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Urinary BPA and Phthalate Metabolite Concentrations and Plasma Vitamin D Levels in Pregnant Women: A Repeated Measures Analysis

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BACKGROUND: In addition to its well-established role in maintaining skeletal health, vitamin D has essential regulatory functions in female reproductive and pregnancy outcomes. Phthalates and bisphenol A (BPA) are endocrine disruptors, and previous research has suggested that these chemical agents may disrupt circulating levels of total 25(OH)D in adults.

OBJECTIVES: We investigated the relationships between repeated measures of urinary phthalate metabolites and BPA and circulating total 25(OH)D in a prospective cohort of pregnant women.

METHODS: The present study population includes participants ($n = 477$) in a nested case–control study of preterm birth drawn from a prospective birth cohort of pregnant women at Brigham and Women's Hospital in Boston, Massachusetts. Urine and blood samples were collected for biomarker measurements at median 10 wk and 26 wk of gestation.

RESULTS: In repeated measures analysis, we observed that an interquartile range (IQR) increase in urinary mono-3-carboxypropyl phthalate (MCPP) was associated with a 4.48% decrease [95% confidence interval (CI): $-7.37, -1.58$] in total 25(OH)D. We also detected inverse associations for metabolites of di(2-ethylhexyl) phthalate (DEHP) [percent difference (% Δ) = -2.83 to -2.16]. For BPA, we observed a nonsignificant inverse association with total 25(OH)D in the overall population. Our sensitivity analysis revealed that the associations for some metabolites (e.g., MEHP) varied by race/ethnicity, which may reflect potential differences in susceptibility. In agreement with findings from repeated measures analysis, we reported that DEHP metabolites and BPA were significantly associated with an approximate 20% increase in the odds of vitamin D deficiency (≤ 20 ng/mL) [odds ratio (95% CI): 1.19 (1.06, 1.35) for molar sum of DEHP metabolites and 1.22 (1.01, 1.47) for BPA] at median 10 wk and 26 wk, respectively.

CONCLUSIONS: Our results provide suggestive evidence of the potential for environmental exposure to phthalates and/or BPA to disrupt circulating vitamin D levels in pregnancy. <https://doi.org/10.1289/EHP1178>

Introduction

Vitamin D is a prohormone that plays an integral role in the regulation of bone metabolism and calcium and phosphorous absorption (Holick 2007; Norman 2008). The major source of vitamin D in humans is exposure to ultraviolet B (UVB) radiation from sunlight, although it can also be obtained through dietary food sources or supplements (Thacher and Clarke 2011). Vitamin D from the skin and diet (vitamin D₂ and D₃) is biologically inactive and is transported to the liver where it is converted to 25-hydroxyvitamin D [25(OH)D], the circulating biomarker of vitamin D nutritional status (Norman 2008; Thacher and Clarke 2011). Further metabolism occurs in the kidneys, wherein 25(OH)D is hydroxylated to its biologically active metabolite, 1,25-dihydroxyvitamin D [1,25(OH)₂D] (Norman 2008; Thacher and Clarke 2011); 1,25(OH)₂D is a secosteroid hormone that initiates biological actions by interacting with its nuclear receptor at target tissues (Bikle 2014; Carlberg 2014; Haussler et al. 2013). Although it is well established that vitamin D plays an essential

role in the development and maintenance of skeletal health, the presence of its nuclear receptor and metabolic enzymes in reproductive tissues, such as the placenta, uterus, and ovaries, indicates that vitamin D may also have regulatory functions in female reproductive and pregnancy outcomes (Grundmann and von Versen 2011; Luk et al. 2012; Ma et al. 2012; Pérez-López 2007).

Maintaining maternal vitamin D homeostasis in pregnancy is necessary for placentation and the maintenance of the pregnancy state as well as for normal fetal growth and development (Luk et al. 2012; Murthi et al. 2016; Ponsonby et al. 2010). Human health studies have shown that reduced levels of 25(OH)D in pregnancy are associated with various maternal and fetal complications, such as preeclampsia, spontaneous preterm birth, and restricted fetal growth (Bodnar and Simhan 2010; Bodnar et al. 2015; Murthi et al. 2016; Robinson et al. 2011). Because pregnancy represents a period of susceptibility during which slight deviations in maternal hormone levels may have detrimental maternal and fetal health consequences, pregnant women are particularly vulnerable to the effects of endocrine-disrupting chemicals.

Phthalates and bisphenol A (BPA) are industrial chemicals found in a wide range of consumer products (Meeker et al. 2009b). Exposure to these agents has been reported in pregnant women worldwide (Cantonwine et al. 2014; Casas et al. 2011; Mortensen et al. 2014; Mu et al. 2015). Both phthalates and BPA may disrupt endocrine systems, and results from epidemiological studies suggest these environmental chemicals may alter sex and thyroid hormone levels in pregnant women (Huang et al. 2007; Johns et al. 2015, 2016a; Sathyanarayana et al. 2014). Given that the active vitamin D metabolite is similar in structure to that of classic sex steroid hormones (Norman 2008), and its nuclear receptor is in the same superfamily of sex steroid and thyroid hormone receptors (Pike and Meyer 2010), it is also plausible that

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phthalates and/or BPA might disrupt the vitamin D endocrine axis. In our recent investigation conducted among a representative sample of U.S. adults, we reported inverse associations between urinary metabolites of di(2-ethylhexyl) phthalate (DEHP) and total 25(OH)D (Johns et al. 2016b). Urinary BPA was inversely associated with total 25(OH)D among women in our sex-stratified analyses (Johns et al. 2016b). Although our previous study showed the potential for phthalates and BPA to alter circulating levels of total 25(OH)D in adult populations, it was limited by its cross-sectional design with single biomarker measurements collected at one time point. Moreover, we are not aware of any studies that have investigated these associations in pregnant women. In the present study, we assessed the associations between environmental exposure to phthalates and BPA and plasma total 25(OH)D levels in a large, prospective cohort of pregnant women.

Methods

Study Population

The present study population includes participants in a nested case-control study of preterm birth drawn from a prospective cohort (LifeCodes) of pregnant women 18 y and older who were recruited early in gestation (<15 weeks) at Brigham and Women's Hospital in Boston, Massachusetts. The only exclusion criterion was higher-order multiple gestations (e.g., triplets or greater). Additional details regarding recruitment and eligibility criteria are described in detail elsewhere (Ferguson et al. 2014a, 2014b; McElrath et al. 2012). In brief, participants completed a questionnaire at the initial study visit (median: 9.7 wk of gestation; range: 4.7–19.1 wk) to collect demographic characteristics (e.g., race/ethnicity, health insurance provider, educational attainment, etc.) and relevant health information (e.g., family health history, tobacco and alcohol use). Participants were followed until delivery and provided health information [e.g., body mass index (BMI)] as well as blood and urine samples for biomarker measurements at three additional study visits: visit 2 (median: 17.9 wk of gestation; range: 14.9–32.1 wk), visit 3 (median: 26.0 wk of gestation; range: 22.9–36.2 wk), and visit 4 (median: 35.1 wk of gestation; range: 33.1–38.3 wk). The present analyses were restricted to visits 1 and 3 because plasma samples collected at only these time points were assayed for total 25(OH)D.

Of the 1,181 pregnant women included in the original birth cohort who were followed until delivery and had a singleton birth, 130 women who delivered a preterm infant (<37 weeks of gestation), and 352 who delivered at or after 37 wk of gestation were included in the nested case-control population. The selection probabilities from the parent cohort population were 90.1% for cases and 33.9% for controls (Ferguson et al. 2015). In the current study, we excluded participants from this population who did not have measurements for urinary phthalate metabolites or BPA ($n = 1$) or 25(OH)D ($n = 4$) at either of the two study visits. The final study population ($n = 477$) included 128 cases of preterm birth and 349 controls. The study protocols were approved by the ethics and research committees of the participating institutions, and all study participants gave written informed consent prior to participation.

Urinary Exposure Measurements

All available urine samples collected at up to two study visits during pregnancy were assayed for nine phthalate metabolites and total (free plus glucuronidated) BPA using isotope dilution-liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS) at NSF International in Ann Arbor, Michigan. Additional

details regarding this analytical method are described elsewhere (Lewis et al. 2013). The nine phthalate metabolites included: mono(2-ethylhexyl) phthalate (MEHP), mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), mono(2-ethyl-5-carboxypentyl) phthalate (MECPP), monobenzyl phthalate (MBzP), mono-*n*-butyl phthalate (MBP), mono-isobutyl phthalate (MiBP), monoethyl phthalate (MEP), and mono (3-carboxypropyl) phthalate (MCP). In addition to analyzing individual phthalate metabolites in our statistical analyses, we created a molar sum ($\mu\text{mol/L}$) measure of the four metabolites of DEHP (MEHP, MEHHP, MEOHP, and MECPP; ΣDEHP). Specifically, we divided each phthalate metabolite concentration by its molecular weight and took the sum of the individual concentrations. Urinary biomarker concentrations less than the limit of detection (LOD) were assigned a value of LOD divided by the square root of 2 (Hornung et al. 1990).

To adjust for urinary dilution in descriptive analyses, phthalate metabolites and BPA were standardized using specific gravity (SG) by the following equation (Meeker et al. 2009a): $\text{PSG} = P[(1.015 - 1)/(SG - 1)]$, where PSG is the specific gravity-adjusted exposure biomarker concentration ($\mu\text{g/L}$), P is the observed exposure biomarker concentration, 1.015 is the specific gravity population median, and SG is the specific gravity of the urine sample. In multivariable analyses, we used unadjusted urinary biomarker concentrations with SG added as a separate covariate because modeling corrected metabolite levels may introduce bias (Barr et al. 2005).

Plasma Vitamin D Measurements

All available plasma samples were assayed for total 25(OH)D, including 25(OH)D₂ plus 25(OH)D₃, using a DiaSorin LIAISON[®] chemiluminescence immunoassay (DiaSorin Inc.) at the Laboratory for Molecular Medicine (Partners Healthcare, Boston, MA) (Ersfeld et al. 2004). The detection range of the assay is 4.0–150 ng/mL, and total coefficients of variation ranged from 9.5% to 12.6%. For quality control, the laboratory uses the U.S. National Institute of Standards and Technology (NIST) level 1 protocol (Burris et al. 2014).

Statistical Analyses

All analyses were performed using R (version 3.3.1; R Development Core Team). We conducted the present study using secondary variables measured under case-control sampling. To make our study population more representative of the original cohort from which the case-control sample arose (i.e., to correct for the over-representation of preterm-birth cases), we applied to all analyses inverse probability weights that represented the inverse sampling fractions for inclusion of controls (Richardson et al. 2007). The distributions of all urinary analytes were right-skewed so we transformed these data in statistical analyses using the natural logarithm (ln). The empirical histogram of total 25(OH)D approximated a normal distribution.

In descriptive analyses, we tabulated weighted means and standard deviations of total 25(OH)D by selected population characteristics. We used the R *nlme* package to fit unadjusted linear mixed models (LMMs) with a subject-specific random intercept, chosen based on Akaike's information criterion (AIC), to account for intra-individual correlation of repeated measures over time. We used unadjusted LMMs to test the differences in mean 25(OH)D concentrations across categorical variables. To investigate the potential effects of gestational weight gain on total 25(OH)D concentrations, we calculated the absolute difference between maternal weight measured at visit 3 (median 26 wk) and prepregnancy, excluding those who lost weight between these

two time points ($n = 16$). We regressed repeated measures of total 25(OH)D on gestational weight gain using LMMs with subject-specific random intercepts, adjusting for gestational age at time of sample collection. We tabulated weighted selected percentiles of all urinary analytes and tested the differences in mean levels between the two study visits of sample collection (visits 1 and 3) using paired t -tests of ln-transformed concentrations. To evaluate the pairwise correlations among urinary phthalate metabolites and BPA, we calculated the Spearman correlation coefficients (r_s) of specific-gravity standardized concentrations by study visit of sample collection.

In repeated measures analyses, we explored the associations between urinary biomarkers and plasma total 25(OH)D using LMMs that included subject-specific random intercepts, with 25(OH)D regressed on one analyte per model. We chose covariates based on biological and statistical considerations. We included maternal age, race/ethnicity (women who identified as white, black, or other regardless of Hispanic origin), and BMI *a priori*. Additional covariates—such as health insurance provider, educational attainment, season at time of sample collection, multivitamin supplement use in pregnancy, parity, fetal sex, and smoking and alcohol use in pregnancy—were added using a forward stepwise selection procedure and were retained in the final models if their inclusion resulted in $\geq 10\%$ change in the main effect estimates.

Crude models included fixed effects terms for gestational age at time of sample collection (continuous) and urinary SG (continuous). Full models were additionally adjusted for maternal age (continuous), BMI at time of enrollment (continuous), race/ethnicity (white, black, other/mixed race), health-insurance provider (private, public), season at time of sample collection (winter, spring, summer, fall), and multivitamin supplement use in pregnancy (yes, no). Participants missing data on key covariates were not included in the final multivariable regression analyses. Final regression models included $n = 459$ women ($n = 837$ samples). All final LMMs were repeated with an interaction term to test whether the effects of phthalates and/or BPA on circulating 25(OH)D levels varied by study visit of sample collection.

Because skin pigmentation is associated with circulating 25(OH)D concentrations (Hall et al. 2010), we performed a sensitivity analysis by stratifying LMMs by race/ethnicity to investigate whether the associations between urinary exposure biomarkers and total 25(OH)D concentrations varied by race/ethnicity. We also assessed whether these effects were modified by race/ethnicity by adding an interaction term in the LMMs for the overall study population. To improve the interpretability of results yielded from models with ln-transformed predictor variables, we presented all regression results as the percent difference ($\% \Delta$) in 25(OH)D associated with an IQR (population-level) increase in urinary biomarker concentrations.

In addition to exploring associations with continuous measures of 25(OH)D, we assessed the relationships between urinary biomarkers and the odds of vitamin D deficiency, defined as total 25(OH)D concentrations ≤ 20 ng/mL (Holick et al. 2011). In this cross-sectional analysis, we stratified logistic regression models by time of sample collection in pregnancy and adjusted all models for the same covariates as those included in repeated measures analysis.

To explore potential nonlinear associations, we fitted generalized additive mixed effects models (GAMM) using the R *mgcv* package. For each model, we regressed repeated measures of total 25(OH)D on a penalized spline of urinary DEHP metabolites and BPA, with one urinary biomarker included per model. These multivariable GAMMs were adjusted for the same covariates as those included in LMMs, and included a random intercept for

each subject. All associations were considered statistically significant at the 5% level.

Results

The population demographic characteristics of the nested case-control study population have been described in detail previously (Ferguson et al. 2014b). Briefly, the present study participants were predominately white and highly educated, and half of the women had a normal BMI (< 25 kg/m²). The distributions of total 25(OH)D by population demographic characteristics are presented in Table 1. Mean 25(OH)D concentrations were significantly higher in all older age groups in comparison with women 18 to 24 y old and in participants who reported multivitamin supplement use during pregnancy in comparison with those who reported no supplement use. Women who identified as black or other race/ethnicity had significantly lower concentrations of 25(OH)D in comparison with concentrations in white women. Significantly lower concentrations were also reported in women who had public health insurance in comparison with private, in those who were overweight (BMI: 25–30 kg/m²) and obese (BMI: > 30 kg/m²) in comparison with women who had a normal BMI, and in all lower educational levels in comparison with college graduates. Absolute weight gain (median = 20.0 lbs) between measurements collected prepregnancy and at visit 3 (median 26 wk of gestation) was not associated with total 25(OH)D concentrations $\{\beta$ [the difference in total 25(OH)D concentration with a 1-lb increase in weight gain] = 0.002; 95% CI: $-0.08, 0.09$ in our study population.

All urinary biomarkers were highly detected in the study population, with urinary phthalate metabolites detected in at least 96% of the samples and BPA detected in 82% of the samples (Table 2). Urinary phthalate metabolites from the same parent compound were strongly correlated at both visits ($r_s = 0.70 - 0.98$ for DEHP metabolites) and were weaker among other metabolites (see Tables S1 and S2). Spearman correlations were weak to moderate between BPA and phthalate metabolites ($r_s \leq 0.28$). Concentrations of urinary MCPP as well as DEHP metabolites, including Σ DEHP, were significantly lower in samples collected at visit 3 (median 26 wk of gestation) in comparison with samples collected at visit 1 (median 10 wk of gestation) (Table 2). Urinary BPA did not significantly differ by study visit of sample collection. Total 25(OH)D concentrations were significantly greater in samples collected at 26 wk of gestation in comparison with those collected at 10 wk (median = 25.6 ng/mL vs. 23.8 ng/mL, respectively) (Table 2).

Results from repeated measures analysis using multivariable LMMs are reported in Table 3. Similar associations were observed between weighted and unweighted analyses (see Table S3). We detected inverse associations between DEHP metabolites and total 25(OH)D, with the strongest associations observed for MEHP ($\% \Delta = -2.76$; 95% CI: $-5.50, -0.01$), MEHHP ($\% \Delta = -2.83$; 95% CI: $-5.60, -0.06$), and MEOHP ($\% \Delta = -2.64$; 95% CI: $-5.28, -0.01$). We also found a significant inverse association between MCPP and 25(OH)D, where an IQR increase in urinary MCPP was associated with a 4.48% decrease in total 25(OH)D (95% CI: $-7.37, -1.58$). For BPA, we observed a non-significant inverse association ($\% \Delta = -2.16$; 95% CI: $-5.78, 1.45$). Our interaction analysis using multivariable LMMs revealed no statistically significant interactions between any of the urinary biomarkers measured and study visit of sample collection (p -value for interaction terms = for BPA, 0.17; and for phthalates, ranged from 0.36 for MiBP to 0.98 for MEHP) (data not shown).

In our sensitivity analysis, associations from race/ethnicity-stratified models were largely inverse (Table 4). An IQR increase in BPA was inversely associated with total 25(OH)D in white

Table 1. Plasma 25(OH)D levels (weighted mean \pm SD) by population demographic characteristics ($n = 477$ pregnant women).

Population characteristics	n (%) ^a	Total 25(OH)D (ng/mL)	p -value ^d
Age (years)			
18–24	52 (12)	20.2 (14.9)	Ref
25–29	95 (20)	23.7 (15.0)	0.01
30–34	188 (39)	25.2 (13.7)	<0.001
≥ 35	142 (29)	26.8 (13.7)	<0.001
Race/ethnicity			
White	280 (59)	27.6 (12.6)	Ref
Black	76 (16)	19.0 (15.3)	<0.001
Other	121 (25)	21.9 (13.9)	<0.001
Education level ^c			
College graduate	186 (41)	26.8 (12.9)	Ref
Junior college or some college	139 (30)	25.6 (13.7)	0.03
Technical school	76 (16)	23.0 (16.0)	<0.001
High school	66 (13)	20.0 (14.9)	<0.001
Health insurance provider ^c			
Private (ref)	381 (81)	25.9 (13.7)	Ref
Public	84 (19)	20.0 (15.3)	<0.001
BMI at initial visit ^c			
<25 kg/m ²	249 (53)	26.8 (14.0)	Ref
25–30 kg/m ²	125 (27)	24.0 (14.0)	<0.001
>30 kg/m ²	100 (20)	20.5 (13.7)	<0.001
Fetal sex			
Male	212 (45)	24.9 (15.5)	Ref
Female	265 (55)	24.7 (13.6)	0.61
Parity			
No previous pregnancies	214 (45)	25.2 (13.9)	Ref
One previous pregnancy	155 (34)	25.4 (15.2)	0.84
More than one previous pregnancy	108 (21)	23.1 (14.0)	0.12
Tobacco use ^c			
Smoked in pregnancy	31 (6)	22.1 (15.3)	Ref
No smoking in pregnancy	440 (94)	25.0 (14.3)	0.20
Alcohol use ^c			
Alcohol use in pregnancy	19 (5)	25.9 (16.1)	Ref
No alcohol use in pregnancy	448 (95)	24.7 (14.3)	0.60
Multivitamin supplement use ^c			
Supplement use in pregnancy	324 (70)	25.9 (13.6)	Ref
No supplement use in pregnancy	147 (30)	22.2 (15.4)	<0.001
Season of sample collection			
Winter (ref)	224 (27) ^b	22.6 (14.2)	Ref
Spring	231 (28)	24.5 (13.9)	<0.001
Summer	185 (22)	27.8 (14.6)	<0.001
Fall	197 (24)	25.1 (14.1)	<0.001

Note: BMI, body mass index; SD, standard deviation; ref, reference category.

^aProportions weighted by preterm birth case-control sampling probabilities to represent the general sampling population.

^bSample size and weighted proportions refer to number of samples (not participants).

^cMissing observations: $n = 10$ for education level; $n = 12$ for insurance provider; $n = 3$ for BMI at initial visit; $n = 6$ for tobacco use; $n = 10$ for alcohol use; $n = 6$ for multivitamin supplement use.

^d p -Value for the difference in mean plasma total 25(OH)D concentrations in the category compared to reference (first category listed) using unadjusted linear mixed models with a random intercept for each subject.

women ($\% \Delta = -4.79$; 95% CI: $-9.78, 0.20$; based on data for 274 women and 506 samples) but did not appear to be associated with total 25(OH)D among women who identified as black ($\% \Delta = -0.60$; 95% CI: $-8.72, 7.51$; 71 women and 121 samples) or other race/ethnicity ($\% \Delta = 0.99$; 95% CI: $-5.62, 7.59$; 114 women and 210 samples). There were no significant differences in the associations for BPA by race/ethnicity, based on p -values for interaction terms (Table 4). Among women who identified as other race/ethnicity, DEHP metabolites were inversely associated with total 25(OH)D, with a significant association observed for MEHP ($\% \Delta = -8.16$; 95% CI: $-13.4, -2.88$) in comparison with a null association for white women ($\% \Delta = -0.18$; 95% CI: $-3.67, 3.31$; p -interaction other race/ethnicity vs. white = <0.01). A weak inverse association for MEHP was observed among black women ($\% \Delta = -1.39$; 95% CI: $-9.38,$

6.60 ; p -interaction black vs. white = 0.06). IQR increases in MCPP were associated with a ~ 8 percent decrease in total 25(OH)D in women of other race/ethnicity ($\% \Delta = -8.33$; 95% CI: $-15.4, -1.26$) and in black women ($\% \Delta = -8.11$; 95% CI: $-20.1, 3.86$), in comparison with a weaker inverse association estimated for white women ($\% \Delta = -3.47$; 95% CI: $-8.94, 2.01$; p -interaction other race/ethnicity vs. white = 0.08 ; p -interaction black vs. white = 0.06).

In our analysis of vitamin D deficiency by study visit of sample collection, we estimated that approximately 35% ($n = 160$) of women were vitamin D deficient at visit 1 (median 10 wk of gestation) and 30% ($n = 117$) at visit 3 (median 26 wk of gestation) (Table 5). We reported from our stratified logistic regression models that a unit increase in urinary DEHP metabolites was associated with a 12% to 19% increase in the odds of vitamin D deficiency at visit 1 [odds ratios (OR) = 1.12; 95% CI: 1.00, 1.25 for MEHP to OR = 1.19; 95% CI: 1.07, 1.34 for MEOHP]. The direction of these relationships remained at visit 3, although none of the estimates were statistically significant. We also found a significant positive association for MiBP at visit 1 (OR = 1.25; 95% CI: 1.04, 1.52). For BPA, we observed a significant increase in the odds of vitamin D deficiency only at visit 3 (OR = 1.22; 95% CI: 1.01, 1.47). Also at visit 3, we reported statistically significant elevated odds ratios for MBzP (OR = 1.27; 95% CI: 1.08, 1.50) and MBP (OR = 1.22; 95% CI: 1.03, 1.45).

Results from our analysis in which we evaluated nonlinear associations using penalized splines for urinary biomarkers in GAMM models are presented in Figure 1. All multivariable associations were found to be linear.

Discussion

In a secondary analysis of 477 pregnant women drawn from a nested case-control study of preterm birth, we found that repeated measures of certain urinary phthalate metabolites, specifically DEHP metabolites and MCPP, were inversely associated with circulating total 25(OH)D levels. A nonsignificant inverse association between urinary BPA and total 25(OH)D was observed in the overall population analysis. Associations varied by race/ethnicity and estimates for white women were more precise than those for black or for women identifying as other race/ethnicity due to differences in the numbers of women in each group. In agreement with findings from repeated measures analysis, we reported that DEHP metabolites and BPA were significantly associated with an approximate 20% increase in the odds of vitamin D deficiency at median 10 wk (Σ DEHP: OR = 1.19; 95% CI: 1.06, 1.35) and 26 wk (BPA: OR = 1.22; 95% CI: 1.01, 1.47), respectively.

We are aware of one previous analysis that has investigated the associations of exposure to phthalates and/or BPA on the vitamin D endocrine system in humans (Johns et al. 2016b). Our results for DEHP metabolites in the current analysis are consistent with those previously reported in a representative sample of U.S. adults 20 y and older (Johns et al. 2016b). In our earlier study utilizing data from participants in the National Health and Nutrition Examination Survey (NHANES) 2005–2010, we found significant inverse associations between urinary DEHP metabolites, including Σ DEHP and circulating total 25(OH)D in adult men and women (Johns et al. 2016b). Furthermore, our exposure-response analysis in that previous NHANES study revealed inverse trends between quintiles of individual DEHP metabolites and total 25(OH)D (Johns et al. 2016b). GAMM model estimates for the present study population also supported linear associations between increasing exposure to DEHP metabolites and decreasing total 25(OH)D concentrations.

Table 2. Weighted median [interquartile range (IQR; 25th–75th percentiles)] of urinary and plasma biomarkers by study visit of sample collection in pregnancy.

Biomarker	LOD	% Detect ^c	Visit 1 (median 10 wk)		Visit 3 (median 26 wk)		<i>p</i> -Value ^d
			# Samples ^b	Median (IQR)	# Samples ^b	Median (IQR)	
Urinary Exposure Biomarkers ^a							
BPA (μg/L)	0.4	82.0	476	1.28 (0.75, 2.08)	409	1.28 (0.84, 2.08)	0.47
MEHP (μg/L)	1.0	96.6	474	10.1 (5.17, 24.7)	409	8.10 (4.65, 16.7)	<0.01
MEHHP (μg/L)	0.1	99.1	474	33.6 (17.4, 80.2)	409	23.9 (12.3, 50.0)	<0.001
MEOHP (μg/L)	0.1	99.2	474	16.9 (8.60, 40.3)	409	14.0 (7.23, 28.7)	<0.01
MECPP (μg/L)	0.2	99.3	474	40.6 (18.9, 107)	409	30.6 (15.0, 72.8)	<0.001
ΣDEHP (μmol/L)	–	–	474	0.37 (0.18, 0.81)	409	0.28 (0.14, 0.58)	<0.001
MBzP (μg/L)	0.2	99.4	474	6.22 (3.36, 13.4)	409	5.87 (3.34, 11.8)	0.83
MBP (μg/L)	0.5	99.3	474	16.1 (10.8, 26.7)	409	16.1 (10.4, 25.5)	0.37
MiBP (μg/L)	0.1	99.2	474	7.14 (4.51, 11.1)	409	7.53 (4.61, 11.6)	0.84
MEP (μg/L)	1.0	99.4	474	124 (49.0, 362)	409	123 (47.2, 363)	0.96
MCPP (μg/L)	0.2	97.7	474	1.68 (1.06, 3.38)	409	1.57 (0.98, 3.13)	0.01
Vitamin D							
25(OH)D (ng/mL)	4.0	100	469	23.8 (17.7, 30.0)	429	25.6 (18.1, 31.5)	<0.001

Note: Analyses were weighted by preterm birth case–control sampling probabilities. LOD, limit of detection.

^aUrinary analyte concentrations corrected for specific gravity.

^bNumber of plasma samples per analyte varied due to limitations in sample volume.

^cPercent of analyte concentrations above the detection limits.

^d*p*-Value for difference between urinary phthalate metabolite or 25(OH)D concentrations between study visits based on a paired *t*-test.

For BPA, we previously reported a statistically significant inverse association with total 25(OH)D when analyses were restricted to women alone (Johns et al. 2016b). The direction of this relationship is similar to those presented in the repeated measures analysis among the overall population of pregnant women in our current study. In race/ethnicity-stratified models, the magnitude of association was larger among white (%Δ = –4.79; 95% CI: –9.78, 0.20) than among women identifying as black (%Δ = –0.60; 95% CI: –8.72, 7.51) or other race/ethnicity (%Δ = 0.99; 95% CI: –5.62, 7.59). These results may reflect racial differences in behaviors, lifestyle factors, and/or metabolic processes that were not captured in the present analyses, thereby potentially leading to residual confounding.

In pregnancy, the fetus relies solely on maternal levels of 25(OH)D, which in turn is converted to 1,25(OH)₂D by a series of hydroxylation steps initiated by cytochrome P450 enzymes found in the fetal-placental unit (Bikle 2014; Rosen et al. 2012). Currently, there is a lack of a consensus on the threshold used to define optimal (or sufficient) serum 25(OH)D concentrations in pregnancy (Thorne-Lyman and Fawzi 2012; Urrutia and Thorp 2012). Furthermore, the optimal threshold may vary by gestational age as the clinical outcomes associated with reduced 25(OH)D likely differ across pregnancy (Aghajafari et al. 2013;

Lucas et al. 2013). Although the data are somewhat conflicting due to the heterogeneity across human health studies, results from meta-analyses suggest that vitamin D insufficiency in pregnancy may be associated with various adverse maternal and neonatal outcomes (e.g., gestational diabetes, preeclampsia, infection, and restricted fetal growth) (Aghajafari et al. 2013; Wei 2014). Some of these effects may be explained by the regulatory role of 1,25(OH)₂D in trophoblast function (Nguyen et al. 2015) and in responding to inflammation and infection in the placenta (Liu et al. 2011). Although the magnitude of estimated differences in 25(OH)D and odds ratios for vitamin D deficiency were relatively small in our analyses, on a population-level these decrements may have significant public health implications, especially if there is a causal association between vitamin D deficiency and adverse maternal and neonatal outcomes. Future research is required to determine the public health impact of sub-clinical changes in circulating 25(OH)D across diverse populations of pregnant women.

Although mechanistic studies are lacking, it is plausible that phthalates and BPA may directly and/or indirectly influence the vitamin D endocrine system at multiple points along its axis. The vitamin D endocrine system is principally regulated by: *a*) 1,25(OH)₂D, which down-regulates its own production; *b*) parathyroid hormone, which in response to low serum calcium levels stimulates hydroxylation enzymes in the kidney to convert 25(OH)D to its active metabolite; *c*) serum calcium and phosphate levels; and *d*) fibroblast growth factor 23 (Henry 2011; Norman 2008). Several animal studies have shown that BPA may disturb calcium metabolism by inducing or inhibiting the renal expression of a vitamin D–dependent calcium-binding protein, calbindin-D9k (CaBP-9k) (Kim et al. 2013; Otsuka et al. 2012) as well as decreasing serum calcium levels (Otsuka et al. 2012) in pregnant mice. However, similar effects have not been reported for phthalates (Hong et al. 2005). These agents may also indirectly influence the vitamin D endocrine system through their effects on the metabolic enzymes involved in the conversion of cutaneous vitamin D to its active metabolite. Animal and *in vitro* studies have demonstrated that phthalates and BPA can alter the expression of cytochrome P450 enzymes involved in steroid and/or thyroid hormone metabolism (Liu et al. 2015; Mathieu-Denoncourt et al. 2015; Quesnot et al. 2014; Sekaran and Jagadeesan 2015). Moreover, increased messenger RNA (mRNA) expression of CYP27B1, the enzyme involved in converting

Table 3. Repeated measures analysis: Percent difference in plasma 25(OH)D associated with an interquartile range (IQR) increase in urinary exposure biomarker concentrations.

Urinary biomarker	IQR	%Δ (95% CI)	<i>p</i> -Value
BPA (μg/L)	1.94	–2.16 (–5.78, 1.45)	0.24
MEHP (μg/L)	17.6	–2.76 (–5.50, –0.01)	0.049
MEHHP (μg/L)	60.2	–2.83 (–5.60, –0.06)	0.046
MEOHP (μg/L)	30.0	–2.64 (–5.28, –0.01)	0.049
MECPP (μg/L)	84.1	–2.25 (–5.31, 0.80)	0.15
ΣDEHP (μmol/L)	0.67	–2.54 (–5.42, 0.34)	0.08
MBzP (μg/L)	13.6	0.88 (–3.29, 5.05)	0.68
MBP (μg/L)	25.4	–2.37 (–5.99, 1.26)	0.20
MiBP (μg/L)	11.0	–0.16 (–4.18, 3.86)	0.94
MEP (μg/L)	336	–0.12 (–3.61, 3.36)	0.94
MCPP (μg/L)	3.02	–4.48 (–7.37, –1.58)	<0.01

Note: Analyses weighted by preterm birth case–control sampling probabilities. Linear mixed models include a random intercept for each subject and are adjusted for specific gravity (continuous), maternal age (continuous), BMI at enrollment (continuous), gestational age at time of sample collection (continuous), race (black, white, other/mixed race), insurance provider (private, public), season at time of sample collection (winter, spring, summer, fall), multivitamin supplement use in pregnancy (yes, no).

Table 4. Race/ethnicity-stratified repeated measures analysis: Percent difference in total 25(OH)D associated with an interquartile range (IQR) increase in urinary exposure biomarker concentrations.

Urinary biomarker	IQR	White women n = 274 women; 506 samples		Black women n = 71 women; 121 samples		Other race/ethnicity n = 114 women; 210 samples		Black vs. white		Other vs. white	
		%Δ (95% CI)	p-Value	%Δ (95% CI)	p-Value	%Δ (95% CI)	p-Value	p-Interaction ^a	p-Interaction ^a		
BPA (μg/L)	1.94	-4.79 (-9.78, 0.20)	0.06	-0.60 (-8.72, 7.51)	0.88	0.99 (-5.62, 7.59)	0.77	0.97	0.71		
MEHP (μg/L)	17.6	-0.18 (-3.67, 3.31)	0.92	-1.39 (-9.38, 6.60)	0.73	-8.16 (-13.4, -2.88)	<0.01	0.06	<0.01		
MEHHP (μg/L)	60.2	-2.11 (-4.98, 0.75)	0.15	0.34 (-6.95, 7.64)	0.93	-4.33 (-9.16, 0.50)	0.08	0.27	0.10		
MEOHP (μg/L)	30.0	-2.20 (-5.66, 1.25)	0.21	-0.58 (-9.12, 7.96)	0.90	-5.19 (-10.9, 0.51)	0.08	0.26	0.11		
MECPP (μg/L)	84.1	-1.05 (-4.60, 2.50)	0.56	-4.28 (-13.0, 4.43)	0.34	-3.96 (-9.51, 1.59)	0.17	0.047	0.11		
ΣDEHP (μmol/L)	0.67	-1.61 (-5.51, 2.28)	0.42	-2.52 (-11.9, 6.85)	0.60	-5.78 (-12.1, 0.49)	0.07	0.09	0.06		
MBZP (μg/L)	13.6	4.02 (-0.97, 9.01)	0.12	-1.54 (-12.0, 8.95)	0.78	-5.27 (-12.6, 2.08)	0.16	0.14	<0.01		
MBP (μg/L)	25.4	-4.61 (-10.5, 1.23)	0.12	2.05 (-11.4, 15.5)	0.77	-3.41 (-11.2, 4.37)	0.39	0.97	0.48		
MIBP (μg/L)	11.0	-3.61 (-8.98, 1.77)	0.19	2.08 (-6.97, 11.1)	0.65	2.79 (-4.93, 10.5)	0.48	0.88	0.96		
MEP (μg/L)	336	-2.24 (-5.13, 0.64)	0.13	3.42 (-2.18, 9.02)	0.24	1.87 (-2.93, 6.67)	0.45	0.12	0.41		
MCCPP (μg/L)	3.02	-3.47 (-8.94, 2.01)	0.22	-8.11 (-20.1, 3.86)	0.19	-8.33 (-15.4, -1.26)	0.02	0.06	0.08		

Note: Analyses weighted by preterm birth case-control sampling probabilities. Linear mixed models include a random intercept for each subject and are adjusted for specific gravity (continuous), maternal age (continuous), BMI at enrollment (continuous), gestational age at time of sample collection (continuous), insurance provider (private, public), season at time of sample collection (winter, spring, summer, fall), multivitamin supplement use in pregnancy (yes, no).
^ap-value for the interaction between ln-transformed urinary biomarkers and race/ethnicity.

Table 5. Adjusted odds ratios (95% CI) of vitamin D deficiency (≤20 ng/mL) associated with a unit increase in urinary biomarkers.

Urinary biomarkers	Odds ratio (95% CI)	p-Value
Visit 1: Median 10 weeks (n = 160 vitamin D deficient women, 292 controls)		
BPA	1.04 (0.87, 1.25)	0.65
MEHP	1.12 (1.00, 1.25)	0.06
MEHHP	1.19 (1.06, 1.33)	<0.01
MEOHP	1.19 (1.07, 1.34)	<0.01
MECPP	1.16 (1.03, 1.30)	0.01
ΣDEHP	1.19 (1.06, 1.35)	<0.01
MBZP	0.95 (0.83, 1.09)	0.49
MBP	0.96 (0.81, 1.14)	0.62
MIBP	1.25 (1.04, 1.52)	0.02
MEP	0.94 (0.84, 1.04)	0.21
MCCPP	1.01 (0.89, 1.14)	0.88
Visit 3: Median 26 weeks (n = 117 vitamin D deficient women, 268 controls)		
BPA	1.22 (1.01, 1.47)	0.04
MEHP	1.12 (0.97, 1.28)	0.11
MEHHP	1.14 (1.00, 1.30)	0.05
MEOHP	1.13 (1.00, 1.29)	0.06
MECPP	1.05 (0.92, 1.18)	0.48
ΣDEHP	1.10 (0.96, 1.26)	0.18
MBZP	1.27 (1.08, 1.50)	<0.01
MBP	1.22 (1.03, 1.45)	0.02
MIBP	1.10 (0.91, 1.32)	0.33
MEP	0.92 (0.83, 1.02)	0.10
MCCPP	1.05 (0.90, 1.21)	0.54

Note: Analyses weighted by preterm birth case-control sampling probabilities. Logistic regression models are adjusted for specific gravity (continuous), maternal age (continuous), BMI at enrollment (continuous), gestational age at time of sample collection (continuous), insurance provider (private, public), season at time of sample collection (winter, spring, summer, fall), multivitamin supplement use in pregnancy (yes, no).

25(OH)D to its active metabolite, was observed in mice treated with BPA (Otsuka et al. 2012). Similar studies assessing the effects of phthalates on enzymes involved in the metabolism of vitamin D have not been conducted to date, and future research is required to elucidate the potential actions of these chemicals on additional components of the vitamin D endocrine system (e.g., vitamin D-binding protein, metabolic enzymes, parathyroid hormone regulation, etc.).

It is also possible that certain lifestyle or physiological factors may partially mediate the associations observed in our study. For example, although studies investigating the relationships between phthalate and BPA exposure and physical activity are lacking, recent animal studies suggest that exposure to endocrine-disrupting chemicals such as phthalates and BPA may reduce or alter voluntary physical activity in mice (Johnson et al. 2015; Schmitt et al. 2016). Because physical activity has been positively associated with 25(OH)D concentrations in pregnant women (Moon et al. 2015; Woolcott et al. 2016), physical activity may be one possible mechanism through which maternal phthalate and/or BPA exposure might contribute to decreased concentrations of 25(OH)D. In the current study, we did not collect data on physical activity from our participants. Additional analyses are required to confirm the role (if any) that physical activity plays in these relationships. It is also possible that phthalate and/or BPA exposure may influence circulating 25(OH)D levels through maternal weight gain in pregnancy. Previous research suggests that exposure to phthalates and BPA may be associated with increased weight gain in women (Song et al. 2014) and that a greater gestational weight gain may lead to a decline in 25(OH)D concentrations in pregnancy (Moon et al. 2015). However, in our study population, absolute weight gain, defined as the difference in measurements collected at visit 3 (median 26 wk of gestation) and prepregnancy, was not associated with repeated measures of 25(OH)D. Further animal and human health studies are needed to characterize the potential vitamin D-disruptive properties of

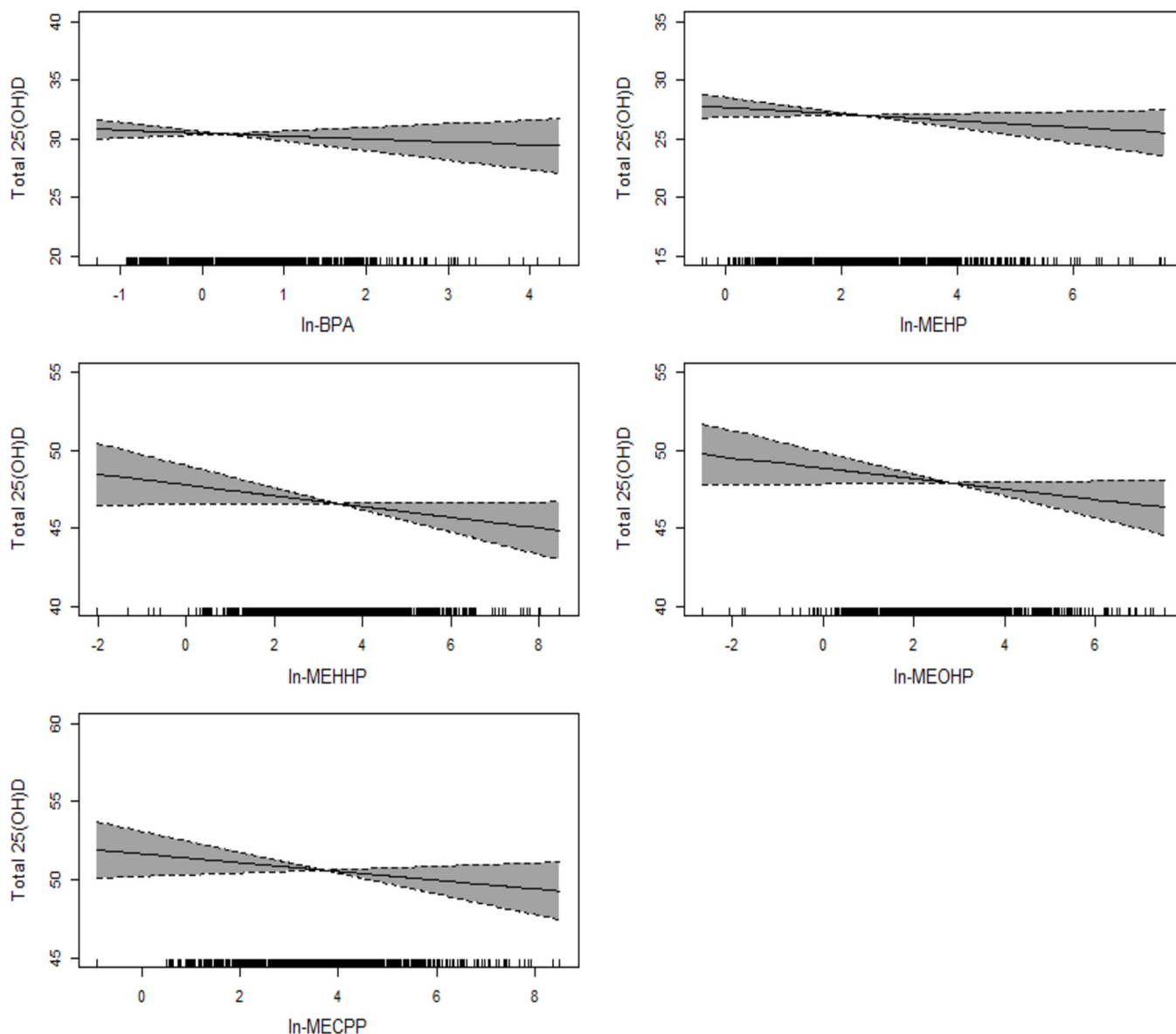


Figure 1. GAMM results for urinary DEHP metabolites and BPA ($\mu\text{g/L}$) and total 25(OH)D (ng/mL), adjusted for specific gravity, maternal age, BMI at enrollment, gestational age at time of sample collection, race, insurance provider, season at time of sample collection, multivitamin supplement use in pregnancy. Analyses weighted by preterm birth case-control sampling probabilities.

phthalates and BPA and to identify their specific mechanisms of action in pregnancy.

One of the main strengths of our study included repeated measures of both the exposures and outcome of interest, which allowed for the use of statistical modeling techniques to more precisely detect the subtle associations of exposure. Nevertheless, our study has several potential limitations. Although the reference assay for measuring 25(OH)D is liquid chromatography tandem mass spectrometry (LC-MS/MS), its time-consuming and laborious procedures limit the efficiency of this method in clinical settings, in comparison with automated immunoassays (Hollis 2010; Wagner et al. 2009). The DiaSorin LIAISON[®] immunoassay, the assay utilized in the present study, is a widely used method in both clinical and research settings (Burriss et al. 2015; Hollis 2010) and has shown excellent agreement with LC-MS/MS methods [concordance correlation coefficient (CCC) = 0.95] (Farrell et al. 2012). Additionally, although we adjusted our statistical analyses for key confounding variables (e.g., season of

sample collection, multivitamin supplement use, and race/ethnicity), we lacked data on dietary food intake and the frequency of use of vitamin D supplements and sunscreen. Concerning the dietary food intake, the dominant exposure pathway for phthalates such as DEHP is ingestion of contaminated food (Wormuth et al. 2006), whereas the major source of vitamin D in humans is exposure to sunlight (Hall et al. 2010; Holick 2004, 2007). Dietary sources of vitamin D are limited but necessary to maintain adequate vitamin D concentrations when sunlight-induced vitamin D synthesis is impaired or in times of insufficient sunlight (Calvo et al. 2004). Few foods naturally contain vitamin D (e.g., oily fish), although in the U.S., some dairy (e.g., milk, yogurt, and cheese), cereal, and juices are fortified with vitamin D (Holick and Chen 2008). Among these foods, dairy consumption has been associated with increased concentrations of urinary DEHP metabolites (Serrano et al. 2014). Unfortunately, we do not have dietary intake data from the women included in our study. Therefore, it is possible that our results may be affected by

unmeasured confounding, particularly if specific dietary sources contributed to phthalate and BPA exposure as well as total 25(OH)D concentrations in our study population. Additionally, although sunscreen use was associated with increased concentrations of urinary phthalate metabolites, particularly MBP, in children who participated in a study of 90 adult-child pairs in California from 2007 to 2009 (Philippat et al. 2015), results from a recent NHANES analysis utilizing data from 2009 to 2012 revealed that sunscreen use was not significantly associated with urinary phthalate metabolite concentrations in adults (Ferguson et al. 2016). We did not collect data on personal care product use for the present study; therefore, we cannot determine whether sunscreen use was associated with urinary phthalate metabolite concentrations in our study participants. Our study may also be limited by our exposure assessment methods. Although we analyzed up to two repeated measures of urinary phthalate metabolite and BPA concentrations per subject, a potential for nondifferential exposure misclassification exists. Additional repeated measurements of exposure may be required to sufficiently reduce bias in our analyses involving short-lived chemicals such as BPA and phthalates (Perrier et al. 2016). Finally, we performed multiple statistical comparisons, and there is the potential that some of the detected associations may have been due to chance.

Conclusions

In conclusion, biomarkers of environmental exposure to phthalates and BPA were associated with reduced circulating total 25(OH)D levels in our study population of pregnant women. Given previous research showing the adverse effects of reduced total 25(OH)D levels in pregnancy on the mother and fetus, future studies are required to confirm these findings in additional cohorts of pregnant women and to determine the potential biological mechanisms through which these agents might influence the vitamin D endocrine system.

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