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Mixtures of urinary concentrations of phenols and phthalate biomarkers in relation to the ovarian reserve among women attending a fertility clinic.

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Abstract

Although prior studies have found associations of the ovarian reserve with urinary concentrations of some individual phenols and phthalate metabolites, little is known about the potential associations of these chemicals as a mixture with the ovarian reserve. We investigated whether mixtures of four urinary phenols (bisphenol A, butylparaben, methylparaben, propylparaben) and eight metabolites of five phthalate diesters including di(2-ethylhexyl) phthalate were associated with markers of the ovarian reserve among 271 women attending a fertility center who enrolled in the Environment and Reproductive Health study (2004-2017). The analysis was restricted to one outcome per study participant using the earliest outcome after the last exposure assessment. Ovarian reserve markers included lower antral follicle count (AFC) defined as $AFC < 7$, circulating serum levels of day 3 follicle stimulating hormone (FSH) assessed by immunoassays, and diminished ovarian reserve (DOR) defined as either $AFC < 7$, $FSH > 10$ UI/L or primary infertility diagnosis of DOR. We applied Bayesian Kernel Machine Regression (BKMR) and quantile g-computation to estimate the joint associations and assess the interactions between chemical exposure biomarkers on the markers of the ovarian reserve while adjusting for confounders. Among all 271 women, 738 urine samples were collected. In quantile g-computation models, a quartile increase in the exposure biomarkers mixture was not significantly associated with lower AFC (OR = 1.10, 95% CI = 0.52, 2.30), day 3 FSH levels (Beta = 0.30, 95% CI = -0.32, 0.93) or DOR (OR = 1.02, 95% CI = 0.52, 2.05). Similarly, BKMR did not show any evidence of associations between the mixture and any of the studied outcomes, or interactions between chemicals. Despite the lack of associations, these results need to be explored among women in other study cohorts.

Keywords: Mixtures; Ovarian reserve; Phenols; Phthalates.

1. Introduction

Over the past decades, the average age of childbearing among women in Western Countries has increased (te Velde and Pearson 2002), mainly due to the opportunity of women to increase their education level and to participate in the labor force, as well as the ability to control their own fertility using more widely available contraceptive methods (Leridon 2006). It is well known that female fecundity, the ability to produce offspring, decreases as women's age increases (Wallace and Kelsey 2010). Consequently, infertility has increased over time (Evers 2002) and is estimated to affect up to 15% of all couples worldwide (Inhorn and Patrizio 2015; Mascarenhas et al. 2012). Approximately 10% of U.S. reproductive aged women are actively trying to get pregnant each year (Curtin et al. 2013; Mosher et al. 2012). Therefore, identifying modifiable factors that can impact the ovarian reserve, which in turns affects fecundity and fertility, have received particular attention.

Among women enrolled in the Environment and Reproductive Health (EARTH) Study, we have previously observed that modifiable factors may affect women's fertility potential, including environmental exposures (Minguez-Alarcon et al. 2016a; Minguez-Alarcon et al. 2016c; Minguez-Alarcon et al. 2017a; Minguez-Alarcon and Gaskins 2017; Minguez-Alarcon et al. 2019; Mínguez-Alarcón et al. 2021), diet (Chavarro et al. 2006, 2008, 2009; Chavarro et al. 2016; Minguez-Alarcon et al. 2016b; Vanegas et al. 2015) and other related variables such as body mass index (BMI), waist circumference and exercise (Chavarro et al. 2012; Gaskins et al. 2015; Gaskins et al. 2016; Li et al. 2019; Minguez-Alarcon et al. 2017b). Specifically, we found that mean urinary concentrations of bisphenol A (Souter et al. 2013), propylparaben (Smith et al. 2013), and several phthalate metabolites (Messerlian et al. 2016) were associated with a lower mean antral follicle count (AFC). However, we did not evaluate chemical mixtures in these previous analyses using data from EARTH and whether endocrine disrupting chemicals (EDCs) measured as a mixture are associated with the ovarian reserve has been less well studied (Bellavia et al. 2023; Pollack et al. 2018; Zhan et al. 2022). The evaluation of the exposure as a group of chemical exposures is important because chemical exposures may be correlated, and may interact with each other in various ways; for example, they may act, additively, synergistically, or even

antagonistically. This is particularly relevant for chemicals like phenols and phthalates that have similar sources of exposure (personal care products). As such, analysis should model exposures simultaneously (as a mixture) and allow for non-linear relationships and interactions. Mixture methods such as BKMR and quantile g-computation are therefore necessary to evaluate complex exposures of the general population to classes of pollutants like phenols and phthalates and their potential health effects (Basso et al. 2022).

To fill this knowledge gap, we evaluated the joint effects of urinary phenol and phthalate metabolite concentrations, as a mixture, in relation to the ovarian reserve among women consulting in a fertility clinic.

2. Material and methods

2.1 Study population

We evaluated women enrolled in the EARTH Study, a prospective cohort established to assess environmental and dietary determinants of fertility at the Massachusetts General Hospital (MGH) Fertility Center (Minguez-Alarcon et al. 2016b). Women between 18 and 45 years old and who were not taking hormones at the time of enrolment seeking fertility care were eligible to participate (there were no criteria for exclusion). Between 2004 and 2019, over 1,000 women enrolled in EARTH. However, to mitigate potential issues of reverse causation, we only included in this study 271 women who had either provided one or several urine samples for exposure assessment before (N = 241) or one sample at the same time (N = 30) as the outcome assessment. The latter included an AFC (N = 262), serum levels of day-3 follicle stimulating hormone (FSH, N = 261) and an infertility diagnosis (N = 271).

The participant's date of birth was collected at entry, and weight and height were measured by trained study staff. BMI was calculated as weight (in kilograms) divided by height (in meters) squared. At enrollment, research staff administered sociodemographic, lifestyle, and medical history questionnaires to participants. Study participants also completed a comprehensive questionnaire on family, medical,

reproductive and occupational history, perceived stress, consumer products use, smoking history, and physical activity. Total physical activity was calculated as the sum of hours of vigorous, moderate and light self-reported leisure exercise as previously explained (Gaskins et al. 2016). Primary infertility type was evaluated using the Society of Assisted Reproductive Technology definitions, and classified as female factor (diminished ovarian reserve (DOR), endometriosis, ovulatory, tubal, uterine and other), male factor, or unexplained (SART 2015). The study was approved by the Human Subjects Committees of the Harvard T.H. Chan School of Public Health, MGH, and the Centers for Disease Control and Prevention (CDC). Participants signed an informed consent after the study procedures were explained by trained research study staff and all the questions were answered.

2.2 Assessment of phenol and phthalate metabolites urinary concentrations

At each clinic visit, women collected one or more spot urine samples in a sterile polypropylene specimen cup. Specific gravity (SG) was measured within hours of collection at the HSPH at room temperature using a handheld refractometer (National Instrument Company, Inc., Baltimore, MD, USA) calibrated with deionized water before each measurement and was used as a covariate for adjustment in the statistical models. 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; Silva et al. 2013; Zhou et al. 2014), we used online solid-phase extraction coupled with isotope dilution-high-performance liquid chromatography-tandem mass spectrometry to quantify the urinary concentrations of four phenol biomarkers (bisphenol A, methylparaben, propylparaben, butylparaben) and eight phthalate metabolites: 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) of biomarkers ranged from 0.1 to 1.2 $\mu\text{g/L}$. Along with study samples, each analytical run included a set of calibrators, reagent blanks, and high- and low-concentration quality control (QC)

materials. Concentrations of the QCs were evaluated using standard statistical probability rules (Caudill et al. 2008).

2.3 Assessments of the ovarian reserve

Ovarian reserve measures included lower AFC, circulating levels of day 3 FSH, and DOR and have already been studied in prior EARTH papers (Ingle et al. 2020; Messerlian et al. 2016; Mínguez-Alarcón et al. 2015). Lower AFC was defined using the European Society of Human Reproduction and Embryology Bologna criteria consensus as $AFC < 7$ (Ferraretti et al. 2011). All women participating in the study underwent an evaluation of ovarian AFC through transvaginal ultrasonography by one of the MGH reproductive endocrinology and infertility physicians in early follicular phase of an unstimulated cycle (Broekmans et al. 2010). No fertility medications were used in the cycle preceding the ultrasonographic determination of the AFC. After blood samples were clotted, tubes were centrifuged at 3000 RPM for 20 minutes. In serum samples, the levels of day 3 FSH were measured with an automated electrochemiluminescence immunoassay using the Elecsys FSH reagent kit at the MGH Core Laboratory (Souter et al. 2013). The intra-assay coefficient of variation (CV) for FSH was $< 3\%$, with an inter-assay CV of $< 9\%$. DOR was defined as $AFC < 7$, $FSH > 10$ UI/L or primary infertility diagnosis of DOR.

2.4 Statistical analysis

The clinical and demographic characteristics of the study population were examined using means \pm standard deviations (SDs) for continuous variables, medians and inter-quartile ranges for discrete variables, and counts and proportions for categorical variables. We used Spearman correlation coefficients to summarize the correlations between urinary biomarker concentrations. Concentrations below the LOD were imputed using multiple imputation through chained equations for multiple LODs (five sets) using a truncated normal imputation (Lapidus et al. 2014). Missing data for covariates (1%) were imputed within this process as well. Covariates were selected a priori through the use of a directed

acyclic graph (Schisterman et al. 2009) (Supplemental Material 1). Chemical metabolites concentrations were averaged for women with more than one urine sample, and subsequently log-transformed. To avoid modeling issues due to multicollinearity, a summary measure of di(2-ethylhexyl)phthalate (DEHP) metabolites (i.e. MEHP, MEHHP, MEOHP, and MECPP) was calculated by dividing each metabolite concentration by their molecular weight and summing them. Mixture analyses were performed using quantile g-computation (Keil et al. 2020) and Bayesian Kernel Machine Regression (BKMR). BKMR is a non-parametric modelling strategy that allows for non-linear exposure-response relationships as well as arbitrary non-additive interactions among exposures. The advantage is that it is highly flexible, making no a priori assumptions about the functional form of the exposure-response relationships. A disadvantage of this flexibility is that it can be inefficient and underpowered to detect small associations. Quantile g-computation, by contrast, is more restrictive, assuming additivity and linearity on the quantile scale, but can be more efficient; it estimates associations with a simultaneous quantile increase in the entire mixture, and thus may be better powered to detect overall mixture association when there are many exposures that each have small individual associations. In BKMR, all effects are calculated as the average change in the outcome for a change in the exposure elements from a particular quantile to another quantile. Quantile g-computation results are presented as odds ratios (ORs) for the binary outcomes (lower AFC and DOR) and as mean differences in concentrations for day 3 FSH, along with corresponding 95% confidence intervals (CIs), for one quartile increase in all serum phenol and phthalate biomarker concentrations. In BKMR analyses, mixture associations are presented as risk differences of lower AFC and DOR or mean differences in day 3 FSH levels corresponding to an increase in all urinary concentrations of the chemical biomarker mixture from the 25th to 75th percentiles, along with corresponding 95% credible intervals (CI). We report posterior inclusion probabilities (PIPs) that represent posterior probability of non-null association between an exposure and the outcome, along with several graphical outputs including (i) the overall association of the mixture corresponding to a simultaneous quantile increase in all mixture components (versus median concentrations), (ii) exposure-response relationships for each individual biomarker while holding all other exposure biomarker at their median levels, (iii) and single-exposure associations (mean difference comparing the 75th to 25th percentile) when all the other exposure

biomarker are fixed at the 25th, 50th, and 75th percentiles. We selected a set of covariates a priori using substantive knowledge and included age (continuous), BMI (categorized as ≤ 20 , between 20 and 25 and between 25 and 55 kg/m²), education level (lower than college graduate, college graduate and graduate degree), and specific gravity (as previously explained) as adjustment variables in all our models. A sensitivity analysis was performed by further adjusting for the number of urine samples per woman (women provided urine samples both at study enrollment and per infertility treatment) since women with more collected urines may be more infertile because they participated longer in the study before achieving a pregnancy. Another sensitivity analysis consisted in modeling AFC as a continuous variable in BKMR after applying a log transformation. Results for each imputed dataset were pooled using Rubin's rules (Rubin 1987). All analyses were performed using R (version 4.2.2). The threshold for significant results for all our analyses was 5%.

3. Results

Women had an average age at enrollment of 35.6 (± 4.6) years (Table 1). Most women were Caucasian (80%), whilst 3% were Black, 9% were Asian and 8% identified with another racial group. The majority of women (61%) had a graduate degree. The main cause of infertility at enrollment was female factor (41%) followed by unexplained (32%) and male factor (24%). The participants provided a median (range) 1 (1-14) urine samples and the average (inter-quartile range) number of days between the collection of urinary samples and the assessment of AFC and day 3 FSH concentrations was 151 (241) days. Among all 271 women, 738 urine samples were collected with biomarker detection frequencies ranging from 72% (Butylparaben) to 100% (Methylparaben, MEP, and MEOHP). Median urinary biomarker concentrations were similar to those found among all female participants in the National Health and Nutrition Examination Survey (NHANES) (CDC 2022). Urinary DEHP metabolite concentrations were highly correlated ($r > 0.95$) as was urinary methylparaben and propylparaben ($r = 0.87$) (Supplementary Material 2). Other urinary biomarkers were moderately correlated with each other (r ranging from 0.17 – 0.80). A

total of 17% women had lower AFC, the mean (\pm standard deviation) day 3 FSH levels was 7.5 (\pm 2.8) UI/L and 21% of women were diagnosed with DOR.

Quantile g-computation results showed no significant associations between a quartile increase in the exposure biomarkers mixture and lower AFC (OR = 1.10, 95% CI = 0.52, 2.30), day 3 FSH levels (mean difference = 0.30, 95% CI = -0.32, 0.93) and DOR (OR = 1.02, 95% CI = 0.52, 2.05) (Supplementary Material 3). The regression results for each imputed dataset were all consistent with the pooled model (data not shown).

These results were confirmed in the BKMR analyses (Supplementary Material 4). The mean/risk difference in outcome when comparing mixture exposure biomarker concentrations at the 75th to the 25th percentile was not associated with lower AFC (Risk difference = -0.01, 95% CI = -0.08, 0.08), or changes in serum day 3 FSH (Mean difference = 0.05, 95% CI = -0.24, 0.35) or DOR (Risk difference = -0.01, 95% CI = -0.08, 0.07). Moreover, we observed no associations between a simultaneous change in mixture component concentration quantiles (relative to medians) and the outcomes (Figures 1A, 2A and 3A). Plots of the exposure response curves while holding all other components at their median levels (Figures 1B, 2B and 3B) did not suggest significant associations of any of the chemicals on the examined outcomes. Similarly, the associations between the individual biomarkers and the outcomes when all other chemicals exposure biomarkers were fixed at different quantiles (.25, .50, .75) did not indicate signs of interactions between chemicals (Figures 1C, 2C and 3C). PIPs (Table 3) were similar for all chemicals for each outcome (7-21% for lower AFC, 23-33% for serum day 3 FSH, and 34-38% for DOR) and were either low (for lower AFC and serum day 3 FSH) or moderate (for DOR). In the sensitivity analysis further adjusting for the number of urine samples per woman, findings remained similar and no associations between mixtures of these biomarkers in relation to the ovarian reserve were observed (data not shown). Modeling AFC as a continuous variable did not change the results (Supplemental Material 5).

4. Discussion

We investigated the associations between a mixture of four phenol and eight phthalate biomarker concentrations measured in multiple urine samples per participant (median of 3) with ovarian reserve markers including lower AFC and day 3 FSH levels, and with DOR among women participating in the EARTH Study. We applied quantile g-computation and BKMR approaches to evaluate the mixture, and overall, we found no evidence of associations between the concentrations of the chemical mixtures and the ovarian reserve. We also observed no evidence of any interaction among EDCs. Given the high prevalence of infertility worldwide (Mascarenhas et al. 2012), the ubiquitous exposure to EDCs among women of reproductive age (CDC 2022) and the scarce literature on mixtures of phenols and phthalates in relation to the ovarian reserve in women, these results need to be verified and validated among women in other study cohorts such as among women from general population rather than fertility clinics.

The results of the current analysis are not all consistent with our previously published EARTH study results for individual biomarkers of phenols and phthalate metabolites in relation to AFC and day 3 FSH levels. Specifically, we previously reported an association of lower mean AFC with higher urinary concentrations of bisphenol A (N = 154, years = 2004-2010, median urinary concentrations = 1.2 µg/L, no association with serum day 3 FSH) (Souter et al. 2013), propylparaben (N = 190, years = 2004-2010, median urinary concentrations = 49.6 µg/L, positively associated with serum day 3 FSH) (Smith et al. 2013), and DEHP metabolites (N = 215, years = 2004-2012, median urinary concentrations = 0.21 µg/L, did not investigate day 3 FSH) (Messerlian et al. 2016). One potential explanation for the different results between previous and the current EARTH analysis may be the higher urinary biomarkers concentrations observed in earlier years of the study compared to those observed in this analysis. As a result, the threshold value necessary for the biomarkers to have an impact on the outcome may not have been reached. In fact, these downward trends in EDCs over time have also been demonstrated among 171 pregnant United States women participating in the Environmental influences on Child Health Outcomes (ECHO) Program, in which authors noted that concentrations of certain chemicals decreased with calendar year, whereas concentrations of their replacements increased (Buckley et al. 2022). Similar trends were reported among women participating in other study cohorts (Ashrap et al. 2018; Kim et al.

2021). Another major difference with our previous publications is the assessment of continuous AFC as an outcome and the assessment as a binary outcome, DOR, in this analysis (BKMR package is not developed for Poisson distribution variables such as AFC). Nonetheless, the associations previously found in EARTH were not consistent across categories of exposure. Additionally, and similar to current results, we previously found no associations of butylparaben and methylparaben (Smith et al. 2013), as well as non-DEHP metabolites (Messerlian et al. 2016) with AFC assessed continuously. Also, urinary bisphenol A concentrations were not related to day 3 FSH levels in earlier years of the study (Souter et al. 2013).

The available epidemiologic literature on mixtures of these EDCs in relation to the ovarian reserve and other fertility measures is still scarce and inconsistent (Bellavia et al. 2023; Pollack et al. 2018; Zhan et al. 2022). Applying BKMR, Bellavia and colleagues evaluated mixtures of phthalates, parabens and PFAS measured in follicular fluid in relation to women's fertility assessed as ovarian sensitive index (OSI) as well as clinical pregnancy and live birth probabilities among 333 women in Sweden and Estonia (Bellavia et al. 2023). While they found negative associations between follicular fluid DEHP and methylparaben with OSI and suggestive with certain PFAS, no associations were found for pregnancy outcomes. In a different study and using principal component analysis, Pollack and colleagues evaluated mixtures of several urinary phenol biomarkers including bisphenol A and parabens among others, in relation to reproductive hormones among 143 premenopausal women (Pollack et al. 2018). The authors found that the ultraviolet protection factor (which was characterized by higher urinary concentrations of benzophenone-1 and benzophenone-3) was negatively associated with FSH levels. In a different investigation applying principal component analysis and BKMR, Zhan et al. explored mixtures of urinary phenols and phthalate metabolites in relation to self-reported infertility among 857 reproductive-aged women participating in 2013-2016 NHANES (Zhan et al. 2022). Authors observed that urinary bisphenol A and certain DEHP metabolites were associated with higher infertility when adjusting for the other examined biomarkers. Of note, none of these mixture studies used quantile-g computation. Clearly, additional studies on mixtures of EDCs and the ovarian reserve as well as fertility endpoints applying

sophisticated approaches will help to increase our understanding of the reproductive health effects of chemical mixtures.

An important strength of this study is the evaluation of the mixture effect of several chemicals on our outcomes of interest using two complementary methods: quantile g-computation and BKMR. Quantile g-computation estimates the ovarian reserve association for a simultaneous increase in all exposure biomarkers by one quantile. This is highly interpretable and efficient, but assumes the association is linear on the quantile scale — i.e. the association comparing the first to second quartile is the same as comparing the third to fourth quartile — and moreover assumes no exposures interact non-additively. BKMR, on the other hand, is not as interpretable or efficient, but it allows each exposure to have its own potentially non-linear exposure response relationship, and further allows exposures to interact synergistically or otherwise. In tandem, BKMR can support the results of the quantile g-computation analysis or indicate that some of its assumptions are not appropriate — e.g. evidence of interactions or of different functional relationships between exposures and outcomes.

The current study has several limitations. First, it may not be possible to generalize our findings to couples from the general population attempting conception. Nevertheless, approximately 15% to 25% of couples trying to achieve pregnancy are diagnosed with infertility (Slama et al. 2012; Thoma et al. 2013), which makes our study findings applicable to a significant proportion of couples seeking treatment. Chemical biomarker concentrations were also similar to U.S. women of the general population for parabens and some of the phthalate metabolites (MiBP, MEHP, MEOHP) although slightly lower for others (MEP, MBzP, MEHHP, MECPP) (CDC 2019). However, not all infertile couples seek treatment, especially in case of secondary infertility implying that our study population is not representative of all couples with fertility issues (Tielemans et al. 2002). Women with secondary infertility who had initially conceived using IVF might also have modified their behaviors regarding personal care products following professional advice given during the first procedure. Furthermore, if exposure to phenols and phthalates is high enough to have contributed to the infertility of male partners with fertile female partners, it is possible that these female partners are more exposed to these chemicals than the general population.

Nevertheless, exposure to this type of chemicals does not appear to be correlated within couples (Buck Louis et al. 2014), presumably because partners do not use the same personal care products. We also considered how the selection process could artificially create non-causal associations between exposures and outcomes using our DAG (Arah 2019) but did not identify clear evidence of such a risk using our set of covariates. Ethnicity has been shown to impact access to any form of infertility treatment in the USA (Dongarwar et al. 2022), suggesting the potential for some form selection bias. However, this relationship is likely confounded by sociodemographic factors such as education, which is included in our models. Second, exposure misclassification is possible given the short biological half-lives (< 24 hours) of these non-persistent chemicals (Koch et al. 2004; Shin et al. 2023; Völkel et al. 2002) and their episodic exposure (Braun et al. 2012). However, this misclassification is likely non-differential. Moreover, we have previously demonstrated in the EARTH cohort that a single urine sample may reasonably represent an individual's exposure to short half-lived chemicals such as BPA and phthalates over several months (Braun et al. 2012; Smith et al. 2012). Nevertheless, we have also employed several methods that have been shown to partially reduce variability, and thus exposure misclassification, including the use of morning voids, the collection of several urine samples to calculate an average concentration (for 89% of the sample), and the correction of concentrations by adjusting for specific gravity (Bastiaensen et al. 2020; Hoppin et al. 2002; Roggeman et al. 2022). Third, residual confounding is still possible because other EDCs, which may be correlated with those included in the current analysis were not measured or excluded a priori from this analysis because of relatively low sample size or detection frequencies (e.g. triclosan, benzophenone-3, bisphenol and phthalate replacements). Fourth, only outcomes measured after the assessment of biomarker exposures were considered in order to avoid issues of reverse causality. Indeed, several studies have looked at the intra-individual variations of AFC between menstrual cycles and have found moderate inter-cycle variations (van Disseldorp et al. 2009). This has nonetheless resulted in the exclusion of individuals from the analyses and thus limited statistical power. In addition, due to the nature of our design we cannot interpret our findings as causal. For this reason, we have interpreted estimates as associations rather than effects. Finally, we only have a few women with data on serum AMH so this ovarian reserve biomarker was excluded from this analysis. The biggest strength of this

study is the use of sophisticated methods to analyze mixtures of exposure biomarkers in relation to the ovarian reserve. Other strengths include the observational design, which minimizes the risk of reverse causation, comprehensive adjustment for other reproductive and lifestyle factors that could result in residual confounding and the collection of multiple urine samples to reduce exposure misclassification.

5. Conclusions

In conclusion, we evaluated mixtures of several endocrine disrupting chemicals in relation to ovarian reserve biomarkers and applied modern approaches to analyze chemical mixtures such as quantile g-computation and BKMR. Overall, we found no associations between mixtures of urinary biomarker concentrations of bisphenol A, parabens and phthalates with lower AFC, day 3 FSH levels and DOR among women attending a fertility center. We also observed no evidence of any interaction between the examined EDCs. Given the high prevalence of infertility worldwide, the ubiquitous exposure to EDCs among women of reproductive age and the scarce literature on mixtures of phenols and phthalates in relation to the ovarian reserve in women, these results need to be explored among women in other study cohorts including among the general population rather than a fertility clinic cohort.

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Table 1. Demographic and reproductive characteristics among 271 women enrolled in the EARTH Study between 2004 and 2017.

Characteristic¹	Study cohort
Age (years), mean (\pm SD)	35.6 (\pm 4.6)
Race	
White/Caucasian	218 (80)
Black	9 (3)
Asian	23 (9)
Other	21 (8)
Education level	
< College graduate	18 (7)
College graduate	86 (32)
Graduate degree	167 (61)
Body mass index (kg/m ²)	
<20	29 (11)
20-25	144 (53)
>25-55	96 (36)
Smoking status	
Never smoker	203 (75)
Past smoker	58 (21)
Current smoker	10 (4)
Infertility diagnosis	
Female Factor	
Diminished ovarian reserve	42 (16)
Endometriosis	26 (10)
Other	8 (3)
Ovulatory	23 (8)
Tubal	14 (5)
Uterine	5 (2)
Male Factor	65 (24)
Unexplained	86 (32)
Previous pregnancy	110 (41)
Previous infertility examination	195 (74)
Previous infertility treatment	108 (49)
Low AFC (<7)	47 (17)
Day 3 FSH Levels (IU/L), mean (\pm SD)	7.5 (\pm 2.8)
Total physical activity (hours per week), mean (\pm SD)	6.5 (7.5)
Menstrual cycle length (days), mean (\pm SD)	29.9 (\pm 7.2)
Regular menstrual cycle	247 (93)
Urine dilution (specific gravity), mean (\pm SD)	1.015 (\pm 0.007)
Number of urine samples, median (IQR)	1.0 (3.0)

Abbreviations: N, number; IQR, interquartile range; SD, standard deviation.

¹Values are presented as N (%) unless otherwise noted.

Missing data for the following characteristics: BMI (N=2), infertility diagnosis (N=2), previous infertility examination (N=6), previous

infertility treatment (N=1), physical activity (N=1), menstrual cycle length (N=3), regular menstrual cycle (N=4).

Table 2. Unadjusted urinary phenol and phthalate biomarker concentrations ($\mu\text{g/L}$) among 271 women contributing 738 urine samples (mean of 3) and enrolled in the EARTH Study between 2004 and 2017.

Biomarker	LOD range	Detection Frequency %	25th	Median	Geometric mean	SD	75th	95th
Bisphenol A	0.1 - 0.4	89	0.5	1.0	1.0	2.7	1.9	5.0
Methylparaben	0.4 - 0.6	100	45.9	125.0	113.8	786.7	294.2	1137.0
Propylparaben	0.1 - 0.2	99	9.0	30.9	22.9	245.8	70.8	280.8
Butylparaben	0.1 - 0.2	72	0.1	0.5	0.7	15.7	3.6	28.0
MBP	0.4 - 0.6	99	4.4	9.5	9.5	59.4	22.1	64.4
MiBP	0.2 - 0.8	99	2.6	5.6	5.5	17.1	11.8	42.1
MEP	0.4 - 0.4	100	17.2	39.5	43.1	539.8	120.5	478.2
MBzP	0.2 - 0.7	99	1.2	2.8	2.6	15.0	6.0	23.2
MEHP	0.5 - 1.2	81	0.9	2.0	2.3	17.0	5.4	22.1
MEHHP	0.2 - 0.7	99	4.1	10.3	11.3	117.8	27.0	125.9
MEOHP	1.2 - 1.2	100	2.7	6.6	7.2	73.4	17.2	76.2
MECPP	0.2 - 0.3	94	7.1	17.6	18.9	144.1	41.9	223.5
ΣDEHP^1	-	-	0.1	0.1	0.1	1.2	0.3	1.5

N: number of participants; LOD: limit of detection; MBP: mono-n-butyl phthalate; MiBP: mono-isobutyl phthalate; MEP: monoethyl phthalate; MBzP: monobenzyl phthalate; MEHP: mono(2-ethylhexyl) phthalate; MEHHP: mono(2-ethyl-5-hydroxyhexyl) phthalate; MEOHP: mono(2-ethyl-5-oxohexyl) phthalate; MECPP: mono(2-ethyl-5-carboxypentyl) phthalate; DEHP: di(2-ethylhexyl) phthalate.

¹ ΣDEHP : Molar sum of DEHP metabolites (MEHP, MEHHP, MEOHP and MECPP) expressed in mmol/l.

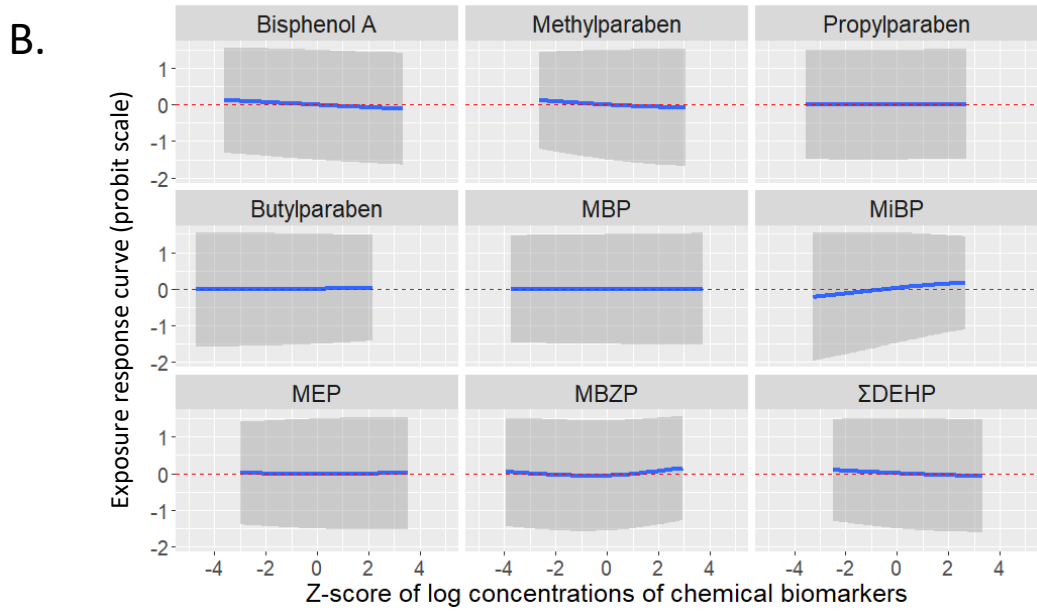
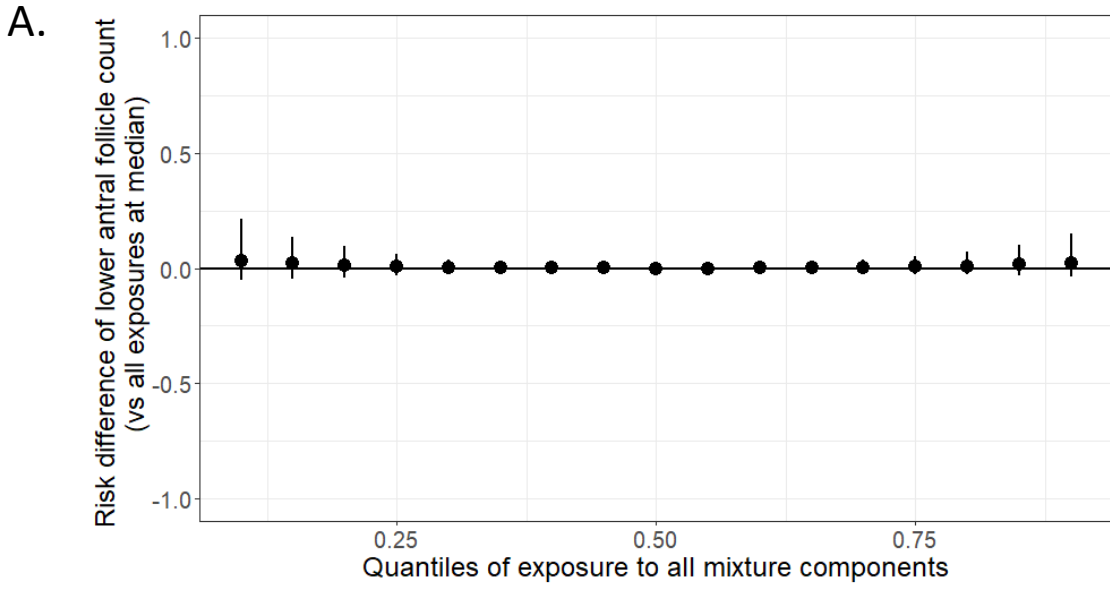
Table 3. Bayesian Kernel Machine Regression posterior inclusion probabilities (%) for the lower antral follicle count (N = 262), day-3 FSH (N = 261) and DOR (N = 271) outcomes

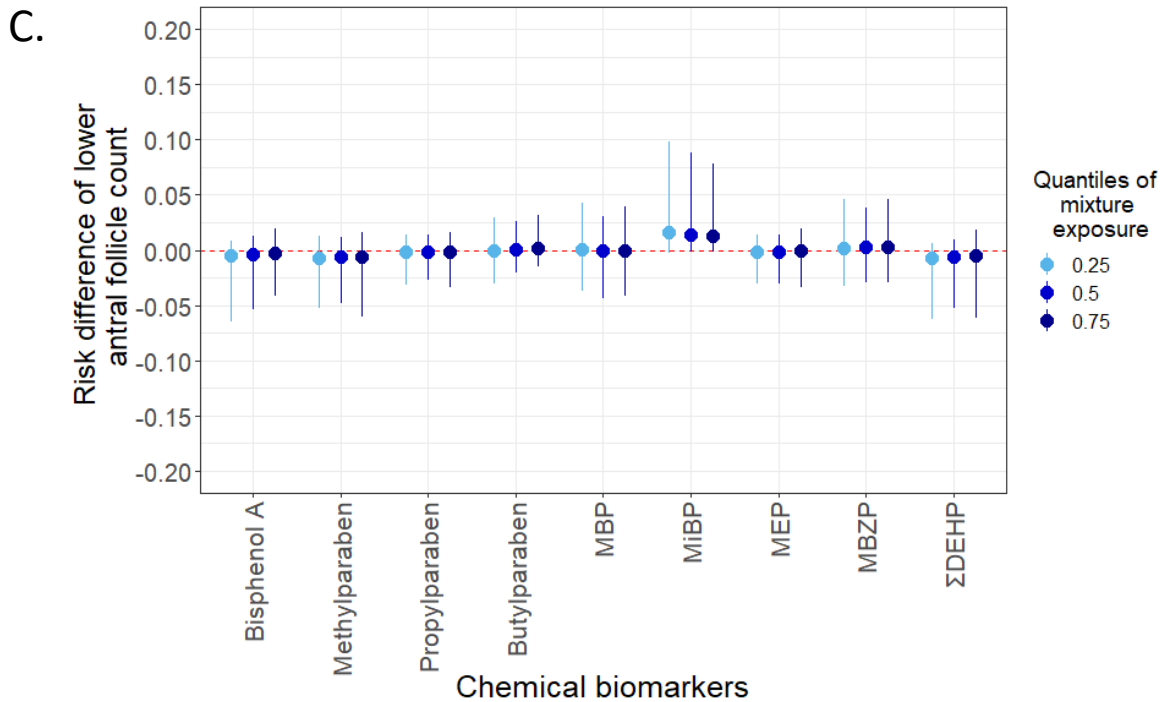
Biomarker	Outcome		
	Lower Antral Follicle Count	Day 3 Follicle Stimulating Hormone levels	Diminished Ovarian Reserve
Bisphenol A	16.2	27.9	36.4
Methylparaben	19.8	24.5	33.7
Propylparaben	9.6	25.2	35.2
Butylparaben	8.3	25.2	36.2
MBP	7.3	26.3	38.2
MiBP	19.2	27.5	34.5
MEP	8.0	32.6	34.0
MBZP	20.5	30.9	37.1
Σ DEHP ¹	10.7	22.9	34.7

MBP: mono-n-butyl phthalate; MiBP: mono-isobutyl phthalate; MEP: monoethyl phthalate; MBzP: monobenzyl phthalate.

¹ Σ DEHP: Molar sum of DEHP metabolites (mono(2-ethylhexyl) phthalate, mono(2-ethyl-5-hydroxyhexyl) phthalate, mono(2-ethyl-5-oxohexyl) phthalate, and mono(2-ethyl-5-carboxypentyl) phthalatePP).

Figure 1. BKMR mixture associations of phenol and phthalate biomarkers with lower antral follicle count (N = 262)

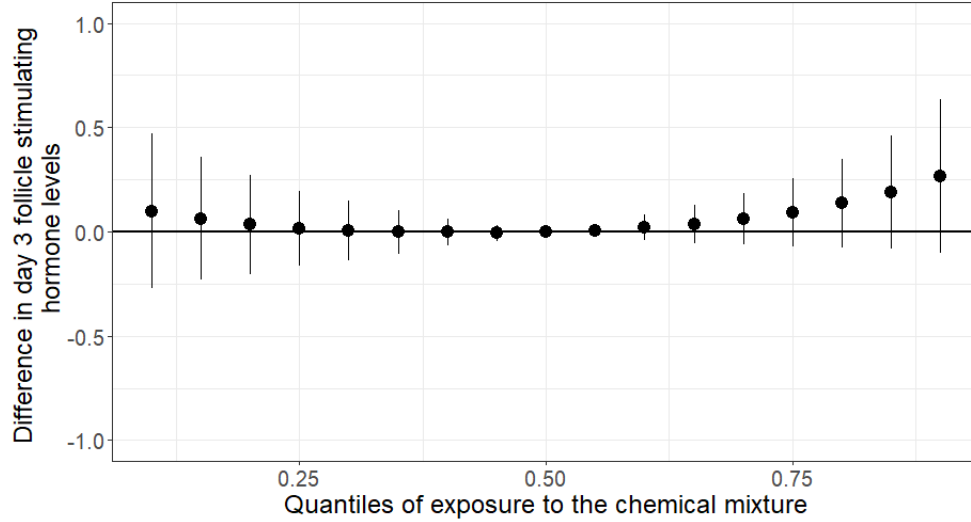




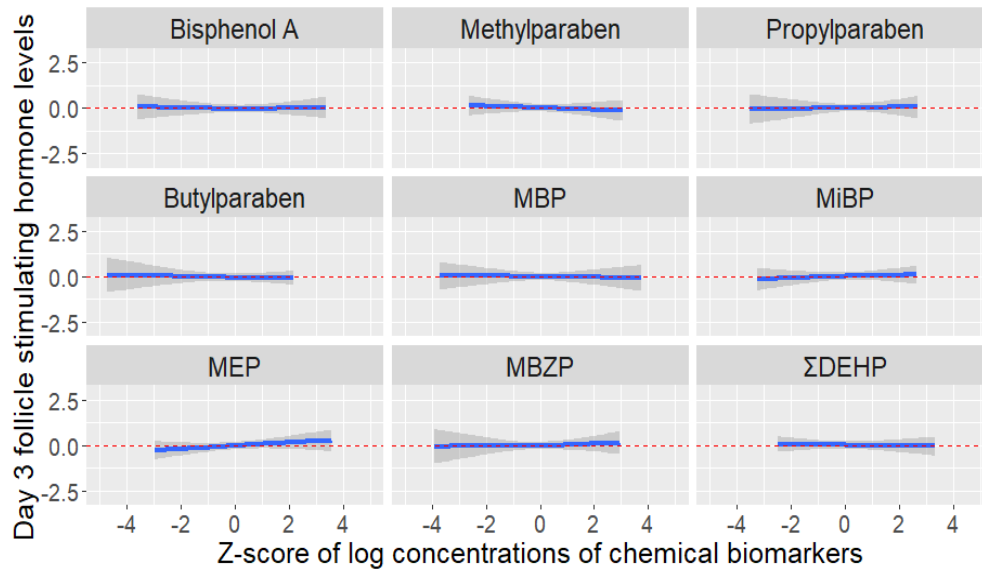
Adjusted for age, body mass index, education level and specific gravity. A. Overall association of the chemical biomarker mixture at different concentration percentiles compared to the 50th percentile and their 95% credible intervals. B. Exposure-response relationships for each chemical biomarker while holding all other biomarkers at their median concentrations. C. Mean difference comparing the 75th to 25th percentile of each exposure (estimates and 95% credible intervals) when all the other biomarker concentrations are fixed at the 25th, 50th, and 75th percentiles. N: number of participants; MBP: mono-n-butyl phthalate; MiBP: mono-isobutyl phthalate; MEP: monoethyl phthalate; MBzP: monobenzyl phthalate; MEHP: mono(2-ethylhexyl) phthalate; MEHHP: mono(2-ethyl-5-hydroxyhexyl) phthalate; MEOHP: mono(2-ethyl-5-oxohexyl) phthalate; MECPP: mono(2-ethyl-5-carboxypentyl) phthalate; ΣDEHP: molar sum of di(2-ethylhexyl) phthalate metabolites.

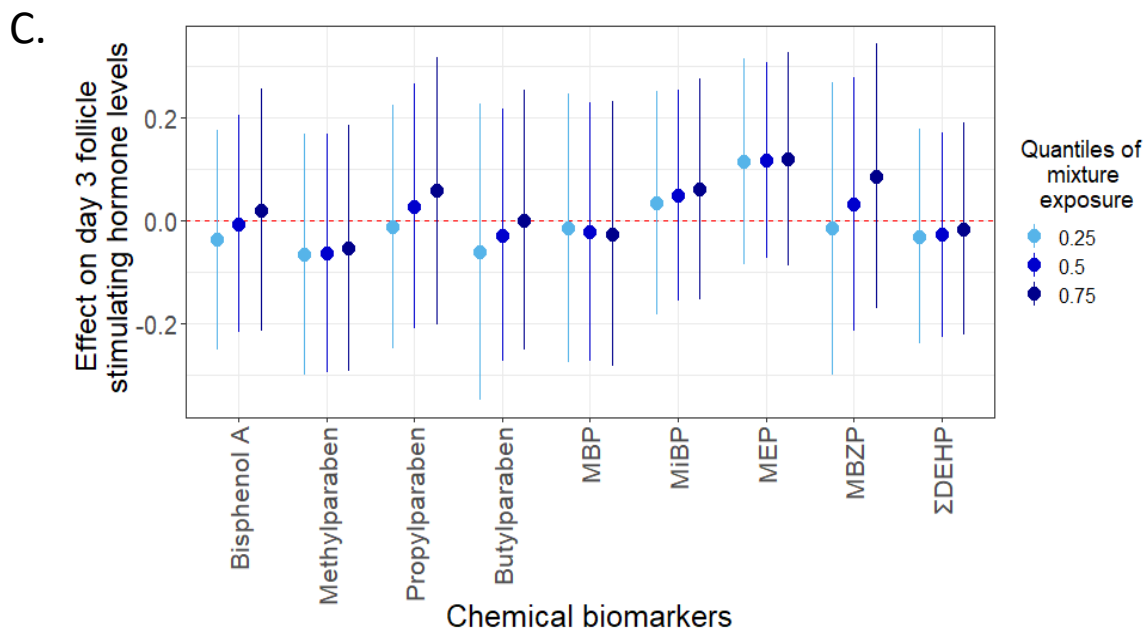
Figure 2. BKMR mixture associations of phenol and phthalate biomarkers with day-3 follicle stimulating hormone levels (N = 261)

A.



B.

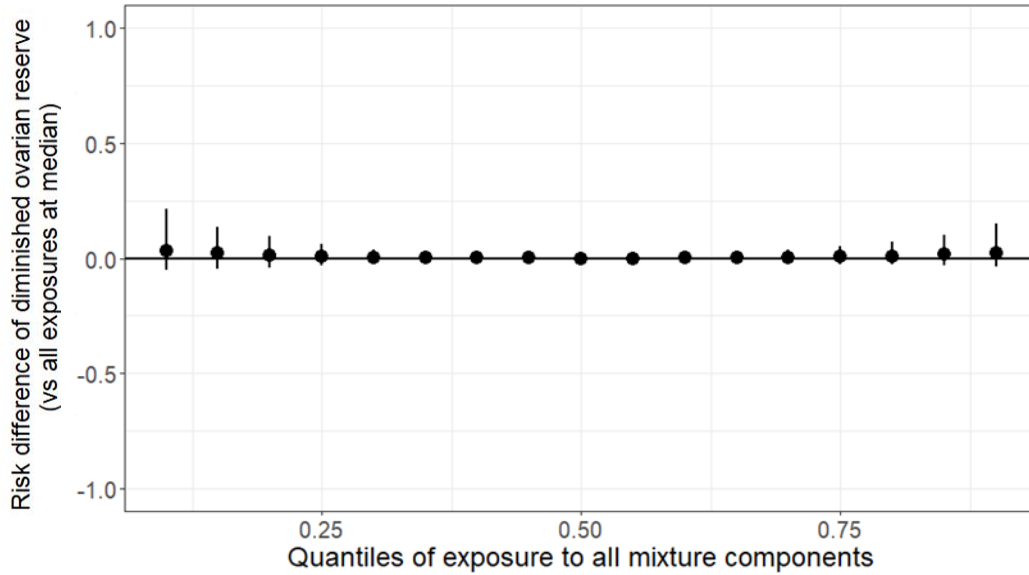




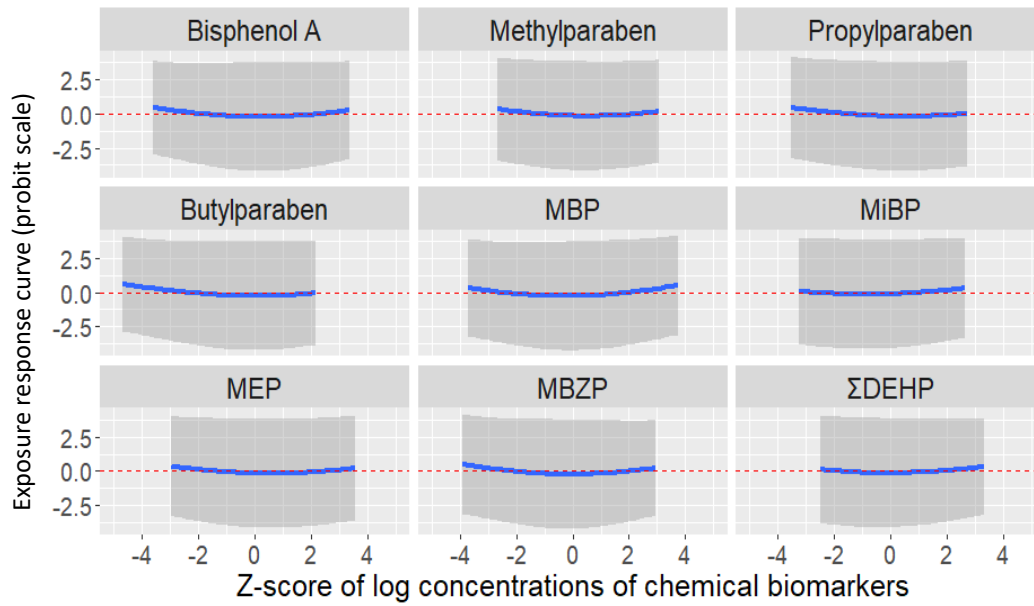
Adjusted for age, body mass index, education level and specific gravity. A. Overall association of the chemical biomarker mixture at different concentration percentiles compared to the 50th percentile and their 95% credible intervals. B. Exposure-response relationships for each chemical biomarker while holding all other biomarkers at their median concentrations. C. Mean difference comparing the 75th to 25th percentile of each exposure (estimates and 95% credible intervals) when all the other biomarker concentrations are fixed at the 25th, 50th, and 75th percentiles. N: number of participants; MBP: mono-n-butyl phthalate; MiBP: mono-isobutyl phthalate; MEP: monoethyl phthalate; MBzP: monobenzyl phthalate; MEHP: mono(2-ethylhexyl) phthalate; MEHHP: mono(2-ethyl-5-hydroxyhexyl) phthalate; MEOHP: mono(2-ethyl-5-oxohexyl) phthalate; MECPP: mono(2-ethyl-5-carboxypentyl) phthalate; ΣDEHP: molar sum of di(2-ethylhexyl) phthalate metabolites.

Figure 3. BKMR mixture associations of phenol and phthalate biomarkers with diminished ovarian reserve (N = 271)

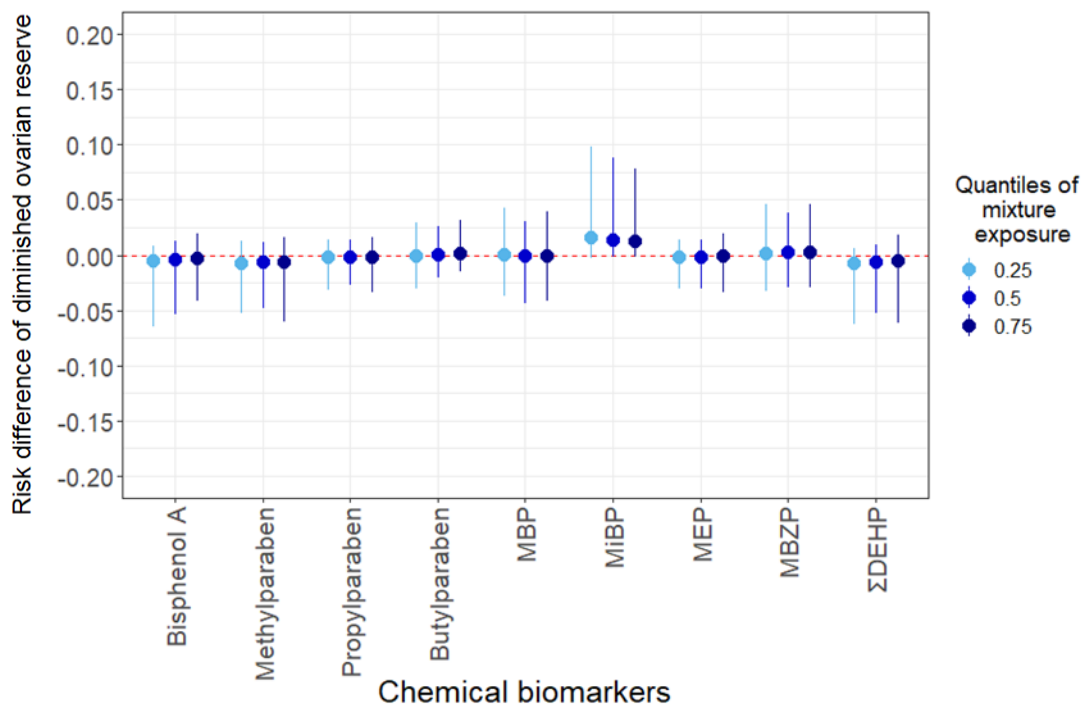
A.



B.



C.



Adjusted for age, body mass index, education level and specific gravity. A. Overall association of the chemical biomarker mixture at different concentration percentiles compared to the 50th percentile and their 95% credible intervals. B. Exposure-response relationships for each chemical biomarker while holding all other biomarkers at their median concentrations. C. Mean difference comparing the 75th to 25th percentile of each exposure (estimates and 95% credible intervals) when all the other biomarker concentrations are fixed at the 25th, 50th, and 75th percentiles. N: number of participants; MBP: mono-n-butyl phthalate; MiBP: mono-isobutyl phthalate; MEP: monoethyl phthalate; MBzP: monobenzyl phthalate; MEHP: mono(2-ethylhexyl) phthalate; MEHHP: mono(2-ethyl-5-hydroxyhexyl) phthalate; MEOHP: mono(2-ethyl-5-oxohexyl) phthalate; MECPP: mono(2-ethyl-5-carboxypentyl) phthalate; Σ DEHP: molar sum of di(2-ethylhexyl) phthalate metabolites.

Author contributions

Study concept and design: Mínguez-Alarcón, Williams, Hauser and Chavarro. Acquisition of data: Souter and Ford. Urine sample analyses: Calafat. Analysis of data: Génard-Walton.

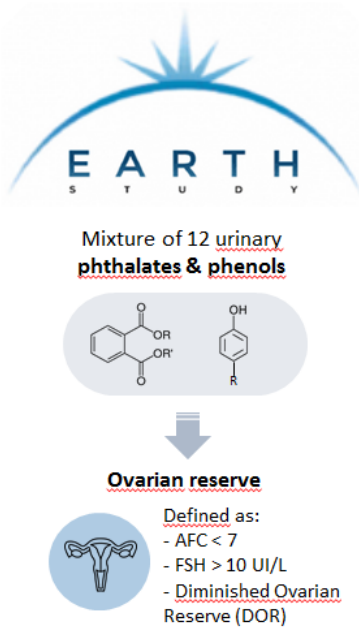
Interpretation of data: All authors. Drafting of the manuscript: Mínguez-Alarcón and Génard-Walton. Critical revision of the manuscript for important intellectual content: All authors.

Declaration of interests

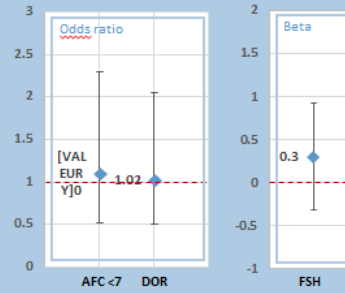
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Graphical abstract



Quantile g-computation effect for a quartile increase in exposure to the mixture (N = 271)



- 1 We found no associations between mixtures of urinary biomarker concentrations of phenols and phthalates and the ovarian reserve
- 2 Results were similar in both quantile g-computation and Bayesian Kernel Machine Regression models
- 3 Other studies on the topic are needed amongst the general population

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Highlights

- We evaluated chemicals mixtures in relation to AFC, FSH and DOR.
- Mixtures of phenols and phthalates were not associated with the ovarian reserve.
- There was no evidence of interaction between biomarkers in the BKMR analysis.

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