Human Reproduction, Vol.31, No.1 pp. 75–83, 2016
Advanced Access publication on November 15, 2015 doi:10.1093/humrep/dev292

human reproduction

ORIGINAL ARTICLE *Infertility*

Urinary phthalate metabolites and ovarian reserve among women seeking infertility care

Carmen Messerlian[1,†*], Irene Souter[2,†], Audrey J. Gaskins[3], Paige L. Williams[4,5], Jennifer B. Ford[1], Yu-Han Chiu[3], Antonia M. Calafat[6], and Russ Hauser[1,2,5] for the Earth Study Team

[1]Department of Environmental Health, Harvard T.H. Chan School of Public Health, Boston, MA, USA [2]Massachusetts General Hospital Fertility Center, Department of Obstetrics and Gynecology, Harvard Medical School, Boston, MA, USA [3]Department of Nutrition, Harvard T.H. Chan School of Public Health, Boston, MA, USA [4]Department of Biostatistics, Harvard T.H. Chan School of Public Health, Boston, MA, USA [5]Department of Epidemiology, Harvard T.H. Chan School of Public Health, Boston, MA, USA [6]National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, GA, USA

*Correspondence address. Harvard T.H. Chan School of Public Health, 665 Huntington Avenue, FXB 102A, Boston, MA, 02115, USA. E-mail: cmesser@hsph.harvard.edu

Submitted on July 22, 2015; resubmitted on September 15, 2015; accepted on October 26, 2015

STUDY QUESTION: Are urinary phthalate metabolites associated with reduced antral follicle growth among women in an infertility setting?

SUMMARY ANSWER: Higher urinary concentrations of di(2-ethylhexyl) phthalate (DEHP) metabolites were associated with significant decreases in antral follicle count (AFC) among women seeking infertility care.

WHAT IS KNOWN ALREADY: Experimental animal studies show that DEHP accelerates primordial follicle recruitment and inhibits antral follicle growth. Whether phthalates also reduce the growing antral follicle pool in humans remains unknown.

STUDY DESIGN, SIZE, DURATION: We examined the association between urinary phthalate metabolites and AFC using prospective data from 215 females recruited between 2004 and 2012 in the Environment and Reproductive Health (EARTH) study.

PARTICIPANTS/MATERIALS, SETTING, METHODS: We quantified the urinary concentrations of 11 phthalate metabolites. We estimated the geometric mean for all urine samples provided prior to unstimulated day 3 AFC assessment for each woman. We evaluated the association of AFC with ∑DEHP (molar sum of four DEHP metabolites) and individual phthalate metabolites using Poisson regression, adjusting for age, BMI and smoking.

MAIN RESULTS AND THE ROLE OF CHANCE: We observed significant decreases in mean AFC for all higher quartiles of ∑DEHP as compared with the lowest quartile. Compared with women in the first quartile of ∑DEHP, women in the second, third and fourth quartiles had a −24% (95% confidence interval (CI): −32%, −16%), −19% (95% CI: −27%, −9%), and −14% (95% CI: −23%, −5%) decrease in mean AFC. The absolute mean AFC in the first quartile was 14.2 follicles (95% CI: 13.2, 15.2) compared with 10.7 follicles (95% CI: 9.9, 11.6) in the second quartile. We observed similar trends among the four individual DEHP metabolites. There was no consistent change in AFC among the remaining phthalate metabolite concentrations evaluated.

LIMITATIONS, REASONS FOR CAUTION: We demonstrated a negative association between DEHP and a well-established marker of ovarian reserve among a subfertile population. However these findings may not be generalizable to women without fertility concerns, and we cannot rule out co-exposure to other chemicals.

WIDER IMPLICATIONS OF THE FINDINGS: Environmental chemicals that inhibit the size of the growing antral follicle pool can impair fertility and reduce fecundity. This study suggests evidence in need of further investigation on the impact of phthalates on the human oocyte and follicular development.

STUDY FUNDING/COMPETING INTERESTS: Work supported by grants ES009718, ES022955, ES000002, and T32ES007069 from the National Institute of Environmental Health Sciences (NIEHS) and grant T32 DK007703-16 from the National Institute of Diabetes and Digestive

[†] The first two authors should be regarded as joint first authors.

and Kidney Diseases (NIDDK). C.M. was supported by a post-doctoral training award from the Canadian Institutes of Health Research. There are no competing interests to declare.

Key words: phthalate metabolites / ovarian reserve / antral follicle count / medically assisted reproduction / assisted reproductive technology / epidemiology

Introduction

Environmental chemicals that interfere with hormonal signaling may disrupt the delicate hormonal balance necessary for healthy reproduction. Epidemiologic evidence now links endocrine disrupting chemicals to adverse reproductive and developmental outcomes (Hauser and Calafat, 2005; Woodruff *et al.*, 2008; Diamanti-Kandarakis *et al.*, 2009; Ehrlich *et al.*, 2012; Toft *et al.*, 2012; Birnbaum, 2013; Swan *et al.*, 2015; Trasande *et al.*, 2015). There is widespread concern over a specific group of chemicals known as phthalates to which most of the general population is exposed. Several phthalates—such as di-n-butyl phthalate (DBP) and di(2-ethylhexyl) phthalate (DEHP)—are established endocrine disruptors and reproductive toxicants in female and male animals (Lovekamp-Swan and Davis, 2003; Gray *et al.*, 2006; Howdeshell *et al.*, 2008; Craig *et al.*, 2014). DEHP and its metabolite mono(2-ethylhexyl) phthalate (MEHP) have been shown to disrupt ovarian functioning and inhibit growth of antral follicles experimentally (Lovekamp-Swan and Davis, 2003; Craig *et al.*, 2014; Hannon *et al.*, 2014).

Primarily used to impart flexibility and durability, phthalates have diverse applications in the manufacturing of plastic. They can be found in a wide variety of products ranging from vinyl tiles and flooring to adhesives, detergents, lubricants, medical devices, pharmaceuticals (in the coating of certain oral medications), clothing, food packing, and toys. As a solubilizing agent, phthalates are also used in the preparation of cosmetics and personal care products (Centres for Disease Control and Prevention, 2009). Such widespread use has led to near universal human exposure (Hauser and Calafat, 2005; Centres for Disease Control and Prevention, 2009). Once ingested, inhaled or absorbed, phthalates have a short half-life, undergoing rapid hydrolysis into bioactive monoesters, some of which may then be further metabolized by oxidation or phase II conjugation. The metabolites are excreted mainly in urine and feces (Wittassek and Angerer, 2008). More than 95% of US and Canadian populations have detectable levels of one or more phthalate metabolites in urine (Saravanabhavan *et al.*, 2013; Zota *et al.*, 2014). Studies show that the fetus is most sensitive to potential adverse effects, and biomonitoring studies have found the highest concentration of many urinary phthalate metabolites in women and children (Hauser and Calafat, 2005; Hogberg *et al.*, 2008; Wittassek and Angerer, 2008; Centres for Disease Control and Prevention, 2009; Trasande *et al.*, 2013).

In experimental animal studies on female reproductive toxicity, the ovary is a suspected target for DEHP. An early *in vivo* study by Davis *et al.* (1994) showed that DEHP decreased estradiol (E_2) production, prolonged estrous cycles, and delayed ovulation in cycling rats (Davis *et al.*, 1994). Gupta and colleagues hypothesized that the antral follicle was the target site of DEHP, and showed that in mice, DEHP and MEHP inhibited the growth of these follicles through reduced E_2 production (Gupta *et al.*, 2010). More recently, Hannon and colleagues (2014) expanded on these finding by demonstrating that exposure of mice to environmentally low levels of DEHP—levels within the range of human exposure—resulted in changes to estrous cycles and accelerated primordial follicle recruitment (Hannon *et al.*, 2014). They furthermore provided evidence of a mechanism by which DEHP may accelerate early folliculogenesis by interfering with cell cycle signaling through altered gene expression. They were the first to report a non-monotonic dose–response for DEHP on the ovary, suggesting that altered ovarian functioning may not be explicitly due to high toxicity levels (Hannon *et al.*, 2014).

Whether phthalates also reduce the growing antral follicle pool in humans remains unknown. While there is substantive experimental evidence linking phthalates to altered ovarian function, very little is known about its impact on the human ovary. We have previously examined the relationship between antral follicle count (AFC) and bisphenol A (Souter *et al.*, 2013). Other researchers have investigated the effect of phthalates on human luteal cell function *in vitro* (Romani *et al.*, 2014). However, we are not aware of any published epidemiological studies investigating the potential effects of phthalates exposures on the growing antral follicle pool. Our primary objective was therefore to examine the prospective association between eleven urinary phthalate metabolites and the AFC among women seeking infertility care.

Materials and Methods

Study participants

The Environmental and Reproductive Health (EARTH) study is a prospective cohort of couples seeking infertility investigation and treatment at the Massachusetts General Hospital (MGH) Fertility Center and is designed to evaluate the effects of diet and environmental exposures on fertility and pregnancy outcomes. Details of the study cohort have been described previously (Ehrlich *et al.*, 2012). The EARTH study has been ongoing since 2004 and has recruited approximately 700 women and 400 men to date. Women between the ages of 18 and 46 years were eligible to participate and were followed from time of entry, throughout their infertility care and eventual pregnancy. The present study included all women enrolled in EARTH between November 2004 and April 2012 with valid ultrasound data ($N = 612$). We excluded *a priori*: women with oophorectomies ($n = 6$), 'difficult to visualize the ovary' scans ($n = 6$), and women with polycystic ovaries ($n = 36$) in whom an AFC value was not entered for either the left or right ovary (or both) due to excessively high counts, leaving 564 eligible women. Among these, we included only women with at least one urine sample collected *prior* to AFC determination ($n = 215$), to ensure temporality of exposure relative to outcome. The final study cohort for statistical analysis consisted of 215 women with results for urinary concentrations of phthalate metabolites and one AFC measurement per participant. The study was approved by the Human Studies Institutional Review Boards of MGH, Harvard T. H. Chan School of Public Health, and the Centers for Disease Control and Prevention (CDC). Prior to signing informed consent, a trained research nurse explained all procedures and answered questions.

Table I Characteristics and primary Society for Assisted Reproductive Technology (SART) diagnosis among 215 women with antral follicle count (AFC) measurement enrolled in the Environment and Reproductive Health (EARTH) study between 2004 and 2012.

Characteristic	Total cohort (*N* = 215) *N* (%)
Age at study entry (years)	
Mean ± SD	35.7 ± 4.6
Range	20–45
Age ≥37	97 (45%)
BMI (kg/m^2)	
Mean ± SD	25.0 ± 5.0
Range	16.1–40.5
Underweight or normal (<25)	128 (60%)
Overweight or obese (>=25)	87 (40%)
Smoking	
Never smoked	159 (74%)
Ever smoked	
Current smoker	11 (5%)
Former smoker	45 (21%)
Race	
Caucasian	175 (81%)
Black/African American	7 (3%)
Asian	16 (7%)
Other	17 (8%)
Nulligravida	143 (67%)
Nullipara	190 (88%)
AFC	
Mean ± SD	12.3 ± 6.9
Range	2–40
FSH IU/l[1]	
Mean ± SD	7.5 ± 2.9
Range	1.0–23.8
Serum day 3 E_2 pmol/l[2]	
Mean ± SD	47.2 ± 28.9
Range	14.0–297
Trying to conceive[3] ≥24 months	55(26%)
Primary SART diagnosis at study entry	
Female factor	90 (42%)
Diminished ovarian reserve	25 (12%)
Ovulatory dysfunction	31 (14%)
Endometriosis	18 (8%)
Uterine factor	4 (2%)
Tubal factor	12 (6%)
Male factor	49 (23%)
Unexplained	75 (35%)
Year at study entry	
2004–2006	45 (21%)
2007–2009	111 (52%)
2010–2012	59 (27%)

[1]FSH measured in 214 women.
[2]Serum estradiol (E_2) levels measured in 211 women.
[3]Number of months trying to conceive was self-reported at time of study entry.

Urinary phthalate metabolite concentrations

EARTH participants provided a urine sample at study entry, and twice during each subsequent treatment cycle, corresponding to Days 3–9 of the monitoring phase of their cycle and again at the time of oocyte retrieval or intrauterine insemination. All urine samples collected prior to the AFC scan date (ranging from 1 to 11 urine samples per woman) were included in the analysis. Urine samples were collected using a sterile phthalate-free polypropylene cup. Each urine sample was analyzed for specific gravity (SG) with a handheld refractometer (National Instrument Company, Inc., Baltimore, MD, USA) divided into aliquots and frozen at −80°C. Samples were shipped on dry ice overnight to the CDC (Atlanta, GA, USA) for quantification of urinary phthalate metabolite concentrations using solid phase extraction coupled with high performance liquid chromatography-isotope dilution tandem mass spectrometry (Silva *et al.* 2007). The eleven phthalate metabolites were: 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), mono(3-carboxypropyl) phthalate (MCPP), monocarboxyisooctyl phthalate (MCOP), monocarboxyisononyl phthalate (MCNP), monobenzyl phthalate (MBzP), monoethyl phthalate (MEP), mono-isobutyl phthalate (MiBP) and mono-n-butyl phthalate (MBP). The limits of detection (LOD) were 0.5–1.2 μg/l (MEHP), 0.2–0.7 μg/l (MEHHP and MEOHP), 0.2–0.6 μg/l (MECPP), 0.1–0.2 μg/l (MCPP), 0.2–0.7 μg/l (MCOP), 0.2–0.6 μg/l (MCNP), 0.2–0.3 μg/l (MBzP), 0.4–0.8 μg/l (MEP), 0.4–0.6 μg/l (MBP), and 0.2–0.3 μg/l (MiBP). We calculated the molar sum of DEHP metabolites (∑DEHP) by dividing each metabolite concentration by its molecular weight and then summing: [(MEHP*(1/278.34)) + (MEHHP*(1/294.34)) + (MEOHP*(1/292.33)) + (MECPP*(1/308.33))]. Values below LOD were assigned the value of LOD divided by the square root of 2 (Hornung and Reed, 1990).

Infertility data and AFC measurement

Routine infertility evaluation at MGH includes the determination of the ovarian AFC through transvaginal ultrasonography on Day 3 of an unstimulated cycle, and measurement of FSH and E_2 concentrations in serum. AFC was defined as the sum of antral follicles in both ovaries as measured by transvaginal ultrasound in the early follicular phase of a cycle (American Society for Reproductive Medicine Practice Committee, 2015). An infertility diagnosis was assigned by the treating infertility physician using the Society for Assisted Reproductive Technology definitions. Other pertinent demographic and clinical information, such as age and race, were obtained from a baseline questionnaire, and parity and gravida were abstracted from the patient's electronic medical records by a trained study staff. Age of participant was collected at time of study enrollment. Height and weight were measured at enrollment by the study nurse. BMI was estimated as weight (kilograms) divided by height (meters) squared.

Statistical analysis

We calculated the geometric mean of each participant's SG-adjusted urinary phthalate metabolite concentrations including all urine samples provided prior to AFC assessment. Urinary phthalate metabolite concentrations were adjusted for urinary dilution by multiplying the metabolite concentration by [(1.015-1)/(SG-1)], where 1.015 is the mean SG level for all included study urine samples, and SG is the specific gravity of the participant's urine sample (Boeniger *et al.*, 1993). We used the geometric mean for each of the phthalate metabolites and for participant's summary estimate of ∑DEHP.

We examined the clinical and demographic characteristics of study participants in the total cohort and by quartiles of ∑DEHP concentration, reported as means (± SD) or number of women (%). We fit multivariable generalized linear models with a Poisson distribution and log-link function

to estimate the mean AFC and 95% confidence interval (CI) by quartile of phthalate metabolite concentration. We estimated the absolute difference and percentage change using quartile one as the reference. We used the adjusted mean ratio (aMR) and corresponding CIs from the parameter estimates to calculate percentage change as: [(aMR − 1)*100]. Statistical tests for trend were conducted across quartiles using the median urinary phthalate metabolite concentration in each quartile in the regression models. We also modeled urinary phthalate metabolite concentrations using the geometric mean as a continuous variable on the log-scale, adjusting for covariates, and assessed the overall linear trend with the outcome. We selected *a priori* a minimum set of covariates, based on substantive knowledge, and included maternal age (continuous), BMI (continuous), and smoking status (never smoked versus ever smoked, defined as a current or former smoker) in adjusted models. In order to explore potential effect modification by age, we included interaction terms in models using the median level of metabolite concentration*age (centered), and further stratified the primary analyses by age into younger women <37 (55%) and older women ≥37 years of age. All tests were two-tailed and the level of statistical significance was set at 0.05. Statistical analyses were performed using SAS v9.4 software (SAS Institute, Inc., Cary, NC, USA).

Sensitivity analysis

As we wanted to examine whether DEHP or its metabolites predicted a potentially relevant clinical marker of diminished ovarian reserve, we dichotomized AFC as low at ≤8 total count compared with an AFC >8. We estimated adjusted risk ratios and 95% CIs for the risk of low AFC across quartiles of ∑DEHP and individual DEHP metabolites with quartile one as the reference using a log-binomial model, in the total sample and stratified by age (<37 versus ≥37 years).

There were 31 participants for whom exposure to ovarian stimulation in the cycle preceding AFC measurement could not be ruled out. In order to assess whether this could have inadvertently biased the results, we conducted a sensitivity analysis by repeating analyses excluding these women. We furthermore assessed the correlation between ∑DEHP and FSH and E_2 levels and examined differences in proportion of infertility diagnosis by quartiles of ∑DEHP.

Results

The study cohort comprised 215 women, predominantly Caucasian (81%) and never-smokers (74%), with an average age of 35.7 years at time of enrollment (Table I). Most women were nulliparous (88%), and 42% had a female factor as the primary cause of infertility. The distribution of the SG-adjusted urinary phthalate metabolite concentrations measured from 471 urine samples provided by 215 women is shown in Table II. The percentage of urine samples with detectable concentrations of phthalate metabolites ranged from 72% for MEHP to 100% for MEP and MECPP. The geometric mean of ∑DEHP did not differ by number of urine samples collected (Supplementary Table SI).

In the Poisson regression models adjusted for age, BMI and smoking status, we observed a decrease in mean AFC across quartiles of ∑DEHP with an association that reached a plateau after the second quartile (Table III). Compared with women in the first quartile of ∑DEHP, women in the second, third and fourth quartiles had a −24% (95% CI: −32%, −16%), −19% (95% CI: −27%, −9%), and −14% (95% CI: −23%, −5%) decrease in mean AFC. The absolute mean AFC in the first quartile was 14.2 follicles (95% CI: 13.2, 15.2) compared with 10.7 follicles (95% CI: 9.9, 11.6) in the second quartile. Similar trends were observed among the four individual DEHP metabolites with a decrease in mean AFC as quartiles increased with a plateau effect seen after the third quartile of MEHP, MEHHP, and MEOHP, and after the second quartile of MECPP. A maximum decrease of −15% (95% CI: −23, −5), −28% (95% CI: −36, −20) and −13% (95% CI: −22, −3) was observed for MEHP quartiles two, three and four, respectively, compared with quartile one. While trend tests by quartile for dose–response were not significant, the log-linear relationships of the geometric mean for ∑DEHP and individual DEHP metabolites in continuous models were significant ($P < 0.05$). With the exception of MBP that exhibited more modest decreases—ranging from −5% in the fourth quartile to −16% in the third quartile—there was no consistent change in mean AFC among the remaining six phthalate metabolites evaluated.

In the age-stratified analysis, we observed somewhat larger decreases in mean AFC among women younger than 37 years of age, than among women 37 years or older (Supplementary Table SII). While there was less evidence of effect modification by age within quartiles of ∑DEHP, we noted more pronounced decreases, both in absolute mean AFC and percentage change for MEHP, MEHHP and MEOHP among women <37 years of age, although interaction terms were not significant. Among younger women, on average those in the second, third and fourth quartile of MEHP had a decrease of −20% (95% CI: −31, −8), −32% (95% CI: −41, −22) and −17% (95% CI: −27, −4), respectively compared with the first quartile. These percentage changes were larger than what was observed among women ≥37 years, where the corresponding differences were: −9% (95% CI: −22, 7), −21% (95% CI: −34, −5) and −8% (95% CI: −22, 10), respectively.

Sensitivity analysis

The risk of lower (or decreased) ovarian reserve, defined as an AFC of ≤8, was modestly increased across higher quartiles of ∑DEHP compared with quartile one in the total sample, although CIs included the null in quartiles three and four. Similar trends of increasing risk across quartiles two and three were observed for the four DEHP metabolites (Supplementary Table SIII). Within the age-stratified analysis, we observed potential evidence of effect modification by age, with stronger associations between risk of AFC ≤8 and quartiles of DEHP metabolites among women <37 years of age, with risk ratios ranging from 1.5 to 4.3 compared with quartile one. Women ≥37 years with higher urinary ∑DEHP or individual DEHP metabolite concentrations appeared not to be at increased risk of low ovarian reserve, with risk ratios generally close to 1 (Supplementary Table SIII).

Excluding the 31 women that may have been exposed to ovarian stimulation in the cycle preceding AFC assessment did not materially change the results of the primary analysis, nor were there any appreciable differences in the study characteristics (data not shown). We also computed the correlation between the geometric mean of ∑DEHP by both FSH and E_2 levels. These were −0.02 and −0.03, respectively. There was furthermore no difference in the distributions of either hormone across quartiles of ∑DEHP (chi-square *P*-value 0.73 and 0.84, respectively). We did not observe a significant difference in the type of infertility diagnosis across quartile of ∑DEHP (chi-square *P*-value 0.40).

Discussion

As far as we are aware, this is the first human study to show a lower number of growing antral follicles across increasing quartiles of urinary DEHP metabolite concentrations among women seeking infertility

Table II Distribution of urinary phthalate metabolite concentrations (metabolite or molar sum) measured among 215 women with AFC measurements and 471 urine samples, enrolled in EARTH between 2004 and 2012.

Urine sample specific				AFC sample specific ($N = 215$) (μg/l)		
Metabolite	**Sample size**	**LOD (μg/l)**	**% Detect[2]**	**SG-adjusted GM[1] (GSD)**	**SG-adjusted Median**	**25th, 75th**
MEP	471	0.4–0.8	100	67.6 (5.9)	54.2	27.6, 139
MBP	471	0.4–0.6	97	12.5 (0.76)	12.8	7.4, 22.5
MiBP	471	0.2–0.3	97	6.3 (0.39)	6.8	3.6, 10.3
MBzP	471	0.2–0.3	93	3.3 (0.24)	3.2	1.7, 6.4
MEHP	471	0.5–1.2	72	3.8 (0.28)	3.5	1.6, 6.7
MEHHP	471	0.2–0.7	99	18.8 (1.6)	17.9	8.2, 41.1
MEOHP	471	0.2–0.7	99	12.1 (0.99)	11.2	5.1, 25.0
MECPP	471	0.2–0.6	100	31.6 (2.4)	29.6	13.5, 59.1
$\sum DEHP$[3]	–	–	–	0.23 (0.02)	0.21	0.10, 0.46
MCPP	471	0.1–0.2	96	3.1 (0.20)	2.7	1.7, 5.6
MCOP	390	0.2–0.7	96	15.6 (1.5)	14.2	5.8, 42.8
MCNP	390	0.2–0.6	93	3.9 (0.27)	3.5	2.1, 6.5

N: number of urinary samples; LOD: limit of detection; SG: specific gravity; GM: Geometric Mean; GSD: Geometric Standard Deviation; MEP: monoethyl phthalate; MBP: mono-n-butyl phthalate; MiBP: mono-isobutyl 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; MCPP: mono(3-carboxypropyl) phthalate; MCOP: monocarboxyisooctyl phthalate; MCNP: monocarboxyisononyl phthalate.
[1]Geometric mean of the specific gravity-adjusted phthalate metabolite concentrations by participant ($N = 215$) was calculated. For MCOP and MCNP, $N = 193$.
[2]Percentage of phthalate metabolite concentrations above the detection limit.
[3]$\sum$DEHP: Molar sum of DEHP metabolites (MEHP, MEHHP, MEOHP and MECPP) expressed in μmol/l.

treatment. The association was non-linear with a similar decrease in AFC for quartiles 2 through 4 for $\sum$DEHP and MECPP, and a plateau after quartile 3 for MEHP, MEHHP, and MEOHP. While reduced AFC with higher quartiles of urinary DEHP metabolites was observed in the total study cohort, these decreases were more pronounced among women <37 years of age compared with ≥ 37. We further observed that the risk of decreased ovarian reserve, defined as an AFC ≤ 8, was modestly increased across quartiles of $\sum$DEHP and DEHP metabolites compared with quartile one in the total cohort. These associations were also stronger among women <37 years of age, further suggesting that age may modify the effect of DEHP on AFC, despite interaction terms ($P > 0.05$).

Our results suggest that DEHP metabolites may adversely impact the size of the growing antral follicle pool among women in this study cohort. Younger women appear to be at higher risk of the potential effects of DEHP exposure. While our study was not designed to elucidate the mechanism through which exposure to phthalates adversely impact follicular development, our results are consistent with evidence from laboratory animal studies (Gupta *et al.*, 2010; Craig *et al.*, 2014; Hannon *et al.*, 2014). However, whether this effect does indeed manifest from a diminished primordial follicle population, a more direct marker of ovarian reserve and ovarian aging, cannot be determined within the parameters of the present study.

While our stratified analysis showed that younger women had larger decreases in mean AFC and this group may be at particular risk of the deleterious effects of DEHP, most of the results from our analysis using AFC as a binary outcome did not reach statistical significance. Although the study was sufficiently powered for the main and stratified analysis using counts, our subgroup analyses looking at low AFC defined as a binary outcome within levels of age had more limited power. This lack of precision, evident in the widened CIs, precluded us from drawing firm conclusions. Nevertheless, the overall trends observed in this sensitivity analysis were consistent with those in the primary analysis. Moreover, it seems intuitive, that among women ≥ 37 years of age, we would not readily observe the influence of an environmental factor exerting an effect on ovarian parameters given that *age* would be the most important predictor and determinant of reduced AFC among older women. However, among younger women, environmental chemicals may indeed play a role and act as a putative risk factor, having an effect comparable to what may be expected through the normal aging process. We also cannot preclude the possibility that in a larger sample, the observed effect among older women may become significant.

Understanding the effect of phthalates on ovarian function has significant implications for human fertility. The ovary's primary functions are folliculogenesis and estrogen synthesis, both critical processes to successful reproduction (Hannon *et al.*, 2014). As the growing antral follicular pool is dependent upon primordial follicle development, the potential to accelerate the depletion of the primary follicle can result in reduced ovarian reserve, premature ovarian aging or infertility. Recently, Hannon and colleagues demonstrated in an *in vivo* mouse model that low—environmentally relevant—levels of DEHP altered estrous cycling in mice and accelerated early folliculogenesis by interfering with PI3K signaling pathways, through altered gene expression or protein levels, in a non-monotonic dose–response (Hannon *et al.*, 2014). PI3 signaling is considered critical to primordial follicle survival and activation (John *et al.*, 2008; Reddy *et al.*, 2009; Zheng *et al.*, 2012). Earlier animal studies used high doses of DEHP (up to 2 g/kg), resulting in decreased

Table III Mean AFC by quartiles of urinary phthalate metabolite concentrations, adjusted for age, BMI, and smoking among 215 women with 215 AFC measurements in EARTH.

Metabolite	Mean AFC (95% CI)[1]	Difference in mean AFC	% Change (95% CI)	*P*-value[2]
∑DEHP (μmol/l)				
Q1 (0.02–0.09)	14.2 (13.2, 15.2)	0 (ref)	–	–
Q2 (0.10–0.21)	10.7 (9.9, 11.6)	−3.5	−24% (−32, −16)	<0.001
Q3 (0.21–0.44)	11.5 (10.7, 12.5)	−2.7	−19% (−27, −9)	0.002
Q4 (0.46–18.88)	12.1 (11.2, 13.1)	−2.1	−14% (−23, −5)	0.004
P-trend[3]				0.34
MEHP (μg/l)				
Q1 (0.21–1.62)	14.1 (13.1, 15.2)	0 (ref)	–	–
Q2 (1.63–3.50)	12.0 (11.1, 13.0)	−2.1	−15% (−23, −5)	0.003
Q3 (3.53–6.53)	10.2 (9.3, 11.0)	−3.9	−28% (−36, −20)	<0.001
Q4 (6.71–265)	12.3 (11.4, 13.2)	−1.8	−13% (−22, −3)	0.009
P-trend				0.33
MEHHP (μg/l)				
Q1 (1.00–7.50)	13.4 (12.5, 14.5)	0 (ref)	–	–
Q2 (8.21–17.70)	11.7 (10.8, 12.6)	−1.7	−13% (−22, −3)	0.01
Q3 (17.90–40.57)	10.9 (10.1, 11.9)	−2.5	−19% (−27, −9)	0.0002
Q4 (41.10–1977)	12.5 (11.6, 13.5)	−0.9	−7% (−16, 4)	0.20
P-trend				0.92
MEOHP (μg/l)				
Q1 (0.94–5.10)	13.9 (12.9, 15.0)	0 (ref)	–	–
Q2 (5.14–11.12)	11.5 (10.7, 12.5)	−2.4	−17% (−26, −7)	0.0009
Q3 (11.22–24.90)	11.4 (10.5, 12.3)	−2.5	−18% (−27, −9)	0.0003
Q4 (25.03–1278)	11.7 (10.9, 12.7)	−2.2	−16% (−24, −6)	0.002
P-trend				0.06
MECPP (μg/l)				
Q1 (3.17–13.40)	14.0 (13.0, 15.1)	0 (ref)	–	–
Q2 (13.50–29.34)	10.9 (10.1, 11.8)	−3.1	−22% (−30, −13)	<0.001
Q3 (29.63–58.45)	12.0 (11.1, 12.9)	−2.0	−14% (−23, −5)	0.004
Q4 (59.10–2109)	11.7 (10.8, 12.6)	−2.3	−17% (−25, −7)	0.001
P-trend				0.08
MEP(μg/l)				
Q1 (3.43–25.20)	12.6 (11.7, 13.6)	0 (ref)	–	–
Q2 (27.60–54.02)	12.5 (11.6, 13.5)	−0.1	−1% (−11, 10)	0.81
Q3 (54.17–138.85)	11.3 (10.4, 12.2)	−1.3	−11% (−20, 0)	0.04
Q4 (139.11–5995)	12.1 (11.2, 13.1)	−0.5	−4% (−14, 7)	0.47
P-trend				0.68
MBP (μg/l)				
Q1 (0.60–7.35)	13.1 (12.2, 14.2)	0 (ref)	–	–
Q2 (7.43–12.75)	11.9 (11.0, 12.8)	−1.2	−10% (−19, 1)	0.07
Q3 (12.79–22.14)	11.0 (10.1, 12.0)	−2.1	−16% (−25, −6)	0.002
Q4 (22.50–140.20)	12.5 (11.6, 13.5)	−0.6	−5% (−15, 6)	0.34
P-trend				0.57
MCPP (μg/l)				
Q1 (0.30–1.70)	12.0 (11.1, 13.0)	0 (ref)	–	–
Q2 (1.71–2.65)	11.7 (10.8, 12.7)	−0.3	−2% (−13, 9)	0.67
Q3 (2.66–5.50)	12.0 (11.1, 13.0)	0	0% (−10, 12)	0.94

Continued

Table III *Continued*

Metabolite	Mean AFC (95% CI)[1]	Difference in mean AFC	% Change (95% CI)	*P*-value[2]
Q4 (5.56–61.70)	12.8 (11.9, 13.8)	0.8	7% (−4, 19)	0.23
P-trend				0.11
MiBP (μg/l)				
Q1 (0.50–3.50)	12.7 (11.8, 13.7)	0 (ref)	--	-
Q2 (3.61–6.75)	12.8 (11.9, 13.8)	0.1	1% (−10, 12)	0.92
Q3 (6.75–10.21)	11.1 (10.3, 12.1)	−1.6	−13% (−22, −2)	0.02
Q4 (10.28–90.0)	11.9 (11.0, 12.9)	−0.8	−6% (−16, 4)	0.23
P-trend				0.12
MBzP (μg/l)				
Q1 (0.22–1.63)	12.1 (11.1, 13.0)	0 (ref)	–	–
Q2 (1.71–3.24)	12.0 (11.1, 13.0)	−0.1	0% (−11, 11)	0.93
Q3 (3.30–6.28)	12.6 (11.7, 13.6)	0.5	4% (−6, 17)	0.44
Q4 (6.39–75.19)	11.9 (11.0, 12.9)	−0.2	−1% (−12, 10)	0.81
P-trend				0.80
MCOP (μg/l)				
Q1 (0.92–5.56)	12.3 (11.4, 13.4)	0 (ref)	–	–
Q2 (5.80–14.19)	11.7 (10.8, 12.7)	−0.6	−5% (−16, 6)	0.36
Q3 (14.23–42.80)	12.3 (11.4, 13.3)	0	0% (−11, 12)	0.96
Q4 (43.22–520.0)	12.8 (11.8, 13.9)	0.5	4% (−7, 16)	0.52
P-trend				0.23
MCNP (μg/l)				
Q1 (0.51–2.06)	12.0 (11.0, 13.0)	0 (ref)	–	–
Q2 (2.07–3.53)	11.6 (10.7, 12.7)	−0.4	−3% (−14, 9)	0.62
Q3 (3.53–6.50)	13.0 (12.0, 14.0)	1.0	8% (−3, 22)	0.16
Q4 (6.75–160.5)	12.6 (11.6, 13.6)	0.6	5% (−6, 18)	0.41
P-trend				0.35

[1]Poisson regression models adjusted for maternal age (continuous), BMI (continuous) and smoking status (never, ever).
[2]*P*-value is for test of significant difference between the quartile of interest and quartile 1.
[3]Tests for linear trend were performed using the median level of urinary phthalate metabolite in each quartile in the regression model, adjusting for covariates.

serum E_2 production, inhibition of antral follicle growth, and subsequent anovulation and estrous cycling disruption (Lovekamp-Swan and Davis, 2003; Howdeshell *et al.*, 2008; Ehrlich *et al.*, 2012). However, the work by Hannon and colleagues has since enhanced our understanding of the effects of DEHP by illustrating that short duration and low levels of exposure result in abnormalities in ovarian functioning. Consequently overt toxicity of very high doses may not be an essential component to the deleterious effects on the ovary.

Consistent with the substantial body of evidence from animal studies, more human studies are emerging linking phthalates and other short-lived chemicals with altered male and female reproductive end-points. Hauser *et al.* recently reported that urinary concentrations of DEHP metabolites were inversely associated with oocyte yield, clinical pregnancy and live births rates following assisted reproduction (Hauser *et al.*, in press). Other studies have shown that phthalates are associated with increased risk of miscarriage (Toft *et al.*, 2012), preterm birth (Ferguson *et al.*, 2014) and reduced anogenital distance (Swan *et al.*, 2015). A study demonstrating reduced couple fecundity through male-mediated exposure underlines the importance of studying the joint effect of a couple's exposure on relevant fertility outcomes (Buck Louis *et al.*, 2014).

Our study provides evidence that the human female ovary may be adversely affected by DEHP exposure. The prospective nature of this design, relying upon an infertile study population from a large academic fertility setting, permitted a careful examination of the direction of the relationship between phthalate metabolite concentrations and the size of the antral follicle pool assessed in the early phase of the menstrual cycle. The urinary concentrations of the phthalate metabolites measured are within the ranges reported for the US general population (Centres for Disease Control and Prevention, 2009). Despite the lack of significant test for a linear dose–response when using quartiles, we observed significant tests of linear trend for DEHP metabolites when assessing the geometric mean concentration as a continuous variable in linear models with the outcome. However, these findings may not be generalizable to women from the general population without fertility concerns, co-exposures to other select chemicals were also not accounted for, exposure to phthalates may be reflective of other unknown lifestyle factors that might influence ovulatory potential, and findings are based on a

relatively small cohort. This study design also does not permit an examination of the potential impact of phthalates on polycystic ovaries. Moreover, we lacked information on anti-Mullerian hormone; however, AFC is considered to be the most well-established marker of ovarian reserve available (Hansen *et al.*, 2011; American Society for Reproductive Medicine Practice Committee, 2015). Furthermore, in the body, phthalates are short-lived chemicals, making the assessment of long-term exposure difficult. We attempted to partially account for this by collecting multiple urine samples that preceded the ovarian ultrasound evaluation, with ~30% of the women having three or more urine samples, and up to 13% having more than five samples (Supplementary Table SI). Multiple phthalate metabolites were also evaluated at the same time to account for various sources of exposure, and all samples were collected in one location and processed under one protocol.

Any environmental chemical that inhibits the size of the growing antral follicle pool, and consequently ovarian reserve, can impair fertility and reduce fecundity. Considering that phthalate exposure is nearly universal, these results may have important clinical and public health relevance. Our findings represent the first human study to suggest that higher urinary concentrations of DEHP metabolites are associated with a negative change in the AFC of women seeking infertility treatment. Because our study suggests probable evidence for the impact of exposure to DEHP on the human oocyte and follicular development, additional research with a larger sample would provide useful information to further elucidate possible mechanisms, and corroborate our findings.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Acknowledgements

The authors gratefully acknowledge Manori Silva, Ella Samandar, Jim Preau and Tao Jia (CDC, Atlanta, GA) for measuring the urinary concentrations of the phthalate metabolites. We also acknowledge all members of the EARTH study team, specifically the Harvard T. H. Chan School of Public Health research nurses Jennifer B. Ford and Myra G. Keller, research staff Ramace Dadd and Patricia Morey, physicians and staff at Massachusetts General Hospital Fertility Center and a special thanks to all the study participants.

Authors' roles

I.S., R.H. and C.M. conceived and designed the study. A.M.C. conducted the chemical analysis of urine samples and produced the chemical database. Statistical analysis of the data was done by C.M. in consultation with P.W., A.G. and Y.-H.C. C.M. drafted the manuscript and all authors critically revised the manuscript for important intellectual content.

Funding

Work supported by grants ES009718, ES022955, ES000002, and T32ES007069 from the National Institute of Environmental Health Sciences (NIEHS) and grant T32 DK007703-16 from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). C.M. was supported by a post-doctoral training award from the Canadian Institutes of Health Research.

Conflict of interest

The authors have no conflict of interests to declare. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention (CDC). The use of trade names and commercial sources is for identification only and does not constitute endorsement by the US Department of Health and Human Services or CDC.

References

American College of Obstetrics and Gynecology Practice Bulletin. Clinical management guidelines for obstetrician-gynecologists. Number 44, July 2003. (Replaces Committee Opinion Number 252, March 2001). *Obstet Gynecol* 2003;**102**:203–213.

American Society for Reproductive Medicine Practice Committee. Testing and interpreting measure of ovarian reserve: a committee opinion. *Fertil Steril* 2015;**103**:1407–1415.

Birnbaum LS. State of the science of endocrine disruptors. *Environ Health Perspect* 2013;**121**:A107.

Boeniger MF, Lowry LK, Rosenberg J. Interpretation of urine results used to assess chemical exposure with emphasis on creatinine adjustments: a review. *Am Ind Hyg Assoc J* 1993;**54**:615–627.

Buck Louis GM, Sundaram R, Sweeney AM, Schisterman EF, Maisog J, Kannan K. Urinary bisphenol A, phthalates, and couple fecundity: the Longitudinal Investigation of Fertility and the Environment (LIFE) Study. *Fertil Steril* 2014;**101**:1359–1366.

Centres for Disease Control and Prevention. Fourth National Report on Human Exposure to Environmental Chemicals (eds). 2009.

Craig ZR, Singh J, Gupta RK, Flaws JA. Co-treatment of mouse antral follicles with 17β-estradiol interferes with mono-2-ethylhexyl phthalate (MEHP)-induced atresia and altered apoptosis gene expression. *Reprod Toxicol* 2014;**45**:45–51.

Davis BJ, Maronpot RR, Heindel JJ. Di-(2-ethylhexyl) phthalate suppresses estradiol and ovulation in cycling rats. *Toxicol Appl Pharmacol* 1994;**128**:216–223.

Diamanti-Kandarakis E, Bourguignon J-P, Giudice LC, Hauser R, Prins GS, Soto AM, Zoeller RT, Gore AC. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endocr Rev* 2009;**30**:293–342.

Ehrlich S, Williams PL, Missmer SA, Flaws JA, Ye X, Calafat AM, Petrozza JC, Wright D, Hauser R. Urinary bisphenol A concentrations and early reproductive health outcomes among women undergoing IVF. *Hum Reprod* 2012;**27**:3583–3592.

Ferguson KK, McElrath TF, Meeker JD. Environmental phthalate exposure and preterm birth. *JAMA Pediatr* 2014;**168**:61–67.

Gray LE Jr, Wilson VS, Stoker T, Lambright C, Furr J, Noriega N, Howdeshell K, Ankley GT, Guillette L. Adverse effects of environmental antiandrogens and androgens on reproductive development in mammals. *Int J Androl* 2006;**29**:96–104; discussion 105–108.

Gupta RK, Singh JM, Leslie TC, Meachum S, Flaws JA, Yao HH. Di-(2-ethylhexyl) phthalate and mono-(2-ethylhexyl) phthalate inhibit growth and reduce estradiol levels of antral follicles in vitro. *Toxicol Appl Pharmacol* 2010;**242**:224–230.

Hannon PR, Peretz J, Flaws JA. Daily exposure to Di(2-ethylhexyl) phthalate alters estrous cyclicity and accelerates primordial follicle recruitment potentially via dysregulation of the phosphatidylinositol 3-kinase signaling pathway in adult mice. *Biol Reprod* 2014;**90**:136.

Hansen KR, Hodnett GM, Knowlton N, Craig LB. Correlation of ovarian reserve tests with histologically determined primordial follicle number. *Fertil Steril* 2011;**95**:170–175.

Hauser R, Calafat AM. Phthalates and human health. *Occup Environ Med* 2005;**62**:806–818.

Hauser R, Gaskins AJ, Souter I, Smith K, Dodge L, Ehrlich S, Meeker JD, Calafat AM, Williams PL. Association of urinary phthalate metabolite concentrations with pregnancy outcomes among women from a fertility center. *Environ Health Perspect*. doi:10.1289/ehp.1509760.

Hogberg J, Hanberg A, Berglund M, Skerfving S, Remberger M, Calafat AM, Filipsson AF, Jansson B, Johansson N, Appelgren M *et al*. Phthalate diesters and their metabolites in human breast milk, blood or serum, and urine as biomarkers of exposure in vulnerable populations. *Environ Health Perspect* 2008;**116**:334–339.

Hornung RW, Reed LD. Estimation of average concentration in the presence of nondetectable values. *Appl Occup Environ Hyg* 1990;**5**:46–51.

Howdeshell KL, Rider CV, Wilson VS, Gray LE Jr. Mechanisms of action of phthalate esters, individually and in combination, to induce abnormal reproductive development in male laboratory rats. *Environ Res* 2008; **108**:168–176.

John GB, Gallardo TD, Shirley LJ, Castrillon DH. Foxo3 is a PI3K-dependent molecular switch controlling the initiation of oocyte growth. *Dev Biol* 2008; **321**:197–204.

Lovekamp-Swan T, Davis BJ. Mechanisms of phthalate ester toxicity in the female reproductive system. *Environ Health Perspect* 2003;**111**:139–145.

Reddy P, Adhikari D, Zheng W, Liang S, Hamalainen T, Tohonen V, Ogawa W, Noda T, Volarevic S, Huhtaniemi I *et al*. PDK1 signaling in oocytes controls reproductive aging and lifespan by manipulating the survival of primordial follicles. *Hum Mol Genet* 2009;**18**:2813–2824.

Romani F, Tropea A, Scarinci E, Federico A, Dello Russo C, Lisi L, Catino S, Lanzone A, Apa R. Endocrine disruptors and human reproductive failure: the in vitro effect of phthalates on human luteal cells. *Fertil Steril* 2014; **102**:831–837.

Saravanabhavan G, Guay M, Langlois E, Giroux S, Murray J, Haines D. Biomonitoring of phthalate metabolites in the Canadian population through the Canadian Health Measures Survey (2007–2009). *Int J Hyg Environ Health* 2013;**216**:652–661.

Silva MJ, Samandar E, Preau JL Jr., Reidy JA, Needham LL, Calafat AM. Quantification of 22 phthalate metabolites in human urine. *J Chromatogr B Analyt Technol Biomed Life Sci* 2007;**860**:106–112.

Souter I, Smith KW, Dimitriadis I, Ehrlich S, Williams PL, Calafat AM, Hauser R. The association of bisphenol-A urinary concentrations with antral follicle counts and other measures of ovarian reserve in women undergoing infertility treatments. *Reprod Toxicol* 2013;**42**:224–231.

Swan SH, Sathyanarayana S, Barrett ES, Janssen S, Liu F, Nguyen RH, Redmon JB, Team TS. First trimester phthalate exposure and anogenital distance in newborns. *Hum Reprod* 2015;**30**:963–972.

Toft G, Jonsson BA, Lindh CH, Jensen TK, Hjollund NH, Vested A, Bonde JP. Association between pregnancy loss and urinary phthalate levels around the time of conception. *Environ Health Perspect* 2012;**120**: 458–463.

Trasande L, Sathyanarayana S, Jo Messito M, Gross RS, Attina TM, Mendelsohn AL. Phthalates and the diets of U.S. children and adolescents. *Environ Res* 2013;**126**:84–90.

Trasande L, Zoeller RT, Hass U, Kortenkamp A, Grandjean P, Myers JP, DiGangi J, Bellanger M, Hauser R, Legler J *et al*. Estimating burden and disease costs of exposure to endocrine-disrupting chemicals in the European union. *J Clin Endocrinol Metab* 2015;**100**:1245–1255.

Wittassek M, Angerer J. Phthalates: metabolism and exposure. *Int J Androl* 2008;**31**:131–138.

Woodruff TJ, Carlson A, Schwartz JM, Giudice LC. Proceedings of the Summit on Environmental Challenges to Reproductive Health and Fertility: executive summary. *Fertil Steril* 2008;**89**:e1–e20.

Zheng W, Nagaraju G, Liu Z, Liu K. Functional roles of the phosphatidylinositol 3-kinases (PI3Ks) signaling in the mammalian ovary. *Mol Cell Endocrinol* 2012;**356**:24–30.

Zota AR, Calafat AM, Woodruff TJ. Temporal trends in phthalate exposures: findings from the National Health and Nutrition Examination Survey, 2001–2010. *Environ Health Perspect* 2014;**122**:235–241.