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Urinary phthalate metabolite concentrations are negatively associated with follicular fluid anti-müllerian hormone concentrations in women undergoing fertility treatment

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Abstract

Exposure to phthalates, endocrine-disrupting chemicals commonly used as plasticizers and in consumer products, has been associated with infertility and premature ovarian failure. Our objective was to investigate whether urinary phthalate metabolite concentrations were associated with pre-ovulatory follicular fluid (FF) anti-müllerian hormone (AMH) concentrations in women undergoing fertility treatment. This cross-sectional analysis included 138 women with urinary phthalate data available in the Environment and Reproductive Health (EARTH) Study (2010–2016) in whom FF AMH concentrations were quantified using a sandwich enzyme-linked

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Declaration of Competing Interest

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envint.2021.106809>.

immunosorbent assay (ELISA). We also quantified 8 phthalate metabolite concentrations using tandem mass spectrometry in 1–2 urine samples per cycle (total 331 urines) and calculated the cycle-specific geometric mean for each metabolite. We applied cluster-weighted generalized estimating equation models (CWGEE) to evaluate the associations of tertiles of urinary phthalate metabolite concentrations with log-transformed FF AMH concentrations adjusting for potential confounders. Study participants had median age of 34.0 years (IQR 32.0, 37.0), 83% were white, and median BMI of 23.1 kg/m² (IQR 21.2, 26.1). The following stimulation protocols were used: luteal phase agonist (70%), antagonist (14%), or flare (16%). Urinary concentrations of select phthalate metabolites were negatively associated with FF AMH. For example, women whose urinary mEOHP was in the lowest tertile (range 0.30–4.04 ng/ml) had an adjusted mean FF AMH of 0.72 ng/mL (95% CI = 0.36, 1.44), compared to women in the highest tertile (range 9.90–235), who had an adjusted mean of 0.24 ng/mL (95% CI = 0.12–0.48, $p < 0.05$). The negative association between urinary concentrations of certain phthalate metabolites with FF AMH concentrations may have implications for antral follicle recruitment and fertility treatment outcomes.

Keywords

IVF; Follicular fluid; AMH; Phthalates

1. Introduction

Phthalates are endocrine disrupting chemicals that are ubiquitous in some plastics and everyday consumer products, such as detergents, medical devices, and food packaging materials (Begum et al., 2020; Hauser and Calafat, 2005; Katsikantami et al., 2016). Phthalates easily leach from plastic products into the environment, leading to daily human exposure. After exposure, high molecular weight phthalates such as di-(2-ethylhexyl) phthalate (DEHP) are rapidly metabolized to hydrolytic monoesters, such as mono-(2-ethylhexyl) phthalate (MEHP), which in turn form oxidative metabolites after enzymatic oxidation of their alkyl chain (Hauser and Calafat, 2005). Four phthalates (DEHP, dibutyl phthalate, benzyl butyl phthalate, di-isobutyl phthalate) are subjects of Annex XVII of Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) Regulation in Europe (Commission Regulation, 2018).

Phthalates have been associated with female reproductive toxicity in animal studies due in large part to their endocrine disrupting properties (Hauser and Calafat, 2005). For example, exposure to DEHP and MEHP has a negative effect on murine reproduction via accelerated primordial follicle recruitment, increased follicular atresia, cell cycle dysregulation, and altered steroidogenesis (Hannon et al., 2015; Craig et al., 2014; Gupta et al., 2010; Wang et al., 2012; Hannon et al., 2015). Many phthalates are considered endocrine-disrupting chemicals (EDCs) in humans as well (Green et al., 2021; Heindel et al., 2017). Human exposure can be quantified by detection of phthalates metabolites in urine (Silva et al., 2004; Silva et al., 2007). Prior epidemiological studies, including those by our group, have demonstrated that maternal urinary phthalate concentrations are inversely associated with ovarian reserve, as assessed by antral follicle count (Messerlian et al., 2016); and also with

in vitro fertilization (IVF) outcomes, specifically oocyte yield, oocyte fertilization, embryo quality, clinical pregnancy, and live birth (Hauser et al., 2016; Mínguez-Alarcón et al., 2019; Begum et al., 2021; Machtinger et al., 2018).

Anti-Müllerian hormone (AMH), also known as Müllerian inhibiting substance (MIS), produced by the granulosa cells of preantral and early antral follicles (Weenen, 2004); is a reliable marker of ovarian reserve and response to gonadotropin medications (Buyuk et al., 2011). We recently reported that AMH in the pre-ovulatory follicular fluid (FF) of women undergoing IVF was positively associated with the probability of clinical pregnancy, even after adjusting for important confounders such as age and stimulation protocol (Sacha et al., 2020). Other studies have also observed positive associations between FF AMH and outcomes such as fertilization rate, embryo quality, and implantation rate (Fanchin et al., 2007; Kim et al., 2014). Therefore, FF AMH, while reflecting other measures of ovarian reserve such as serum AMH, is an indicator of granulosa cell function and plays an important physiologic role in oocyte development in the follicle. Exposure to DEHP and mono-(2-ethylhexyl) phthalate (MEHP) (Gupta et al., 2010; Davis et al., 1994) was shown to reduce estradiol production by granulosa cells of antral follicles, which led us to hypothesize that AMH production could also be reduced due to granulosa cell toxicity. Examining FF AMH is thus crucial to understand the mechanisms by which phthalate exposure effects the local ovarian environment and folliculogenesis.

In this study, we evaluated whether maternal urinary phthalate metabolite concentrations were associated with pre-ovulatory FF AMH concentrations. We hypothesized that urinary phthalate metabolites would be negatively associated with FF AMH concentrations.

2. Materials and methods

2.1. Study design

Participants included in this analysis were women seeking fertility care at the Massachusetts General Hospital (MGH) Fertility Center, a single academic fertility center in Boston, Massachusetts, who enrolled in the Environment and Reproductive Health (EARTH) Study (2004–2019). The EARTH Study was a prospective cohort study that aimed to investigate environmental and dietary factors in relation to fertility and reproductive health (Mínguez-Alarcón et al., 2016). Women between the ages of 18 and 45 years old with any infertility diagnosis were eligible to enroll. The MGH Fertility Center additionally has a maximum body mass index (BMI) threshold of 40 kg/m² to undergo treatment. FF AMH concentrations were quantified from 162 women contributing 217 IVF cycles in which at least two FF samples were collected (between 2010 and 2016). Of those, 138 women who underwent 179 fresh IVF cycles had urinary phthalate metabolite concentration available and are included in the present cross-sectional analysis, since both urine and FF samples were collected the same day. We have previously shown that women in the EARTH study with FF AMH concentrations measured had similar demographic and reproductive characteristics compared to women who did not (Sacha et al., 2020). Participants signed an informed consent prior to study enrollment. The EARTH Study is approved by the institutional review boards at MGH (Partners IRB #1999P008167) and Harvard T.H. Chan School of Public Health.

2.2. Ovarian stimulation protocols

All study participants underwent a standard infertility work-up as previously described (Messerlian et al., 2018; Souter et al., 2013). Infertility diagnosis was assigned according to previously described definitions of the Society for Assisted Reproductive Technology (SART) (SART, 2015). SART diagnoses include: 1) male factor infertility; 2) female factor infertility which included endometriosis, diminished ovarian reserve, tubal or ovulatory disorders, or other causes; and 3) unexplained infertility (idiopathic).

Patients then underwent controlled ovarian hyperstimulation by luteal-phase gonadotropin-releasing hormone (GnRH) agonist, GnRH-antagonist downregulation, or GnRH agonist flare protocol, with follicular synchronization and pituitary down-regulation as clinically indicated and previously described (Souter et al., 2013; Chavarro et al., 2012; Gaskins et al., 2019; Sacha et al., 2019). During treatment with recombinant gonadotropins (follitropin beta, Follistim, Merck, Kenilworth, NJ, or follitropin alpha, Gonal-F, EMD-Serono; and menotropins, Menopur or Repronex, Ferring Pharmaceuticals, Parsippany, NJ), patients were serially monitored with transvaginal ultrasound and serum estradiol (E₂) to assess follicular measurements and endometrial thickness. Once at least three follicles reached 16 mm or more in diameter and the E₂ level was > 600 pg/mL, intramuscular human chorionic gonadotropin (hCG) (10,000 IU, Novarel, Ferring Pharmaceuticals or 10,000 IU, Pregnyl, Merck) was administered to induce final oocyte maturation. The peak serum E₂ concentration was defined as the highest level of E₂ preceding the oocyte retrieval and obtained on the day of hCG administration. The patients underwent a transvaginal ultrasound-guided oocyte retrieval 35–37 h later (Chavarro et al., 2012). Intramuscular progesterone, 50 mg per day, was begun on the day after oocyte retrieval and continued until 10 weeks gestation if the patient conceived.

2.3. FF collection and AMH quantification

FF was collected as previously described from the first three (N = 102 women, 74%) or two (N = 36 women, 26%) dominant (>16 mm) follicles punctured during each patient's egg retrieval (Sacha et al., 2020). The fluid from each follicle was separately aspirated into a tube with 1 ml sterile culture media so that FF volume could be accurately calculated. After identification and transfer of the oocytes by the embryologist, the FF from each follicle was transferred to a 15 ml tube and the collection time and sample volumes logged. The FF was then centrifuged at 3000 rpm for 10 min to remove material such as red blood cells and granulosa cells, and the supernatant was stored in aliquots at – 80C, taking care not to pool FF from different follicles.

AMH concentration was quantified in FF of the study patients using a sandwich enzyme-linked immunosorbent assay (ELISA) with mouse monoclonal anti-human recombinant antibody (6E11), which binds to the AMH homodimer, as the primary antibody, and rabbit polyclonal anti-AMH antibody (MGH6), as the secondary antibody (Sacha et al., 2020; Kano et al., 2017; Hudson et al., 1990). The standard curve was created using a recombinant AMH protein “LRMIS” (created from AMH/MIS cDNA with human albumin leader sequence (L) and modified cleavage site (R)) in blocking buffer (1% BSA/PBS and Tween 20 (PBST)), which has been proposed as an international standard by the World Health

Organization (WHO) (Pepin et al., 2013; Ferguson et al., 2018). This ELISA produces absolute AMH concentrations that strongly correlate with the Gen II Elisa commercial assay used by the MGH clinical laboratories during the study time period (Spearman $r^2 = 0.81$, evaluated for a subset of 22 women with serum AMH concentrations previously performed for their fertility work up (Sacha et al., 2020). Intra-assay variability was 12.0% and inter-assay variability was 15.5% with an assay limit of detection (LOD) of 0.034–0.068 ng/mL based on the standard curve values. Values below the levels represent extrapolation from the standard curve. Prior to analysis, FF AMH concentration was corrected for sample culture media volume using the formula: corrected AMH concentration = (measured AMH concentration * measured volume)/(measured volume – 1 ml).

2.4. Urine collection and phthalate metabolite quantification

Women provided one (23%) or two (77%) spot urine samples per IVF cycle, with the first one collected between days 3 and 9 of the follicular phase (not necessarily a fasting sample), and the second one collected as a fasting sample in the morning prior to the oocyte retrieval, approximately one week apart. Urine was collected in a sterile polypropylene specimen cup. Specific gravity (SG), used as a covariate in the models to account for urine dilution, was measured at room temperature using a handheld refractometer (National Instrument Company, Inc., Baltimore, MD, USA) calibrated with deionized water before each measurement. The urine was divided into aliquots, frozen, and stored at $-80\text{ }^{\circ}\text{C}$. Urine samples were shipped on dry ice overnight to the Center for Disease Control (CDC) where they were stored at or below $-40\text{ }^{\circ}\text{C}$ until analysis. The CDC laboratory is certified by the Health Care Financing Administration to comply with the requirements set forth in the Clinical Laboratory Improvement Amendments of 1988 (CLIA '88). All analytical measurements follow strict quality control/quality assurance according to CLIA guidelines. For example, along with study samples, each analytical run includes 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).

As previously described (Hauser et al., 2016); we used online solid-phase extraction coupled with isotope dilution-high-performance liquid chromatography-tandem mass spectrometry to quantify the urinary concentrations of phthalate metabolites, including monoethyl phthalate (MEP); mono-n-butyl phthalate (MBP); monoisobutyl phthalate (MiBP); monobenzyl phthalate (MBzP); MEHP; mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP); mono(2-ethyl-5-oxohexyl) phthalate (MEOHP); and mono(2-ethyl-5-carboxypentyl) phthalate (MECPP) (Silva et al., 2007). LOD ranged from 0.1 to 1.2 ng/mL. Concentrations below the LOD were assigned a value equal to the LOD divided by the square root of 2. The geometric mean of the phthalate metabolite concentrations from two spot urine samples collected during each IVF cycle was used as a measure of cycle-specific urinary biomarker concentration, except for cycles with only one urine sample for which the single biomarker concentration was used as the cycle-specific urinary biomarker concentration. A similar approach was used to calculate the cycle-specific SG. Because of the potential for bias, rather than correcting by SG using a formula, we adjusted for SG by including this as a covariate in the statistical models (Schisterman et al., 2005; Barr et al., 2005). We

calculated the molar sum of DEHP metabolites (Σ DEHP) by dividing each DEHP metabolite concentration by its molecular weight (g/mol) and then summing: $([MEHP \times (1/278.34)] + [MEHHP \times (1/294.34)] + [MEOHP \times (1/292.33)] + [MECPP \times (1/308.33)])$. Similarly, we calculated the molar sum of non-DEHP metabolites (Σ non-DEHP) by dividing each non-DEHP metabolite concentration by its molecular weight and then summing: $([MEP \times (1/194.18)] + [MBP \times (1/222.24)] + [MiBP \times (1/222.24)] + [MBzP \times (1/256.25)])$.

2.5. Statistical analyses

We present demographic and reproductive characteristics of the study participants using median \pm interquartile ranges (IQRs) or counts (%), and distribution of urinary concentrations of phthalate metabolites using percentiles and geometric means \pm standard deviations (SDs). Mean intra-cycle FF AMH concentrations were calculated as the sum of FF AMH concentrations in the two or three selected follicles, divided per cycle by the number of follicles. Due to right skewness, we log-transformed the mean intra-cycle FF AMH concentrations and this variable was used as the outcome in the statistical models. Urinary concentrations of phthalate metabolites were investigated individually as well as the Σ DEHP and Σ non-DEHP, based on similar exposure sources previously found among women in the EARTH Study (Souter et al., 2020). Then, exposure variables were categorized into tertiles, with the lowest tertiles considered as the reference group. We applied cluster-weighted generalized estimating equation models (CWGEE) to evaluate the associations of tertiles of urinary phthalate metabolite concentrations with log-transformed FF AMH concentrations adjusting for potential confounders. As previously noted in an application to women in EARTH, CWGEE is predicted to provide efficient estimates in the presence of a different number of IVF cycles contributed per woman, as women with more cycles have more difficulty getting pregnant, provided that the lack of balance can be accurately predicted by all measured covariates in the adjusted model (Yland et al., 2019; Williamson et al., 2003).

To enhance the interpretation of the results, population marginal means (Searle et al., 1980) were presented adjusting for all the covariates in the model (at the mean level for continuous variables and weighted according to their relative frequencies for categorical variables). Confounding was assessed using prior knowledge on biological relevance and descriptive statistics from our study population using a directed acyclic graph (DAG) (Supplemental Fig. 1). The variables considered as potential confounders included factors previously related to female reproductive endpoints (Rooney and Domar, 2014; Sharma et al., 2013), and factors associated with urinary phthalate metabolites and reproductive outcomes, including FF AMH concentrations, in this study. In addition, we included well-known predictors in the fully adjusted models regardless of statistical significance since it has been demonstrated that this can improve the precision of the exposure estimates in the model (Schisterman et al., 2009). Final models were adjusted for urinary dilution (SG), age, race, body mass index (BMI), and stimulation protocol type. Statistical analyses were conducted with SAS (version 9.4; SAS Institute Inc., Cary, NC, USA). Statistical tests were two-tailed and all p-values < 0.05 were regarded as statistically significant.

3. Results

The 138 women included in this analysis had a median (interquartile range [IQR]) age of 34.0 (32.0, 37.0) years and BMI of 23.1 (21.2, 26.1) kg/m², were predominantly white (83%), and 24% ever smoked (Table 1). The most common infertility diagnosis was male factor (42%). Most female participants underwent a luteal phase GnRH agonist stimulation protocol (70%) during their IVF cycles, of which over half (54%) utilized intracytoplasmic sperm injection (ICSI). Median (IQR) FF AMH concentrations were 0.61 (0.23, 1.55) ng/mL.

A total of 331 urine samples were collected from the 138 women undergoing 179 fresh IVF cycles and included in this analysis (Table 2). The detection frequencies for urinary concentrations of phthalate metabolites ranged from 75% to 100%, and were similar to those found in U.S. females from the general population (CDC, 2018). Urinary concentrations of the DEHP metabolites MEHP, MEHHP, MEOHP and MECPP were highly correlated with each other ($r = 0.80$ to 0.89) (Supplemental Table 1). Urinary concentrations of MEP, MBP, MiBP, and MBzP were moderate-to-weakly correlated with all other phthalate DEHP and non-DEHP metabolites ($r < 0.52$). Urinary phthalate metabolites among women who provided two urine samples were moderately ($r = 0.25$ for mEHP) to highly ($r = 0.65$ for mEP) correlated. We found low-to-moderate across-women variability for the measured urinary phthalate metabolites [ICC (95% CI) ranged from 0.05 (0.01–0.32 for mEHP) to 0.44 (0.33–0.56) for mBzP].

In adjusted models, urinary MEOHP concentrations were negatively associated with FF AMH concentrations ($p = 0.01$, Table 3). Specifically, compared to women in the first tertile of MEOHP, women both in the second and in the third tertiles, had on average, 30% lower FF AMH. We also observed other suggestive negative dose–response between urinary MECPP, DEHP as well as MBzP, and FF AMH concentrations. For example, women in the first tertile of urinary MECPP had, on average, 0.56 ng/mL of FF AMH concentrations, compared to 0.28 ($p = 0.15$) and 0.25 ng/mL ($p = 0.09$) for women in the second or third tertiles, respectively.

Although associations did not reach statistical significance, we also observed decreasing FF AMH with increasing tertiles of urinary MBP, MiBP and MEHHP concentrations. Tertiles of urinary MEHP concentrations were unrelated to FF AMH among women in our study.

4. Discussion

This study findings support prior literature demonstrating that phthalate exposure in women is associated with altered intrafollicular steroidogenesis (Hannon and Flaws, 2015) and is the first, to our knowledge, showing negative associations between select urinary phthalate metabolite concentrations and FF AMH concentrations. Among women undergoing IVF in EARTH, we have reported that FF AMH concentrations are positively associated with the probability of clinical pregnancy (Sacha et al., 2020), and also that urinary phthalate metabolite concentrations are negatively related to pregnancy outcomes, such as probability of clinical pregnancy (Hauser et al., 2016). Therefore, findings from the current study, if

replicated in longitudinal studies, could provide the basis of a potential mechanism by which phthalates may affect pregnancy outcomes in women undergoing fertility treatment. From a clinical perspective, a better understanding of the role that certain environmental exposures play in conditions such as diminished ovarian reserve may help inform management of these patients and provide opportunities for guidance on ways to reduce exposure to environmental chemicals such as phthalates.

The correlations observed in our study between urinary phthalate metabolites and FF AMH in preovulatory follicles are consistent with prior studies in both humans and animals suggesting that ovarian steroid production is susceptible to phthalate exposure in women with infertility (Hannon et al., 2015; Gupta et al., 2010; Hannon et al., 2015; Hauser et al., 2016; Du et al., 2019; Svechnikova et al., 2007). For example, DEHP-treated preovulatory follicles in rats were smaller due to smaller granulosa cell areas, and produced less estrogen, leading to a lack of LH surge and anovulation (Davis et al., 1994). We report that MEOHP in particular may affect granulosa cells in women based on the inverse association with FF AMH. Altered preovulatory steroidogenesis may contribute to the observations by Hauser et al. in this same cohort and by Machtinger et al. of decreased total, mature, and fertilized oocytes, as well as fewer high quality embryos, with increased urinary MEOHP concentrations (Hauser et al., 2016; Machtinger et al., 2018). It remains unclear why associations differed among phthalates from the same diester, for instance for MEHP, MEOHP and MEHHP. It may relate to differences in biological activity of the metabolites or the metabolism of the diester into the monoester and oxidative metabolites.

Altered FF AMH production may shed light on the negative effects of phthalate exposure on the antral follicle pool (Hannon et al., 2015; Hannon et al., 2015; Messerlian et al., 2016). Animal studies show that low level daily exposure of female mice to DEHP leads to increased PI3K signaling, a proposed mechanism for the observed acceleration of primordial follicle recruitment (Hannon et al., 2014). Over time, accelerated recruitment could lead to a prematurely exhausted cohort of antral follicles, diminished ovarian reserve, and infertility (Hannon and Flaws, 2015; Hannon et al., 2014). AMH plays a key role in inhibiting primordial follicle recruitment, and AMH gene expression in granulosa cells is induced by Bone morphogenetic protein 15 (BMP15) and Growth differentiation factor 9 (GDF9) via both Smad2/3 and PI3K/Akt pathway signalling (Roy et al., 2018). Interestingly, DEHP has been shown to decrease *gdf9 mRNA* expression in female mice (Liu et al., 2018); and MEHP appears to inhibit FSH-stimulated cAMP accumulation in rat granulosa cells (Treinen et al., 1990; Davis et al., 1994). Reductions in GDF9 and cAMP could reduce AMH expression (Roy et al., 2018; Taieb et al., 2011). Thus, phthalates are likely toxic to key growth factors in granulosa cells, leading to reduced production of AMH in FF, which could promote accelerated recruitment of primordial follicles and over time gradual depletion of ovarian reserve and antral follicles pools. Within the context of an IVF cycle, this may also lead to early recruitment of a dominant follicle or a limited pool of follicles, which may ultimately reduce IVF success. Finally, dysregulated PI3K signaling may be a result of reduced AMH expression rather than a direct mechanism for accelerated primordial follicle recruitment by phthalates; this requires further investigation.

The EARTH study cohort and study design have numerous strengths. Study participants were treated at a single institution with a consistent method for FF collection following EARTH study protocols. Urine is considered the optimal matrix for assessment of biomarkers of exposure for non-persistent chemicals and so our work improves on prior literature examining the association between phthalate metabolites and hormone concentrations in FF (Du et al., 2019; Calafat et al., 2015). In our study, urine for phthalate assessment was collected at two time points during the IVF cycle, which improves the exposure assessment given the relatively short half-life of phthalates and the episodic nature of the exposures. Unless patients drastically altered their activities of daily life during their IVF cycle, urinary phthalate metabolite concentrations could adequately reflect long-term exposure. However, we are not able to confirm a causative temporal association since we do not know the extent of past cumulative phthalate exposure and its impact FF AMH.

There are several important limitations to our work. First, we assess a select group of primarily white, highly educated women with infertility, who do not reflect all women with infertility and may be more susceptible to endocrine disruption than fertile women. Studying the effects of phthalate exposure in fertile women undergoing fertility treatment (i.e. for egg freezing, egg donation, or genetic reasons) would be an interesting future study. Furthermore, because there is no clinical indication for FF collection in the general population, it is not possible to compare our findings to the general population, thus limiting the generalizability of our study. To address potential varied effects of ovarian stimulation on FF AMH, we controlled for stimulation protocol in our models. Furthermore, we were unable to assess how increased phthalate exposure impacts women with polycystic ovarian syndrome (PCOS) and diminished ovarian reserve due to low numbers of women with these diagnoses. This question could be evaluated in a larger study, as well. The cross-sectional design of this study using urine samples provided during the IVF cycle prevents establishing that exposure to phthalates occurred before changes in FF AMH, which is needed to perform a proper mediation analysis. However, results from this analysis are expected to motivate further research on the topic that should include longitudinal analyses.

Our sample size was modest, though we still observed dose–response relationships between phthalate exposure and FF AMH concentrations. Available FF volumes restricted our ability to perform more than two replicates for each FF AMH measurement; more replicates may have reduced assay variability and improved our ability to detect associations with phthalate concentrations. In addition, the women who chose to participate in the EARTH study had often previously undergone IVF, and so this cohort may be biased towards patients with a poorer prognosis compared to our total clinic population. We also included only cycles with at least two FF samples for AMH quantification and phthalates measured in urine, creating the possibility of selection bias. However, the characteristics of the women in our analysis were similar to the overall EARTH study cohort, reducing the chances of selection bias. Furthermore, we still observed inverse correlations between specific phthalate metabolites and FF AMH. Importantly, contamination of FF with blood is a limitation common to all studies analyzing components of FF obtained during oocyte retrieval. Finally, assessing other intrafollicular hormones such as estradiol and progesterone, were beyond the scope of the current work but would be interesting to measure as other markers of follicle health and ovarian aging.

5. Conclusions

In this study, we demonstrate that increased urinary concentrations of selected phthalate metabolites were associated with decreased FF AMH. These findings establish an important role for FF AMH in understanding the potential effects of phthalate exposure on ovarian physiology, reproductive potential, and IVF outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

AMH	Anti-Müllerian hormone
MIS	Müllerian inhibiting substance
FF	follicular fluid
IVF	in vitro fertilization
EARTH	Environment and Reproductive Health
DEHP	Study di-(2-ethylhexyl) phthalate
BMI	body mass index
SART	Society for Assisted Reproductive Technology
GnRH	gonadotropin-releasing hormone
hCG	human chorionic gonadotropin
ELISA	enzyme-linked immunosorbent assay
PBST	1% BSA/PBS and Tween 20
WHO	World Health Organization
CDC	Center for Disease Control
MEP	monoethyl phthalate
MBP	mono-n-butyl phthalate
MiBP	monoisobutyl phthalate

MBzP	monobenzyl phthalate
MEHP	mono(2-ethylhexyl) phthalate
MEHHP	mono(2-ethyl-5-hydroxyhexyl) phthalate
MEOHP	mono(2-ethyl-5-oxohexyl) phthalate
MECPP	and mono(2-ethyl-5-carboxypentyl) phthalate
DEHP	molar sum of DEHP metabolites
SG	specific gravity
IQRs	interquartile ranges
LOD	limits of detection
SDs	standard deviations
CWGEE	cluster-weighted generalized estimating equation models
ICSI	intracytoplasmic sperm injection

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Table 1

Demographic and reproductive characteristics [median (IQR) or N (%)] among 138 women undergoing 179 IVF cycles in the Environment and Reproductive Health (EARTH) Study.

Per woman	
Age, years	34.0 (32.0, 37.0)
Race, N (%)	
White	114 (83)
Black	4 (3)
Asian	14 (10)
Other	6 (4)
Body Mass Index, kg/m ²	23.1 (21.2, 26.1)
Ever smoked, N (%)	33 (24)
Education, N (%)	
High school/some college	11 (8)
College graduate	40 (29)
Graduate degree	87 (63)
Per cycle Infertility diagnosis, n (%)	
Male factor	76 (42)
Female factor	53 (30)
Diminished Ovarian Reserve	11 (6)
Endometriosis	8 (4)
Ovulatory Disorders	19 (11)
Tubal	14 (8)
Uterine	1 (1)
Idiopathic	50 (28)
Treatment protocol, n (%)	
Antagonist	25 (14)
Flare	28 (16)
Luteal phase agonist	126 (70)
Estradiol Trigger Levels, pmol/L	2026 (1542, 2614)
ICSI ^a cycles, n (%)	97 (54)
Day 3 FSH ^b Levels, IU/L	6.5 (5.8, 7.8)
FF ^c AMH ^d concentrations, ng/mL	0.61 (0.23, 1.55)

^aIntracytoplasmic sperm injection

^bFollicular stimulating hormone

^cFollicular fluid

^dAnti-Müllerian hormone

Distribution of urinary concentrations ($\mu\text{g/L}$) of phthalate metabolites among 138 women contributing 331 urine samples in the EARTH Study.

Table 2

	Detection Frequency ^a %	GM (SD)	10th	25th	50th	75th	95th
Mono-n-butyl phthalate (MBP)	98	10.1 (0.73)	1.70	4.80	11.9	25.4	66.1
Mono-isobutyl phthalate (MIBP)	99	7.87 (0.54)	1.60	3.40	9.50	18.9	47.4
Monoethyl phthalate (MEP)	100	38.6 (3.11)	6.60	14.5	33.1	98.3	477
Monobenzyl phthalate (MBzP)	95	3.50 (0.28)	0.50	1.22	3.20	8.70	34.2
Mono-2-ethylhexyl phthalate (MEHP)	75	1.70 (0.12)	0.35	0.35	1.60	3.60	20.6
Mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP)	100	9.11 (0.62)	1.90	3.90	8.70	19.7	78.8
Mono-2-ethyl-5-oxohexyl phthalate (MEOHP)	100	6.49 (0.43)	1.30	3.00	6.70	13.5	47.7
Mono-2-ethyl-5-carboxypentyl phthalate (MECPP)	100	16.0 (0.97)	3.90	7.20	16.0	30.5	116

Per IVF cycle (N total = 179), 1 (N = 27, 15%) or 2 (N = 152, 85%) urine samples (N = 331 total) were collected.

^aLimits of detection (LOD) ranged from 0.1 to 1.2 ng/ml.

Table 3

Adjusted (adjusted mean, 95% CI) FF AMH concentrations by tertiles of urinary phthalate metabolite concentrations among 138 women undergoing 179 IVF cycles in the EARTH Study.

FF AMH levels, ng/mL		FF AMH levels, ng/mL	
Range, ng/mL	Adjusted for Specific gravity (SG)	Range, ng/mL	Adjusted for SG
	Adjusted for SG, age, BMI, race and protocol.		Adjusted for SG, age, BMI, race and protocol.
mEHP			
T1 (0.35–0.90)	0.34 (0.17, 0.69)	T1 (0.28–6.29)	0.43 (0.20, 0.90)
T2 (0.94–2.34)	0.46 (0.23, 0.92)	T2 (6.30–16.6)	0.31 (0.16, 0.62)
T3 (2.40–102)	0.27 (0.14, 0.54)	T3 (16.7–215)	0.32 (0.15, 0.67)
T2-T3 (0.94–102)	0.35 (0.21, 0.58)	T2-T3 (6.30–215)	0.32 (0.19, 0.52)
mEHHP			
T1 (0.56–5.92)	0.44 (0.22, 0.90)	T1 (0.14–5.13)	0.47 (0.22, 0.99)
T2 (6.01–13.9)	0.37 (0.19, 0.75)	T2 (5.16–13.1)	0.35 (0.17, 0.70)
T3 (14.0–317)	0.25 (0.12, 0.51)	T3 (13.2–86.6)	0.26 (0.12, 0.53)
T2-T3 (6.01–317)	0.31 (0.19, 0.51)	T2-T3 (5.16–86.6)	0.30 (0.18, 0.50)
mEOHP			
T1 (0.30–4.04)	0.77 (0.38, 1.56)	T1 (0.80–19.0)	0.56 (0.28, 1.11)
T2 (4.13–9.81)	0.24 (0.12, 0.47)*	T2 (19.3–63.1)	0.22 (0.11, 0.44)‡
T3 (9.90–235)	0.23 (0.11, 0.47)*	T3 (65.2–2610)	0.34 (0.17, 0.68)
T2-T3 (4.13–235)	0.23 (0.14, 0.38)*	T2-T3 (19.3–2610)	0.28 (0.17, 0.45)‡
mECP			
T1 (1.51–10.3)	0.58 (0.28, 1.18)	T1 (0.21–1.77)	0.61 (0.30, 1.24)
T2 (10.4–23.0)	0.30 (0.15, 0.58)	T2 (1.78–5.88)	0.28 (0.14, 0.57)
T3 (23.1–338)	0.24 (0.12, 0.50)‡	T3 (6.00–136)	0.24 (0.12, 0.49)‡
T2-T3 (10.4–338)	0.27 (0.16, 0.44)‡	T2-T3 (1.78–136)	0.26 (0.16, 0.43)‡
DEHP			
T1 (0.01–0.07)	0.54 (0.27, 1.10)	non-DEHP	
T2 (0.08–0.16)	0.25 (0.12, 0.49)‡	T1 (0.01–0.19)	0.62 (0.31, 1.24)
T3 (0.17–3.34)	0.32 (0.15, 0.65)	T2 (0.20–0.54)	0.19 (0.10, 0.39)*
		T3 (0.55–13.8)	0.35 (0.18, 0.69)
			0.58 (0.29, 1.15)
			0.20 (0.10, 0.39)*
			0.35 (0.18, 0.68)

FF AMH levels, ng/mL		FF AMH levels, ng/mL	
Range, ng/mL	Adjusted for Specific gravity (SG) protocol.	Range, ng/mL	Adjusted for SG protocol.
T2-T3 (0.08–3.34)	0.28 (0.217 0.46) [‡]	T2-T3 (0.20–13.8)	0.26 (0.16, 0.43) [‡]

* p-value < 0.05 when comparing that tertile/group with the lowest tertile/group.

[‡] p-value < 0.10 when comparing that tertile/group with the lowest tertile/group.