliparous women. Body mass index (BMI, time-varying) was calculated as weight in kilograms divided by height in meters squared. Menopausal status (time-varying) was classified into 3 groups: premenopausal, early perimenopausal (change in length of bleed or interbleed interval) or late perimenopausal (no bleeding for 3-11 months), and postmenopausal (natural or surgical) [27].

Statistical Analyses

The distributions of participant characteristics were examined at the MPS baseline (1999-2000). Medians (interquartile ranges, IQRs) were calculated for continuous variables with skewed distributions. Categorical variables were expressed as percentages (%). Spearman correlation coefficients were computed for urinary phthalate concentrations between 2 time points. In repeated-measures analyses, we explored the associations between phthalate metabolites and hormones and AMH using multivariable linear mixed-effect models with random intercepts. For the associations between phthalate metabolites and timing of natural menopause, we used Cox proportional-hazards models. Time-varying phthalate metabolites were fit for both models with adjustment for age (time-varying), race/ethnicity, study site, education, smoking status (time-varying), physical activity (time-varying), parity, BMI (time-varying), menopausal status (time-varying), and urinary creatinine (time-varying). To evaluate effect modification by menopausal status, we repeated analyses stratified by menopausal status. Urinary concentrations of phthalate metabolites and hormones with skewed distributions were log-transformed to ensure normality. Log base 2 was applied to phthalate metabolites so that all regression coefficients and associated 95% CIs were expressed as percentage change in hormone levels for a doubling in urinary phthalate metabolite concentrations.

In addition to examining individual phthalate metabolites, we converted each phthalate metabolite to its molecular equivalent [28]. $\sum^{LMW}$ phthalate metabolites included MEP, MnBP, and MiBP, and were expressed as the molar sum of MEP. $\sum^{HMW}$ phthalate metabolites combined MBzP, MCNP, MCOP, MCPP, MEHP, MEHHP, MEOHP, and MECPP, and were expressed as the molar sum of MEHP. $\sum^{DEHP}$ metabolites included MEHP, MEHHP, MEOHP and MECPP, and were expressed as the molar sum of MEHP. $\sum^{Anti}$-androgenic phthalate metabolites included MBzP, MnBP, MEHP, MEHHP, MEOHP, and MECPP, and were expressed as the molar sum of MEHP [29]. $\sum^{Estrogenic}$ phthalate metabolites combined MEP, MnBP, MiBP, and MBzP, and were expressed as the molar sum of MEP [30]. Given the relatively large number of associations examined for hormones and timing of natural menopause, we addressed multiple comparisons at an false discovery rate (FDR) of 5% using the Benjamini-Hochberg method [31]. The validity of the Benjamini-Hochberg test depends on the hypothesis testing being independent [31]. Thus, we conducted multiple comparisons separately in the primary and secondary subgroup (or stratified) analyses. All data analyses were performed using SAS version 9.4 (SAS Institute Inc).

Results

Participant Characteristics and Urinary Concentrations of Phthalate Metabolites

The characteristics of the study population at SWAN visit 3 (1999-2000) are presented in Table 1. The median age was 49.4 years with an IQR of 47.3 to 51.5 years. The median (IQR) of sex hormone concentrations were 33.1 (IQR: 19.6 to 75.2) pg/mL for estradiol, 33.8 (IQR: 24.0 to 47.3) ng/dL

Table 1. Distributions of participant characteristics among the Multi-Pollutant Study population at Study of Women's Health Across the Nation visit 3 (1999-2000, N = 1189)

Characteristic	Median (IQR) or N (%)
Sex hormone	
Estradiol, pg/mL	33.1 (19.6-75.2)
Testosterone, ng/dL	33.8 (24.0-47.3)
FSH, IU/L	26.2 (13.5-63.7)
SHBG, nM	37.7 (24.9-53.6)
Covariate	
Age, y	49.4 (47.3-51.5)
Race/ethnicity	
White	565 (47.5%)
Black	271 (22.8%)
Chinese	159 (13.4%)
Japanese	194 (16.3%)
Study site	
Southeast MI	215 (18.1%)
Boston, MA	206 (17.3%)
Oakland, CA	263 (22.1%)
Los Angeles, CA	317 (26.7%)
Pittsburgh, PA	188 (15.8%)
Education	
High school or less	215 (18.2%)
Some college	374 (31.6%)
College degree	296 (25.0%)
Post-college degree	297 (25.1%)
Smoking status	
Never smoker	756 (63.6%)
Former smoker	313 (26.3%)
Current smoker	120 (10.1%)
Physical activity	7.8 (6.6-9.0)
Parity	
Nulliparous	234 (19.7%)
Parous	955 (80.3%)
BMI	26.1 (22.5-31.8)
Menopausal status	
Premenopause	167 (14.0%)
Early perimenopause	735 (61.8%)
Late perimenopause	120 (10.1%)
Natural postmenopause	158 (13.3%)
Surgical postmenopause	9 (0.8%)

Abbreviations: BMI, body mass index; CA, California; FSH, follicle-stimulating hormone; IQR, interquartile range; MA, Massachusetts; MI, Michigan, PA, Pennsylvania; SHBG, sex hormone–binding globulin.

for testosterone, 26.2 (IQR: 13.5 to 63.7) IU/L for FSH, and 37.7 (IQR: 24.9 to 53.6) nM for SHBG. The study population included 47.5% White women, 22.8% Black women, 13.4% Chinese women, and 16.3% Japanese women. Women tended to be never smokers (63.6%), parous (80.3%), receive college degrees or above (50.1%) and enter early perimenopause (61.8%). Detection rates, distributions, and molecular weights of phthalate metabolites at visits 3 and 6, and correlations of each metabolite between visits 3 and 6, are shown in Table 2. Phthalates were moderately and positively correlated over time, with Spearman correlation ranging from 0.31 to 0.44. Compared with visit 3 (1999-2000), we observed statistically significantly decreased urinary concentrations of MEP, MBzP, MCPP, MCOP, and MCNP. We detected significant increases in urinary concentrations of MiBP and almost all DEHP metabolites, except for MEHP ($P = .77$). MnBP also remained similar over time ($P = .86$).

Associations of Phthalate Metabolites With Testosterone, Estradiol, Follicle-stimulating Hormone, and Sex Hormone–binding Globulin

Higher urinary concentrations of MCOP, MCPP, and MnBP were independently associated with lower serum testosterone concentrations in the total population (Table 3). The fully adjusted percentage change in testosterone associated with a doubling in phthalate metabolites was −2.08% (95% CI, −3.66 to −0.47) for MCOP, −2.00% (95% CI, −3.94 to −0.02) for MCPP, and −1.99% (95% CI, −3.82 to −0.13) for MnBP. We detected statistically significant inverse associations of MCOP, MECPP, MEHHP, MEOHP, MiBP, $\sum^{DEHP}$, $\sum^{HMW}$, and $\sum^{Anti}$-androgenic phthalate metabolites in postmenopausal women (see Table 3). In particular, a doubling in the molar sum of phthalate metabolites was related to lower testosterone with a percentage change of −3.83% (95% CI, −6.97 to −0.59) for $\sum^{DEHP}$, −3.52% (95% CI, −7.01 to −0.10) for $\sum^{HMW}$, and −4.18% (95% CI, −8.00 to −0.20) for $\sum^{Anti}$-androgenic phthalate metabolites. In addition, MCOP was independently associated with lower testosterone in premenopausal women (percentage change: −4.99%; 95% CI, −9.41 to −0.36). MCPP was also inversely related to testosterone during early and late perimenopause (percentage change: −2.99%; 95% CI, −5.40 to −0.51). After adjusting for multiple comparisons with FDRs less than 5%, the associations between MCPP and testosterone became insignificant.

Furthermore, a doubling of MiBP concentrations was associated with lower estradiol (percentage change: −3.64%; 95% CI, −6.83 to −0.34) in the total population (Table 4). Similar to the total population, MiBP was also associated with lower estradiol in perimenopausal women. In contrast, MEHP was inversely associated with estradiol only among premenopausal women (percentage change: −7.81%; 95% CI, −14.68 to −0.38), while MEOHP was associated with lower estradiol among postmenopausal women (percentage change: −4.16%; 95% CI, −7.85 to −0.33). The results for estradiol were not statistically significant after correcting for multiple comparisons. No significant associations were observed of phthalate metabolites with FSH (Supplementary Table S1) or SHBG (Supplementary Table S2) in the total population [22].

Associations Between Phthalate Metabolites and Anti-Müllerian Hormone

Higher urinary concentrations of phthalate metabolites, especially DEHP and HMW metabolites, were associated with lower AMH concentrations (Fig. 1). Changes in AMH related to a doubling increase in urinary phthalate metabolites were −11.96% (95% CI, −22.45 to −0.06) for MBzP, −14.26% (95% CI, −24.10 to −3.14) for MECPP, −15.58% (95% CI, −24.59 to −5.50) for MEHHP, −10.83% (95% CI, −19.84 to −0.80), and −13.50% (95% CI, −22.93 to −2.90) for MEOHP. The results for MBzP and MEHP became insignificant after multiple comparison adjustments. For the

Table 2. Detection rates, distributions, and molecular weights of phthalate metabolites measured in the Study of Women's Health Across the Nation at visits 3 and 6, and correlations of each metabolite between visits 3 and 6

Phthalate parent compound	Phthalate metabolite	MW, g/mol	V3 (1999-2000)		V6 (2002-2003)		Difference between V3 and V6 P^a	Spearman correlation	
			% > LOD	Median (IQR), ng/mL	% > LOD	Median (IQR), ng/mL		rho	*P*
DEP	MEP	194.2	99.9%	83.5 (36.0-218.3)	99.8%	60.2 (23.7-164.6)	< .0001	0.44	< .0001
DiBP	MiBP	222.2	98.0%	2.8 (1.4-5.5)	99.9%	3.3 (1.8-6.3)	< .0001	0.35	< .0001
DBP	MnBP	222.2	100%	20.2 (9.8-39.3)	99.8%	18.5 (9.2-37.7)	.86	0.41	< .0001
DEHP	MEHP	278.3	84.5%	3.3 (1.5-7.0)	82.6%	2.9 (1.4-7.0)	.77	0.38	< .0001
	MEHHP	294.3	99.9%	17.2 (8.1-36.4)	100%	23.4 (11.2v51.4)	< .0001	0.37	< .0001
	MEOHP	292.3	99.9%	10.4 (4.8-21.8)	99.9%	11.3 (5.3-25.1)	< .0001	0.37	< .0001
	MECPP	308.3	100%	19.0 (9.3-37.9)	100%	25.1 (12.7-55.1)	< .0001	0.35	< .0001
BBzP	MBzP	256.3	99.8%	11.1 (5.2-23.0)	99.6%	7.6 (3.5-15.4)	< .0001	0.42	< .0001
	MnBP	222.2	100%	20.2 (9.8-39.3)	99.8%	18.5 (9.2-37.7)	.86	0.41	< .0001
DnOP	MCPP	252.2	98.6%	2.8 (1.6-5.1)	97.3%	1.6 (0.9-3.0)	< .0001	0.39	< .0001
DiDP	MCOP	322.4	99.7%	4.9 (2.3-9.5)	99.0%	3.4 (1.6-6.7)	< .0001	0.31	< .0001
	MCNP	336.4	99.7%	2.8 (1.5-5.5)	99.0%	2.0 (1.0-4.1)	< .0001	0.35	< .0001
	MiNP	292.4	1.1%	< LOD	0.7%	< LOD	NA	NA	NA

Abbreviations: BBzP, butyl benzyl phthalate; DBP, di-n-butyl phthalate; DEP, diethyl phthalate; DEHP, di-(2-ethylhexyl) phthalate; DIBP, diisobutyl phthalate; DiDP, diisodecyl phthalate; DiNP, diisononyl phthalate; DnOP, di-n-octyl phthalate; IQR, interquartile range; LOD, limit of detection; MBzP, mono-benzyl phthalate; MCNP, mono-carboxyisononyl phthalate; MCOP, mono-carboxyoctyl phthalate; MCPP, mono-(3-carboxypropyl) phthalate; MECPP, mono-2-ethyl-5-carboxypentyl phthalate; MEHHP, mono-(2-ethyl-5-hydroxyhexyl) phthalate; MEHP, mono-(2-ethyl)-hexyl phthalate; MEOHP, mono-(2-ethyl-5-oxohexyl) phthalate; MEP, mono-ethyl phthalate; MiBP, mono-isobutyl phthalate; MiNP, mono-isononyl phthalate; MnBP, mono-n-butyl phthalate; NA, not available; V, visit.
[a]Wilcoxon signed rank test to compare urinary phthalate metabolite concentrations between visit 3 and visit 6 while accounting for urinary creatinine.

molar sum of phthalate metabolites, a doubling increase in the molar sum was related to lower AMH with a percentage change of −15.01% (95% CI, −24.56 to −4.26) for $\sum^{DEHP}$, −17.01% (95% CI, −26.98 to −5.68) for $\sum^{HMW}$, and −17.11% (95% CI, −28.16 to −4.34) for $\sum^{Anti}$-androgenic phthalate metabolites. No statistically significant findings were observed for other compounds, especially LMW phthalate metabolites.

Associations Between Phthalate Metabolites and Timing of Natural Menopause

We found no statistically significant associations between phthalate metabolites and timing of natural menopause (Fig. 2). After adjusting for age, race/ethnicity, study site, education, smoking status, physical activity, parity, BMI, and urinary creatinine, the HRs of natural menopause was 1.04 (95% CI, 0.98-1.10) for MBzP, 1.03 (95% CI, 0.97-1.09) for MCOP, 1.02 (95% CI, 0.96-1.09) for MCNP, 1.04 (95% CI, 0.97-1.11) for MCPP, 0.99 (95% CI, 0.93-1.01) for MECPP, 1.00 (95% CI, 0.95-1.05) for MEHHP, 0.98 (95% CI, 0.94-1.03), and 0.99 (95% CI, 0.94-1.05) for MEOHP. Similar to HMW metabolites, no results were detected for LMW metabolites and the molar sums of phthalate metabolites.

Discussion

In the present study, we observed statistically significant associations of several phthalate metabolites with lower serum concentrations of testosterone in repeatedly collected samples among women without HT. Stratification by menopausal status revealed inverse relationships between several phthalate metabolites (particularly DEHP, HMW, and antiandrogenic metabolites) and testosterone in postmenopausal women. We also observed statistically significant associations of DEHP metabolites and lower AMH concentrations. By contrast, we found no statistically significant associations of phthalate metabolites with other hormones or timing of natural menopause. Overall, these results suggest that exposure to some HMW phthalates including DEHP metabolites may alter testosterone and diminish the ovarian reserve in midlife women. Although testosterone has been understudied in women, previous research has suggested associations of lower testosterone with muscle loss [32] and sexual dysfunction [33]. Most pressing is the need for research to clarify whether the effect on hormones from phthalate exposure leads to subsequent adverse health outcomes.

Several biological mechanisms may underlie the associations between phthalates and sex hormones. Our results are consistent with toxicological studies showing that phthalates have antiandrogenic properties [34–36]. Exposure to phthalates may reduce ovarian steroidogenesis by targeting the expression of hormone-producing enzymes [9, 10]. For instance, female mice treated with 2.6 mg/kg/d of a mixture of 4 phthalates (MBP, MBzP, MEHP and MiNP) in utero had a decreased expression of 17α-hydroxylase-17, 20-desmolase (CYP17), which could consequently reduce ovarian testosterone concentrations [37]. In addition to its effects on the expression of these enzymes, phthalates can activate PPARα and PPARγ isoforms [38]. PPARα and PPARγ are both expressed along the hypothalamic-pituitary-ovarian axis [39]. PPAR activation decreased testosterone secretion by reducing the expression and activity of CYP17, 17β-hydroxysteroid dehydrogenase (17βHSD), and had no effects on estradiol in the porcine ovarian follicles [40]. PPARs may also disrupt ovarian steroidogenesis by decreasing the production of androgenic precursors in the theca cells and antagonizing the stimulation of androstenedione [41].

Table 3. Percentage change (95% CI) in serum concentrations of testosterone for a doubling in urinary phthalate metabolite concentrations and molar sums of phthalate metabolites, in total population and by menopausal status

Phthalate metabolites	Total	Stratification by menopausal status		
	Percentage change (95% CI)	Premenopausal Percentage change (95% CI)	Early and late perimenopausal Percentage change (95% CI)	Postmenopausal Percentage change (95% CI)
HMW metabolites				
MBzP	−0.34 (−1.97 to 1.32)	−0.68 (−5.73 to 4.65)	−0.51 (−2.57 to 1.59)	−0.39 (−4.04 to 3.39)
MCOP	**−2.08 (−3.66 to −0.47)**[a]	−4.99 (−9.41, −0.36)	−1.90 (−3.93 to 0.18)	**−3.66 (−7.11 to −0.07)**[a]
MCNP	−0.37 (−1.87 to 1.16)	−2.97 (−8.03 to 2.35)	−0.48 (−2.41 to 1.48)	0.35 (−3.02 to 3.83)
MCPP	−2.00 (−3.94 to −0.02)	−2.43 (−8.67 to 4.22)	−2.99 (−5.40 to −0.51)	−0.26 (−4.86 to 4.55)
DEHP metabolites				
MECPP	−0.54 (−1.97 to 0.91)	1.20 (−5.99 to 3.84)	0.39 (−1.42 to 2.23)	**−3.24 (−6.44 to −0.05)**[a]
MEHHP	−0.29 (−1.62 to 1.06)	−0.83 (−5.17 to 3.71)	0.87 (−0.81 to 2.58)	**−3.92 (−6.92 to −0.83)**[a]
MEHP	−0.38 (−1.69 to 0.93)	−0.54 (−5.12 to 4.27)	0.98 (−0.67 to 2.66)	−2.63 (−5.50 to 0.33)
MEOHP	−0.88 (−2.22 to 0.49)	−1.65 (−6.05 to 2.95)	0.21 (−1.50 to 1.95)	**−4.22 (−7.21 to −1.14)**[a]
LMW metabolites				
MEP	−0.64 (−1.84 to 0.56)	0.55 (−3.34 to 4.59)	−0.46 (−1.98 to 1.08)	−1.32 (−3.89 to 1.31)
MiBP	−1.17 (−2.95 to 0.64)	−1.39 (−6.74 to 4.27)	−0.63 (−2.96 to 1.75)	−3.71 (−7.41 to −0.13)
MnBP	**−1.99 (−3.82 to −0.13)**[a]	−2.64 (−8.44 to 3.52)	−2.07 (−4.33 to 0.23)	−2.04 (−6.31 to 2.41)
Sum of metabolites				
∑DEHP	−0.50 (−1.89 to 0.92)	−1.23 (−5.89 to 3.67)	0.65 (−1.12 to 2.45)	**−3.83 (−6.97 to −0.59)**[a]
∑LMW	−0.91 (−2.30 to 0.50)	0.38 (−4.21 to 5.19)	−0.76 (−2.52 to 1.04)	−1.72 (−4.74 to 1.40)
∑HMW	−0.52 (−2.06 to 1.06)	−0.86 (−6.01 to 4.61)	0.48 (−1.49 to 2.48)	**−3.52 (−7.01 to −0.10)**[a]
∑Antiandrogenic	−0.94 (−2.63 to 0.77)	−1.29 (−6.93 to 4.68)	0.11 (−2.02 to 2.29)	**−4.18 (−8.00 to −0.20)**[a]
∑Estrogenic	−0.83 (−2.29 to 0.64)	0.87 (−4.09 to 6.09)	−0.75 (−2.59 to 1.12)	−1.71 (−4.85 to 1.55)

Models were adjusted for age (time-varying), race/ethnicity, study site, education, smoking status (time-varying), physical activity (time-varying), parity, body mass index (time-varying), menopausal status (time-varying), and urinary creatinine (time-varying).
Abbreviations: DEHP, di-(2-ethylhexyl) phthalate; HMW, high-molecular weight; LMW, low-molecular weight; MBzP, mono-benzyl phthalate; MCNP, mono-carboxyisononyl phthalate; MCOP, mono-carboxyoctyl phthalate; MCPP, mono-(3-carboxypropyl) phthalate; MECPP, mono-2-ethyl-5-carboxypentyl phthalate; MEHHP, mono-(2-ethyl-5-hydroxyhexyl) phthalate; MEHP, mono-(2-ethyl)-hexyl phthalate; MEOHP, mono-(2-ethyl-5-oxohexyl) phthalate; MEP, mono-ethyl phthalate; MiBP, mono-isobutyl phthalate; MnBP, mono-n-butyl phthalate.
False discovery rate [a]*P* less than .05 (in bold).

Despite plausible pathological pathways linking phthalates and sex hormones, previous studies have been contradictory. Our results of phthalate metabolites and testosterone agree with previous cross-sectional studies in reproductive-aged women [15], middle-aged women [14], and men [11, 14]. In a cross-sectional study in China (n = 194 women aged 20-45 years undergoing in vitro fertilization), higher follicle fluid concentrations of MEHHP were associated with lower testosterone concentrations [15]. In the National Health and Nutrition Examination Survey (NHANES) 2011 to 2012, higher urinary concentrations of total DEHP, MBzP, MiBP, MnBP, MEP, and MCPP were associated with lower testosterone among 230 women aged 40 to 60 years; and higher MnBP was associated with lower testosterone among 221 men aged 40 to 60 years [14]. Furthermore, in the NHANES 2013 to 2016, higher DEHP, HMW, and LMW phthalate metabolites were associated with lower testosterone in older men [11]. By contrast, in a cross-sectional study of 718 premenopausal and perimenopausal women from the Midlife Women's Health Study, a statistically significant positive relationship between MiBP and testosterone was found [17]. Moreover, in the NHANES 2013-2016, Long et al [16] reported no association between phthalate metabolites and total testosterone. Similarly, phthalates were not related to testosterone in 1377 pregnant women [42] and 295 men aged 18 to 54 years [12].

With respect to estrogen, a case-control study of women with and without premature ovarian failure reported inverse associations of MiBP, MnBP, MEHHP, LMW, HMW, and total phthalate metabolites with estradiol [43]. Lower DEHP metabolites were also associated with lower estradiol in postmenopausal women [16]. On the contrary, other studies found no associations [11, 13, 44] or positive associations [15, 17]. As for FSH, our results are consistent with those of Chiang et al [17], which reported no association between phthalates and FSH in midlife women, whereas other studies reported inverse associations [12] or positive associations [43]. Regarding SHBG, in a repeated analysis of 677 women with samples collected at up to 2 time points during pregnancy, higher MCOP was associated with lower SHBG [45]. Hart et al [42] found an inverse association between MEHP and SHBG but a positive association between MECPP and SHBG in pregnant women. Other studies reported no associations [13, 17].

Several methodological differences could explain the discrepancies in the associations observed in previous studies. First, previous studies have implemented different study designs, with mostly cross-sectional [11–17]. Second, the study populations were very diverse, with children [13], pregnant women [42, 44, 45], reproductive-aged women [15, 43], midlife women [14, 16, 17], or men [11, 12, 14]. In particular,

Table 4. Percentage change (95% CI) in serum concentrations of estradiol in relation to a doubling increase in urinary phthalate metabolite concentrations and molar sums of phthalate metabolites, in total population and by menopausal status

Phthalate metabolites	Total	Stratification by menopausal status		
	Percentage change (95% CI)	Premenopausal Percentage change (95% CI)	Early and late perimenopausal Percentage change (95% CI)	Postmenopausal Percentage change (95% CI)
HMW metabolites				
MBzP	0.44 (−2.56 to 3.53)	−4.22 (−12.25 to 4.55)	1.45 (−2.65 to 5.72)	−0.03 (−4.49 to 4.63)
MCOP	0.51 (−2.64 to 3.76)	1.71 (−6.88 to 11.09)	0.23 (−4.06 to 4.72)	1.88 (−2.65 to 6.62)
MCNP	1.79 (−1.17 to 4.85)	−2.18 (−10.50 to 6.91)	2.15 (−1.91 to 6.37)	1.70 (−2.58 to 6.16)
MCPP	−0.46 (−4.15 to 3.38)	0.73 (−9.91 to 12.63)	1.24 (−3.79 to 6.54)	−3.23 (−8.72 to 2.59)
DEHP metabolites				
MECPP	−0.73 (−3.44 to 2.04)	−5.33 (−12.97 to 2.97)	−0.17 (−3.81 to 3.61)	−2.52 (−6.50 to 1.63)
MEHHP	−0.32 (−2.83 to 2.27)	−5.77 (−12.60 to 1.60)	0.99 (−2.43 to 4.53)	−3.70 (−7.41 to 0.15)
MEHP	−0.98 (−3.40 to 1.51)	−7.81 (−14.68 to −0.38)	−0.25 (−3.53 to 3.15)	−2.54 (−6.08 to 1.14)
MEOHP	−0.86 (−3.41 to 1.01)	−6.40 (−13.37 to 1.12)	0.59 (−2.90 to 4.20)	−4.16 (−7.85 to −0.33)
LMW metabolites				
MEP	0.62 (−1.59 to 2.89)	3.75 (−2.77 to 10.72)	1.32 (−1.73 to 4.47)	−2.07 (−5.21 to 1.17)
MiBP	−3.64 (−6.83 to −0.34)	3.10 (−6.21 to 13.34)	−4.91 (−9.24 to −0.38)	−3.71 (−8.30 to 1.10)
MnBP	−3.36 (−6.65 to 0.01)	−5.71 (−14.91 to 4.50)	−3.58 (−7.91 to 0.95)	−2.45 (−7.70 to 3.09)
Sum of metabolites				
∑DEHP	−0.58 (−3.24 to 2.14)	−6.18 (−13.52, 1.78)	0.54 (−3.06 to 4.27)	−3.49 (−7.37 to 0.56)
∑LMW	0.23 (−2.35 to 2.88)	4.12 (−3.57 to 12.42)	0.86 (−2.68 to 4.54)	−2.74 (−6.42 to 1.09)
∑HMW	−0.08 (−3.03 to 2.96)	−7.48 (−15.49 to 1.29)	1.52 (−2.50 to 5.71)	−3.34 (−7.64 to 1.16)
∑Antiandrogenic	−0.41 (−3.61 to 2.90)	−8.14 (−16.80 to 1.41)	1.11 (−3.23 to 5.64)	−3.80 (−8.52 to 1.15)
∑Estrogenic	0.39 (−2.31 to 3.17)	3.66 (−4.54 to 12.56)	1.19 (−2.53 to 5.05)	−2.56 (−6.40 to 1.43)

Models were adjusted for age (time-varying), race/ethnicity, study site, education, smoking status (time-varying), physical activity (time-varying), parity, body mass index (time-varying), menopausal status (time-varying), and urinary creatinine (time-varying).
Abbreviations: DEHP, di-(2-ethylhexyl) phthalate; HMW, high-molecular weight; LMW, low-molecular weight; MBzP, mono-benzyl phthalate; MCNP, mono-carboxyisononyl phthalate; MCOP, mono-carboxyoctyl phthalate; MCPP, mono-(3-carboxypropyl) phthalate; MECPP, mono-2-ethyl-5-carboxypentyl phthalate; MEHHP, mono-(2-ethyl-5-hydroxyhexyl) phthalate; MEHP, mono-(2-ethyl)-hexyl phthalate; MEOHP, mono-(2-ethyl-5-oxohexyl) phthalate; MEP, mono-ethyl phthalate; MiBP, mono-isobutyl phthalate; MnBP, mono-n-butyl phthalate.

Chiang et al [17] did not include postmenopausal women in their study population. In addition, Meeker and Ferguson [14] did not consider menopausal status, which affects circulating levels of sex hormones. It should be noted that urinary concentrations of phthalate metabolites in the present study are comparable to those among women in other studies, for example, NHANES (Supplementary Fig. S2 [22]). We also used time-varying exposures measured at baseline and in the middle of the follow-up to account for temporal variations in exposure. We observed moderate temporal correlations, especially LMW phthalate metabolites such as MEP, which is consistent with the previous literature [46]. We also observed different temporal trends in different phthalate metabolites: MEP, MBzP, MCPP, MCOP, and MCNP declined whereas MiBP and DEHP metabolites increased from baseline (1999-2000) to the follow-up (2002-2003). These trends are consistent with those found in the general US population [47]. Regulation of the use of phthalates through legislation, the campaign by environmental health organization, and awareness of consumers may explain the observed declines during this period. On the other hand, increasing trends of some phthalates may be because these phthalates may have been used as substitutes for other compounds (eg, DiBP as a substitute for DnBP) [47, 48].

The most pronounced associations between phthalates and testosterone were generally observed in women during the postmenopausal period. While Long et al [16] suggests postmenopausal women were more susceptible to endocrine disruption by phthalates than premenopausal women, most studies do not address why and how those women become especially vulnerable to the effects of those plasticizers. It is unlikely due to exposure levels because urinary concentrations of phthalate metabolites were similar across menopausal stages in our study population (data not shown). Alternatively, the observed findings in postmenopausal women may represent a distinct phenomenon caused by changes in the reproductive hormonal environment to which women are particularly sensitive; this agrees with previous findings in older women and men [11, 14]. Postmenopausal women have lower testosterone concentrations than premenopausal women, but the decline is gradual and results from declining ovarian functions with reproductive aging. Low testosterone levels have been associated with higher risks of decreased bone mineral density, osteoporosis, sexual dysfunction, and depression [49, 50].

AMH is a member of the transforming growth factor β (TGF-β) family of growth and differentiation factors [51]. AMH is released from the granulosa cells of growing follicles, and its concentrations are proportional to the number of early-stage follicles in the ovaries. Accordingly, circulating AMH concentrations decline with age in women until they become undetectable after menopause [52]. Therefore, AMH is considered a marker for the growing follicle pool and the

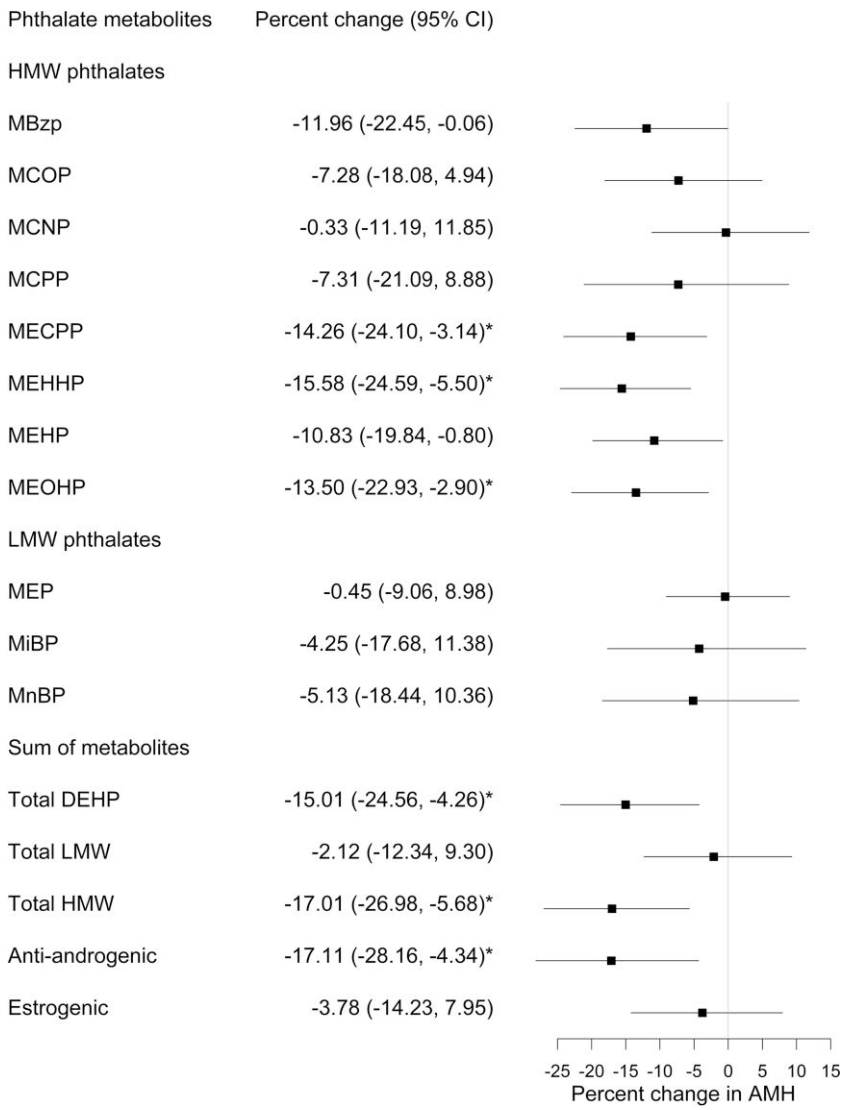

Figure 1. Percentage change (95% CI) in serum concentrations of anti-Müllerian hormone (AMH) in relation to a doubling increase in urinary phthalate metabolite concentrations and molar sums of phthalate metabolites, in total population and by menopausal status. Models were adjusted for age (time-varying), race/ethnicity, study site, education, smoking status (time-varying), physical activity (time-varying), parity, body mass index (time-varying), menopausal status (time-varying), and urinary creatinine (time-varying). False discovery rate *P less than .05.

process of ovarian aging [53]. The associations observed in our study between phthalate metabolites and AMH are consistent with prior studies both in humans and animals suggesting ovarian folliculogenesis is susceptible to endocrine disruption by phthalate exposure [9]. For instance, chronic exposure to DEHP accelerated primordial follicle recruitment, followed by a decrease in primordial follicles in adult mice [54]. A recent study of 138 women undergoing fertility treatment showed inverse associations between select urinary phthalates, particularly MEOHP, and preovulatory follicular fluid AMH concentrations [55]. By contrast, we observed no statistically significant associations between phthalate metabolites and timing of natural menopause even though there is an apparent association between AMH levels and the time to FMP in this population (data not shown). We conducted further analysis to examine the associations between phthalate metabolites and age at natural menopause among women with complete information on AMH, and we found no associations. It is unlikely that the different study populations for AMH and natural menopause could account for the differences in study results. Future studies are warranted to confirm the relationships between phthalate exposures and the onset of natural menopause.

Our study has many strengths, such as our large, diverse, well-characterized multiracial/multiethnic group of midlife women. The collection of repeated measurements of phthalate metabolites during the menopausal transition allows for the use of statistical modeling techniques to detect associations more powerfully. However, our results should be considered in the context of some limitations. First, we used a single spot urine at each time point for phthalate assessment, which does not allow us to address within-person variability of phthalate exposure. These chemicals are quickly metabolized and have been found to be variable day to day. However, phthalate metabolite concentrations in the present study measured at 2 time points, 3 years apart, showed moderate correlations, suggesting that phthalate exposure may be relatively reliable over time. This may be because phthalates are

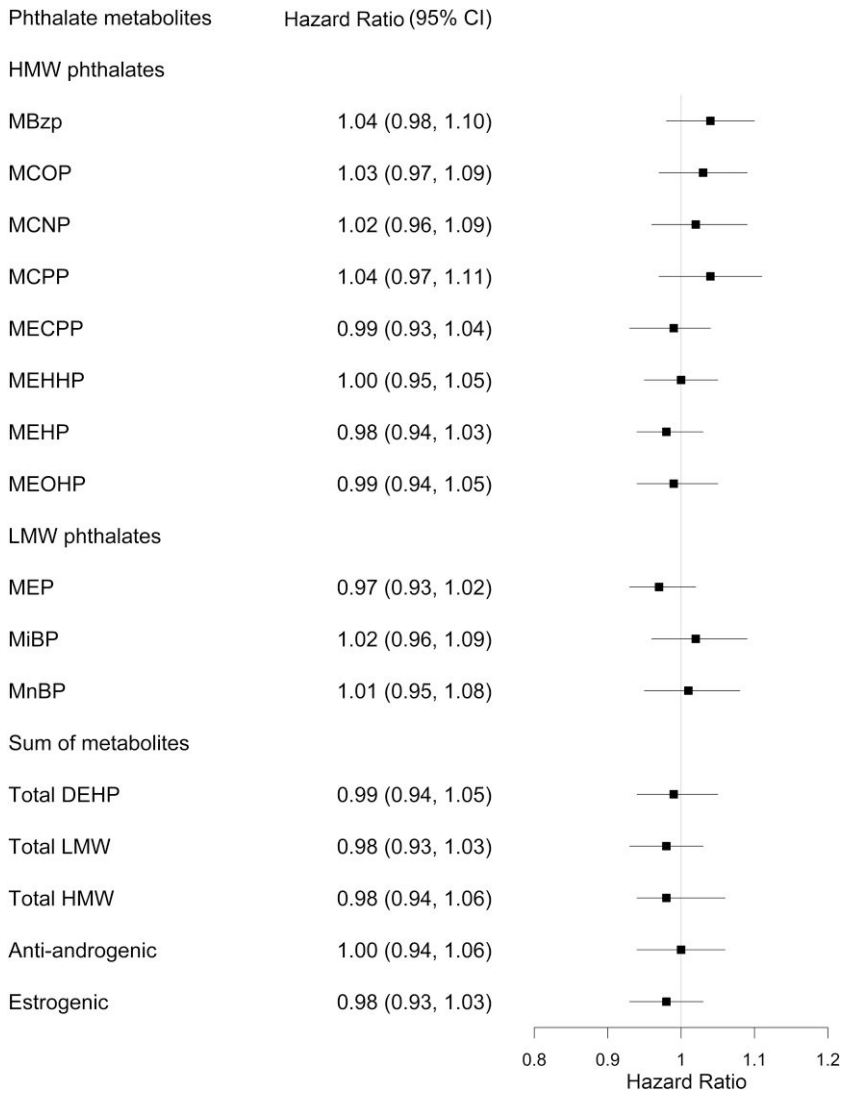

Figure 2. Adjusted hazard ratios (95% CI) for incident natural menopause with a doubling increase in urinary concentrations of urinary concentrations of phthalate metabolites. Models were adjusted for age (time-varying), race/ethnicity, study site, education, smoking status (time-varying), physical activity (time-varying), parity, body mass index (time-varying), and urinary creatinine (time-varying). False discovery rate *P less than .05.

ubiquitous and individuals can be exposed to the use of everyday products including personal care products and food contact materials. Second, women were censored if they started receiving HT. If women with higher phthalate metabolite concentrations had more rapid reproductive aging and were more likely to have hormone use, our findings may be biased toward the null. Third, although we adjusted for a number of potential confounders, we cannot exclude the possibility of residual confounding (eg, dietary intake and use of personal care products). Finally, not all women had 2 study visits in this study because of loss to follow up, which may have affected the study results.

Overall, our study suggests that environmental phthalate exposure may disturb circulating levels of sex hormones, in particular testosterone, in midlife women who are not on HT. Phthalate metabolites, especially DEHP, HMW, and anti-androgenic metabolites, may decrease women's testosterone concentrations after menopause. Exposure to phthalates, especially DEHP, may also contribute to declines in the ovarian reserve, as indicated by lower AMH concentrations. Additional epidemiologic and toxicological studies are warranted to ascertain the direction of specific associations and to further elucidate time windows of susceptibility to endocrine disruption by these plasticizers. Nonetheless, midlife women, particularly postmenopausal women, may avoid exposure to phthalates by reducing phthalate exposure sources such as plastic containers, food packaging materials, personal care products, and cosmetics.

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Disclosures

The authors have nothing to disclose.

Data Availability

Restrictions apply to the availability of some or all data generated or analyzed during this study to preserve patient confidentiality or because they were used under license. The corresponding author will on request detail the restrictions and any conditions under which access to some data may be provided.

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