Phthalate exposure and reproductive hormone concentrations in pregnancy

Sheela Sathyanarayana^{1,2}, Emily Barrett³, Samantha Butts⁴, Christina Wang⁵
and Shanna Helen Swan⁶

¹Department of Pediatrics, University of Washington, 2001 West 8th Avenue, Seattle, Washington 98121, USA, ²Seattle Children's Research Institute, Center for Child Health, Behavior and Development, 2001 West 8th Avenue, Seattle, Washington, USA, ³Department of Obstetrics and Gynecology, University of Rochester School of Medicine and Dentistry, 601 Elmwood Avenue Box 668, Rochester, New York 14642, USA, ⁴Department of Obstetrics and Gynecology, University of Pennsylvania School of Medicine, 3701 Market Street, Philadelphia, Pennsylvania 19104, USA, ⁵Division of Endocrinology, Department of Medicine, Harbor-UCLA Medical Center and Los Angeles Biomedical Research Institute, 1124 West Carson Street, RB-1, Torrance, California 90502, USA and ⁶Department of Preventive Medicine, Icahn School of Medicine at Mount Sinai, 17 East 102 Street, Floor 3, D3-135, New York, New York 10029, USA

Correspondence should be addressed to S Sathyanarayana; Email: sheela.sathyanarayana@seattlechildrens.org

Abstract

Some phthalate chemicals can affect hormone physiology *in utero*, resulting in adverse reproductive health outcomes in animal models. It is unknown whether these exposures are related to circulating maternal hormone concentrations during pregnancy. We used multivariate linear regression to estimate associations between phthalate metabolite concentrations and concurrent serum-free and total testosterone and estradiol (E₂) levels in 180 pregnant women in the Study for Future Families. We also examined associations between prenatal serum hormone concentrations and anogenital outcome in infants. All analyses were adjusted for appropriate confounding variables. Total testosterone, free testosterone, and E₂ concentrations ranged from 8 to 406 ng/dl, 0.03 to 1.2 ng/dl, and 529 to 40 600 pg/ml respectively. We observed an inverse association between log-sum di-2-ethylhexyl phthalate (DEHP) metabolite concentrations and lower log-total testosterone concentrations (−0.15, 95% CI −0.26, −0.04) and log-free testosterone (−0.15, 95% CI −0.27, −0.03). This relationship persisted regardless of fetal sex. Similarly, we observed an inverse association between log monobutyl phthalate (MBP) concentrations and log-total and -free testosterone concentrations in women carrying male fetuses. Monoethyl phthalate (MEP) concentrations were positively associated with log-total and -free testosterone concentrations in women carrying male fetuses (0.09, 95% CI 0.003, 0.17 and 0.10, 95% CI 0.01, 0.19 respectively). Prenatal hormone concentrations were not significantly associated with infant anogenital outcomes. Our preliminary data suggest that DEHP metabolite, MBP, and MEP exposures during pregnancy are associated with prenatal sex steroid hormone concentrations, but sex steroid hormone concentrations were not associated with infant reproductive outcomes.

Reproduction (2014) **147** 401–409

This paper forms part of a special issue of *Reproduction* on Endocrine Disruptors. This article was presented at the 7th Copenhagen Workshop on Endocrine Disruptors, 28–31 May 2013. The meeting was supported by the Danish Ministry of the Environment – Environmental Protection Agency as an activity under the Danish Centre on Endocrine Disruptors. Publication of this special issue has been supported by the Society for Reproduction and Fertility. The opinions or views expressed in this special issue are those of the authors, and do not necessarily reflect the opinions or recommendations of the Danish Ministry of the Environment – Environmental Protection Agency or the Society for Reproduction and Fertility. The Guest Editors for this special issue were Anna-Maria Andersson, Hanne Frederiksen, Niels Erik Skakkebaek, Rigshospitalet, Denmark, Kenneth M Grigor, Western General Hospital, Edinburgh, UK and Jorma Toppari, University of Turku, Finland.

Introduction

Phthalates are synthetic endocrine-disrupting chemicals that can affect sex steroid hormone concentrations, signaling, and function during gestation in rodent models (Fisher 2004). Changes in hormone concentrations during this important reproductive programming and developmental window can lead to significant downstream birth defects in the offspring in animal models (Gray *et al.* 2006). In humans, phthalate exposure during pregnancy is related to a number of adverse childhood health outcomes that may be hormone mediated, including reduced anogenital distance (AGD) and changes in sex-specific childhood

behavior (Swan *et al.* 2005, Engel *et al.* 2009, 2010). Whether phthalate exposure during pregnancy is related to sex steroid hormone concentrations remains undetermined but may have important implications for fetal development and sex steroid-mediated health outcomes.

Exposure to di-2-ethylhexyl phthalate (DEHP), dibutyl phthalate (DBP), and benzylbutyl phthalate (BBzP) during gestation leads to reductions in testosterone concentrations in both the pregnant dam and in male offspring and subsequent adverse reproductive abnormalities in males (Parks *et al.* 2000, Kavlock *et al.* 2002, Mylchreest *et al.* 2002, Foster 2005). In humans, prenatal exposure to DEHP has been negatively associated with free testosterone concentrations in the cord blood (Lin *et al.* 2011). DEHP has also been negatively associated with estradiol (E_2) concentrations in adult female rodents and *in vitro* in ovarian granulosa cells via decreased aromatase transcription, but this relationship remains to be explored in pregnant women (Laskey & Berman 1993, Davis *et al.* 1994, Lovekamp-Swan & Davis 2003). Studies examining the impact of diethyl phthalate (DEP) exposure during gestation have observed reduced testosterone concentrations but not reproductive tract defects in rodent offspring. (Fujii *et al.* 2005, Howdeshell *et al.* 2008).

The maternal, placental, and fetal compartments work in concert to produce circulating reproductive hormones during pregnancy, and the influence of each compartment changes depending on the gestational period (Tulchinsky *et al.* 1972, Speroff & Marc 2005, Strauss 2009). In early gestation, both the maternal and fetal compartments contribute to circulating hormone concentrations. In later gestation, the fetal adrenal glands provide the precursors for maternal estrogen and androgen production (Siiteri & MacDonald 1966, Speroff & Marc 2005). Therefore, circulating maternal hormone concentrations can reflect fetal production, and the influence of the fetal compartment increases as gestation progresses (Speroff & Marc 2005, Strauss 2009). Total and free testosterone concentrations rise throughout pregnancy but not to the same extent as estrogens, and sources of the increased testosterone are not well characterized but are likely fetal in origin (Bammann *et al.* 1980, Toriola *et al.* 2011). The fetus controls estrogen production by producing androgens that are delivered to the placenta and aromatized, and the concentrations rise rapidly as pregnancy progresses (Siiteri & MacDonald 1966, Madden *et al.* 1978). Results on whether reproductive hormone concentrations differ by fetal sex in the first trimester of pregnancy are conflicting (Klinga *et al.* 1978, Glass & Klein 1981), but late in gestation, reproductive hormones in the fetal compartment and likely, to a great extent, those in the maternal compartment are reflective of fetal production and control (Speroff & Marc 2005, Faupel-Badger *et al.* 2011).

E_2 and testosterone are important sex steroid hormones that can impact maternal and fetal health (Melmed *et al.* 2011). Estrogens are important for many homeostatic functions as well as for normal utero-placental blood flow, mammary gland development, and fetal adrenal gland function during pregnancy (Speroff & Marc 2005, Melmed *et al.* 2011). Similarly, testosterone plays a role in several body functions and is important for reproductive function and appropriate development of the male genital tract during pregnancy (Speroff & Marc 2005, Melmed *et al.* 2011). The focus of this study is to examine the relationship between phthalate exposure and testosterone and E_2 concentrations in pregnancy as well as the relationship between prenatal hormones and infant reproductive outcomes.

Materials and methods

Study participants

Pregnant women were originally recruited in the first phase of the Study for Future Families (SFFI), a multicenter pregnancy cohort study, at prenatal clinics in Los Angeles, California, Minneapolis, Minnesota, Columbia, and Missouri, from September 1999 through August 2002. Methods are described in detail elsewhere (Swan *et al.* 2005). Briefly, couples whose pregnancy was not medically assisted were eligible unless the woman or her partner was <18 years of age, either partner did not read and speak Spanish or English, or the father was unavailable or unknown. All participants completed a questionnaire and gave blood and urine samples on the same day, and the majority of samples were collected in the second or third trimester of pregnancy (98%). Eligibility criteria for the current analysis included data on prenatal urinary phthalate measurement, prenatal hormone measurement, and complete data on covariates. AGD is defined as the distance from the anus to anterior base of the penis in males and the distance from the anus to the anterior base of the clitoris in females. Anoscrotal distance (ASD) is the distance from the anus to the base of the scrotum, and ano-fourchette distance (AFD) is the distance from the anus to the base of the posterior fourchette. All measurements were performed in infants born to mothers in SFFI according to a standardized protocol described previously (Swan 2006). In total, we analyzed data for 180 mothers who met these parameters. SFFI obtained human subjects approval, and all participants signed informed consents. The present analyses were deemed exempt for human subjects review, as all data were de-identified.

Serum hormone measurements

All hormone measurements were carried out at the Endocrine and Metabolic Research Laboratory at Los Angeles Biomedical Research Center, Harbor-UCLA Medical Center, using assays that have been fully validated and reported. Serum testosterone was measured by liquid chromatography tandem mass spectrometry (LC-MS/MS; Shiraishi *et al.* 2008). Testosterone (>99% pure Sigma-Aldrich) was used as a calibration standard. 1,2 Deuterated (D_2)-testosterone (>98% pure,

Cambridge Isotope Laboratories, Inc., Andover, MA, USA) was used as the internal standard for T measurements. LC–MS/MS runs were carried out with a Shimadzu HPLC System (Columbia, MD, USA) attached to an Applied Biosystems API5000 LC–MS/MS equipped with a TurbolonSpray source. The calibration standards showed a linear response from 1 ng/dl (0.35 nmol/l) to 2000 ng/dl (69.3 nmol/l) for testosterone. The within- and between-run precision was <5% and the recovery of samples spiked with the steroids was between 100 and 113% for testosterone. The lower limit of quantification for testosterone was 2 ng/dl or 0.069 nmol/l (Shiraishi *et al.* 2008). Free testosterone was measured by equilibrium dialysis using labeled testosterone as described previously (Qoubaitary *et al.* 2006).

Serum E₂ concentrations for all mothers of boys were measured with a validated LC–MS/MS method (Rothman *et al.* 2011). Calibration standards and test samples were prepared for LC–MS/MS. First the proteins in the sera were precipitated using acetonitrile. The supernatant was dried and reconstituted with PBS and then extracted with 2 ml diethyl ether. LC–MS/MS was carried out with a Shimadzu HPLC system attached to an Applied Biosystems API5000 LC–MS/MS equipped with a TurbolonSpray source. The calibration standards showed a linear response from 1.0 to 1000 pg/ml for E₂. Analysis of four pools with E₂ concentrations ranging from about 10 to 500 pg/ml showed the within-run precision range to be from 2.78 to 3.58%. The between-run precision for E₂ (computed over 27 runs) ranged from 3.7 to 9.8%. The recovery of samples spiked with the steroids was between 84.4 and 129% with a mean recovery of 101.8% for E₂. The LOQ was 2 pg/ml for E₂. Serum E₂ concentrations for mothers of girls were measured in 2003 in a different laboratory by RIA (Pantex, Santa Monica, CA, USA). The limit of detection (LOD) was 4.9 pg/ml and the inter-assay coefficient of variation was below 9%. We applied a correction factor to the concentrations of mothers of girls by RIA in order to use E₂ concentrations for all mothers in the analysis. The adjustment factor was created based on 156 samples of overlapping E₂ concentrations from 2003 and 2011. The values from 2003 were lower compared with those from 2011, but despite the differing concentrations, the data were highly correlated (0.99). We calculated the E₂ concentrations as (2011 E₂ concentration = 1.33063 × E₂ (2003) – 0.00001007 (E₂ (2003))²).

Phthalate metabolite measurements

The Division of Laboratory Sciences within the National Center for Environmental Health, Centers for Disease Control, conducted the analyses and had no access to participant data. The analytical method for urinary phthalate metabolites involved the enzymatic deconjugation of the metabolites from their glucuronidated form, followed by concentration of the analytes of interest by solid-phase extraction, separation with HPLC, and detection by isotope-dilution tandem mass spectrometry (Silva *et al.* 2004, Kato *et al.* 2005). This approach allows for the simultaneous quantification in human urine of the following phthalate metabolites reported in this work: monoethyl phthalate (MEP), monobutyl phthalate (MBP), monomethyl phthalate (MMP), MBzP, monoisobutyl phthalate

(MiBP), mono-2-ethylhexyl phthalate (MEHP), and two oxidative metabolites of DEHP, mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP) and mono-2-ethyl-5-oxo-hexyl phthalate (MEOHP). Isotopically labeled internal standards and conjugated internal standards were used to increase precision and accuracy of the measurements. Quality control and reagent blank samples were analyzed along with unknown samples to monitor performance of the method. Urinary creatinine was measured at the time of phthalate analysis.

Statistical analysis

We examined the range of all exposure and outcome variables under consideration including concentration and distribution of maternal serum hormone concentrations and urinary phthalate concentrations. Because the distributions for total testosterone, free testosterone, and E₂ were right skewed, we used log transformations in all statistical analyses. Most phthalate metabolite concentrations were above the LOD, which was between 0.95 and 1.07 µg/l, depending upon the analyte. Concentrations below the LOD were assigned a value equal to the LOD divided by the square root of two (Hornung & Reed 1990). The percent of samples below the LOD for each of the phthalate metabolites was <15% except for MMP, which was over 50%. We chose not to include MMP in the statistical analyses. All phthalate metabolite concentrations were logarithmically transformed to normalize distributions. MEHP, MEOHP, and MEHHP are metabolites of a single parent compound (DEHP). Therefore, we used the molar sum of these metabolites to reflect total DEHP exposure.

We examined the bivariate relationship between the covariates chosen a priori and log hormone concentrations. We chose these factors based on past studies examining prenatal hormone concentrations as well as studies examining phthalate exposures. Based on this analysis, we included maternal age, gestational age at blood draw, and urinary creatinine as continuous covariates. We included study center, parity, and education as categorical covariates. We used linear regression to explore the associations between log-transformed prenatal phthalate metabolite concentrations and concurrent log-transformed maternal total testosterone, free testosterone, and E₂ concentrations in individual models.

We fit two models with and without sex-by-phthalate interactions. We reported both sets of results regardless of the significance of the interaction, as others have done (Sagiv *et al.* 2012). The first model assumes a common slope for boys and girls. The second model allows for separate slopes for boys and girls with the inclusion of sex by phthalate interaction terms (see equation 1). We used this model in order to avoid a loss of power from traditional stratification methods.

Sex-specific linear regression model*

$$Y = \beta_0 + (SEX)(\log(MEHP))\beta_1 + (1 - SEX)(\log(MEHP))\beta_2 \\ + (SEX)\beta_3 + COVARIATES$$

*where sex is coded as 0=boys, 1=girls.

We used multivariate linear regression to explore the associations between log-transformed prenatal hormone concentrations and AGD, ASD, and AFD in male and female

Table 1 Demographic characteristics for 180 pregnant women.

	<i>n</i> (%)
Ethnicity	
White	143 (79)
Hispanic	24 (13)
Asian	10 (6)
African-American/other	3 (2)
Maternal age (years)	
<20–30	84 (46)
>30–42	96 (54)
Study center	
MN	83 (86)
CA	44 (24)
MO	53 (29)
Education	
Grade school only/high school only/some college	58 (32)
Graduated college or technical school	64 (36)
Some graduate work or graduate degree	58 (32)
Parity	
Nulliparous	99 (55)
Parous	81 (45)
Gestational age at blood draw (weeks)	
0–20	4 (2)
>20–30	68 (38)
>30	108 (60)
Infant sex in second trimester	
Male	31 (46)
Female	37 (54)
Infant sex in third trimester	
Male	62 (57)
Female	46 (43)

infants separately. All analyses were adjusted for weight percentile of infant, age of infant, study center, gestational age at blood draw, and maternal age.

Results

The 180 pregnant women included in the analyses were primarily white (79%) and aged between 20 and 40 years (97%). Women were highly educated (>68% with at least a college degree), and 98% were in their second or third trimester of pregnancy at the time of blood and urine collection (Table 1). Within this group, the highest urinary phthalate concentrations were observed for MEP, and the lowest for MIBP (Table 2). Total testosterone concentrations ranged from 8 to 406 ng/dl, and free testosterone concentrations ranged from 0.03 to 1.2 ng/dl. E₂ concentrations ranged from 560 to 40 600 pg/ml (log values are shown in Figs 1a, b and 2a, b).

In bivariate analysis of participant characteristics and hormone concentrations, increased maternal age and parity were associated with reduced log testosterone and log-free testosterone. Ethnicity was not significantly associated with any hormone concentrations. Having graduated college or technical school was significantly associated with lower testosterone concentrations in pregnant women, but this relationship was not significant in the higher educated women. Increasing gestational age was significantly associated with E₂ concentrations but not testosterone concentrations.

In the third trimester of pregnancy, female sex was associated with reduced log-total and log-free testosterone concentrations and increased log E₂ concentrations (Table 3).

Within the model that estimated sex-specific slopes, we observed a lower log-total concentration in relation to a log unit increase in the sum of DEHP metabolites in women with both male (−0.07, 95% CI −0.20, 0.06) and female fetuses (−0.15, −0.26, −0.04), but this result was only statistically significant for women carrying female fetuses (Table 4). We observed similar results with log-free testosterone in relation to the sum of DEHP metabolites. We observed some evidence for effect modification for MBP and MBZP in relation to log-total and -free testosterone concentrations, with positive estimates for women with male fetuses and negative estimates for women with female fetuses. We also observed a higher log-total testosterone concentration in relation to a log unit increase in MEP (0.09, 95% CI 0.003, 0.17) in women with male fetuses only (Table 4). None of the prenatal log hormone concentrations were associated with AGD, ASD, or AFD in male or female infants (Table 5).

Discussion

We report an association between increased prenatal DEHP metabolite exposure and lower testosterone concentrations in women carrying both male and female fetuses, and MBP was also associated with lower testosterone concentrations but only in women carrying female fetuses. We found the opposite relationship with increasing MEP exposure associated with higher testosterone concentrations, and this relationship was strongest in women with male fetuses. We did not find an association between prenatal hormone concentrations and infant anogenital outcomes. These findings are preliminary and based on a small sample size but suggest that exposure to some phthalates during pregnancy may affect sex steroid hormone concentrations.

Free testosterone is unbound and is bioavailable to peripheral tissues while total testosterone reflects both bound and free testosterone concentrations (Speroff & Marc 2005). The Pearson's correlation coefficient between the total and free testosterone was >0.9, and regression results were similar for both, which supports

Table 2 Distribution of phthalate metabolite concentrations in µg/l (*n* = 180).

	Geometric mean	25%	50%	75%
MBP	16.43	9.20	17.35	54.85
MBzP	9.80	4.50	11.00	38.60
MEP	148.55	47.00	126.40	529.60
MIBP	2.64	1.15	2.70	4.85
Sum DEHP metabolites (µM/l)	10.87	5.53	9.99	21.05

Figure 1 Log free and total testosterone concentrations in mothers with male fetuses (a) and female fetuses (b).

the validity of both the hormonal and statistical analyses. The origin of prenatal circulating testosterone concentrations in humans is unclear but is thought to be primarily directed by the fetus as pregnancy progresses via fetal testicular Leydig cell production (Bammann *et al.* 1980, Kerlan *et al.* 1994). In women carrying female fetuses, testosterone is likely produced in a similar manner to nonpregnant women, through ovarian, adrenal gland, and peripheral production (Speroff & Marc 2005). From our results, it may be that DEHP metabolites are both exerting an effect on adrenal as well as Leydig cell production of testosterone because we see reductions in testosterone concentration in women carrying both male and female fetuses. Testosterone plays an important role in sex differentiation during early human gestation. In male fetuses, increasing testosterone concentrations lead to virilization and normal development of male genital structures. In females, estrogen is the predominant sex steroid hormone and increased testosterone concentrations can lead to abnormal virilization (Speroff & Marc 2005). In addition, normal estrogen and testosterone play an integral role in an adult female's general endocrine homeostasis and are known to be associated with a variety of health outcomes (Longcope 1986).

In animal studies, DEHP and DBP exposure in pregnant dams leads to lower intra-testicular testosterone concentrations in male offspring via a direct testicular toxic effect on Leydig cells (Parks *et al.* 2000, Foster 2005, Howdeshell *et al.* 2008). Therefore, our study may suggest that DEHP could be acting via a direct testicular toxic effect on fetal Leydig cells leading to reductions in circulating maternal testosterone concentrations. It is unclear whether DEHP exposure could also affect ovarian, adrenal, or androstenedione production of testosterone as there are no studies of this mechanism in the literature. Several studies suggest that DEP, an endocrine-disrupting chemical, is not as potent as DEHP or DBP, but one study did observe reduced testosterone concentrations in male offspring of pregnant dams exposed to DEP at 3000 and 15 000 ppm during gestation (Gray *et al.* 2000, Fujii *et al.* 2005, Howdeshell *et al.* 2008), and some human epidemiologic studies have found relationships between DEP exposure and adverse endocrine and reproductive health outcomes (Lopez-Carrillo *et al.* 2010, Tranfo *et al.* 2011). Results of our study suggest that DEP may be acting via a novel mechanism in relation to increased prenatal circulating

Figure 2 Log E2 concentrations in mothers with male fetuses (a) and female fetuses (b).

Table 3 Bivariate relationships between participant characteristics and log hormone concentrations (point estimate, 95% CI) for 180 pregnant women.

	Log-total testosterone	Log-free testosterone	Log estradiol
Ethnicity			
White	Ref	Ref	Ref
Hispanic	0.15 (−0.26, 0.55)	0.15 (−0.30, 0.60)	0.21 (−0.32, 0.76)
Asian	0.26 (−0.01, 0.53)	0.18 (−0.12, 0.48)	−0.15 (−0.57, 0.28)
African–American/other	0.29 (−0.43, 1.01)	0.08 (−0.72, 0.88)	0.71 (−0.41, 1.8)
Maternal age (years)			
<20–30	Ref	Ref	Ref
>30–42	−0.31* (−0.49, −0.13)	−0.30* (−0.51, −0.10)	−0.20 (−0.46, 0.05)
Study center			
MN	Ref	Ref	Ref
CA	0.19 (−0.04, 0.42)	0.07 (−0.18, 0.33)	−0.11 (−0.48, 0.25)
MO	−0.04 (−0.26, 0.17)	−0.08 (−0.32, 0.16)	−0.08 (−0.37, 0.20)
Education			
Grade school only/high school only/ some college	Ref	Ref	Ref
Graduated college or technical school	−0.32* (−0.54, −0.10)	−0.29* (−0.54, −0.05)	0.09 (−0.23, 0.41)
Some graduate work or graduate degree	−0.15 (−0.37, 0.08)	−0.10 (−0.35, 0.16)	−0.01 (−0.31, 0.32)
Parity			
Nulliparous	Ref	Ref	Ref
Parous	−0.40* (−0.57, −0.22)	−0.36* (−0.56, −0.16)	−0.27* (−0.52, −0.12)
Gestational age at blood draw (weeks)			
0–24	Ref	Ref	Ref
>24–41	0.03 (−0.16, 0.22)	0.08 (−0.13, 0.29)	1.10* (0.91, 1.30)
Infant sex in second trimester			
Male	Ref	Ref	Ref
Female	0.09 (−0.28, 0.46)	−0.30 (−0.66, 0.06)	0.16 (−0.22, 0.54)
Infant sex in third trimester			
Male	Ref	Ref	Ref
Female	−0.23* (−0.44, −0.02)	−0.48* (−0.71, −0.24)	0.04 (−0.15, 0.24)

* $P < 0.05$.

testosterone concentrations given that we observed an increase in testosterone concentrations in women carrying male fetuses. Another possibility is that the DEP finding is due to chance alone.

During gestation, E_2 production occurs primarily in the placenta through aromatization of androgens (Speroff & Marc 2005). BBzP and DBP have been associated with weak estrogenic activity, but other

studies have documented an anti-estrogenic effect in the peripheral tissues (Jobling *et al.* 1995, Lee *et al.* 2004). In two studies of rodent models, circulating estrogen concentrations of adult females were decreased in relation to high-dose DEHP exposure (Davis *et al.* 1994, Hirosawa *et al.* 2006). Overall, the impact of phthalate exposure on estrogen steroidogenesis is not well understood, with conflicting results in the literature.

Table 4 Linear regression analysis with sex-specific slopes of phthalate metabolites in relation to log-total testosterone, -free testosterone, and estradiol concentrations in pregnant women ($n=180$).

Serum hormone	Phthalate or BPA metabolite	Women with male fetuses ($n=94$)			Women with female fetuses ($n=86$)		
		Coefficient	95% CI	P value	Coefficient	95% CI	P value
Log-total testosterone	Log mbp	0.15	−0.04, 0.33	0.12	−0.20	−0.39, −0.01	0.04
	Log mbzp	0.06	−0.07, 0.19	0.37	−0.13	−0.26, 0.01	0.06
	Log mep	0.09	0.003, 0.17	0.04	0.03	−0.06, 0.11	0.56
	Log mibp	−0.03	−0.18, 0.13	0.75	−0.10	−0.28, 0.07	0.23
	Log sumdehp	−0.07	−0.20, 0.06	0.30	−0.15	−0.26, −0.04	0.01
Log-free testosterone	Log mbp	0.13	−0.07, 0.33	0.22	−0.21	−0.42, 0.004	0.05
	Log mbzp	0.07	−0.07, 0.21	0.35	−0.10	−0.25, 0.04	0.16
	Log mep	0.10	0.01, 0.19	0.04	−0.02	−0.10, 0.08	0.75
	Log mibp	−0.03	−0.20, 0.14	0.77	−0.11	−0.30, 0.08	0.26
	Log sumdehp	−0.04	−0.18, 0.10	0.55	−0.15	−0.27, −0.03	0.01
Log estradiol	Log mbp	0.04	−0.10, 0.18	0.57	−0.002	−0.18, 0.17	0.97
	Log mbzp	−0.03	−0.12, 0.07	0.62	−0.10	−0.23, 0.03	0.14
	Log mep	−0.03	−0.10, 0.04	0.38	0.01	−0.07, 0.10	0.78
	Log mibp	0.003	−0.12, 0.12	0.95	0.03	−0.14, 0.20	0.73
	Log sumdehp	−0.06	−0.17, 0.04	0.21	−0.08	−0.18, 0.01	0.08

Bold type indicates statistically significant relationships.

Table 5 Linear regression analysis slopes of anogenital outcomes in infants in relation to log-total testosterone, -free testosterone, and estradiol concentrations in pregnant women ($n=180$).

Anogenital outcome	Serum hormone	Male newborns ($n=94$)			Female newborns ($n=86$)		
		Coefficient	95% CI	<i>P</i> value	Coefficient	95% CI	<i>P</i> value
AGD	Log testosterone	-1.50	-4.55, 1.55	0.33	0.33	-1.17, 1.83	0.66
	Log-free testosterone	-1.16	-3.98, 1.67	0.42	0.30	-1.09, 1.70	0.67
	Log estradiol	1.98	-1.44, 5.39	0.25	-0.43	-3.54, 2.68	0.78
ASD/AFD	Log testosterone	-0.18	-3.37, 3.73	0.92	-0.56	-2.44, 1.32	0.56
	Log-free testosterone	0.04	-3.24, 3.33	0.98	-0.33	-2.08, 1.43	0.71
	Log estradiol	1.38	-2.60, 5.35	0.49	-0.97	-4.69, 2.75	0.60

We observed the suggestion of lower E_2 concentrations in relation to increased DEHP exposure (as well as in the sex-specific analyses), but this association was not statistically significant.

We examined the relationship between prenatal sex steroid hormones and sex-specific AGD within the SFFI dataset but did not observe any statistically significant relationships. However, this analysis was limited in several ways including exposure misclassification as well as a lack of precision in outcomes. The timing of serum collection was not appropriate. The genital reproductive programming window is early in pregnancy and the majority of the SFFI (>90%) serum samples were taken late in the second trimester or within the third trimester of pregnancy when they are less likely to have impacted genital development. The age of the infants at AGD exam was highly variable (9–36 months), making it more difficult to detect associations if they existed. Future studies would need to assess hormone concentrations in the first trimester of pregnancy when genital structures develop and then examine genital outcomes at birth.

Limitations of our study included spot urine samples to reflect urinary phthalate exposures. Although there can be variability in phthalate concentrations over time, several studies suggest that a spot sample can be representative of a 3-month period of exposure in adult women (Hoppin *et al.* 2002). One study of phthalate variability in pregnancy showed that DEHP metabolites decreased as the pregnancy progressed while MEP and MiBP concentrations increased (Braun *et al.* 2012). If these findings are generalizable, urine DEHP metabolite concentrations in our study may have been an underestimate of exposure during the first trimester while MEP concentrations could have been an overestimate. Urine and serum were collected at the same time for each woman. Ideally, we would like to have serum collected after the urine samples as it is not clear whether an effect would be seen concurrently or after hours or days. We did not have multiple measurements within the same women, which would allow for longitudinal analysis to examine changes in concentrations. We had samples from different time points during pregnancy and attempted to correct this by adjusting for gestational age at blood draw in our statistical analyses. Regardless,

residual confounding by covariates in the analysis could introduce bias in the analysis. We conducted two sensitivity analyses. The first excluded the first trimester samples ($n=4$), and results were unchanged. We then restricted analyses to women in the third trimester only ($n=96$) and found that all estimates were in the same direction and of similar magnitude to the primary analysis. We were unable to directly assess fetal hormone concentrations in this study, and methodology to do this (amniotic fluid collection) would not be feasible in the general population.

Conclusion

Exposure to DEHP, MBP, and MEP during pregnancy may be related to changes in circulating prenatal testosterone concentrations. If confirmed, these findings are significant because testosterone is involved in the development of maternal and infant health outcomes including cardiovascular disease, hormone-mediated reproductive disease, neurodevelopmental outcomes, and fetal genital tract development. These results are preliminary and should be confirmed in future larger human cohort studies.

Discussion from meeting

Maria Andersson (Copenhagen, Denmark): You discovered two gene variants, one associated with decreased anogenital distance (AGD) and one with anoscrotal distance (ASD). There is no change in the coding regions of the genes, but is there any functional difference due to altered expression?

Sheela Sathyanarayana (Seattle, USA): There is increased expression of the ATF3 gene in rats and this is validated in studies of human foreskin cells.

Anna-Maria Andersson: There is increased testosterone in the serum of young mothers under the age of 20 years. Is this associated with smoking or social class? Smoking is known to increase testosterone.

Sheela Sathyanarayana: There were very few smokers in our cohort, and these tended to be older women. There were too few smokers to be included in statistical analysis.

Joe Braun (Providence, USA): Your gene–environment interaction study indicated that joint exposure to a

mixture of phthalates was associated with reduced AGD and minor changes in the minor allele of the AFT3 gene. This did not reach statistical significance but it was a pilot study with low power. Animal studies indicate that MEP and DEP phthalates are not antiandrogenic.

Sheela Sathyanarayana: The lack of antiandrogenicity published for MEP and DEP does not make sense with our findings, however, some publications are more in keeping with our results. We shall continue our studies to see if the effect is persistent.

Joe Braun: Have you performed genotyping on the women who have a positive association between MEP and testosterone? There might be a subgroup with the inverse relationship, which could be consistent with your AGD correlations.

Sheela Sathyanarayana: We have not yet performed genotyping, but this is planned for mothers and fathers in future in the GEAR study (genetics and the environment associated with male reproduction), which will be an improvement on the pilot study.

Hagai Levine (Jerusalem, Israel): In your mixtures study, did you see combined interaction with different genes?

Sheela Sathyanarayana: We have not looked at that in the pilot study because the pool is too low. It might be possible in the bigger cohort of 400 men, but for genetic studies, 400 individuals might not be a large enough number for statistical significance.

Ewa Rajpert-De Meyts (Copenhagen, Denmark): Your experimental design was good when you looked at the level of maternal hormones separately for male and female fetuses. Your cross-sectional assessment of mothers exposed to phthalates indicated reduced maternal serum testosterone in association with male fetuses. Is this due to a combination of effects on the mother and fetus, or is it mainly due to reduced testosterone production by the fetus?

Sheela Sathyanarayana: That is difficult to assess because not many studies have been performed on phthalate exposure in adult women. In the bivariate analysis, pregnant mothers with female fetuses had lower testosterone levels. This might be due to reduced maternal production of testosterone, but there might also be a fetal component in the levels.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported. Dr S H Swan and Dr S Sathyanarayana were both invited to give talks at the 7th Copenhagen Workshop on Endocrine Disruptors meeting, and their travel expenses were paid by the organizers.

Funding

This study was primarily supported by a NIH NICHD K-12 Award HD053984-02. This study was supported by grants from

the US Environmental Protection Agency; National Institutes of Health grants R01-ES09916 to the University of Missouri, MO1-RR00400 to the University of Minnesota, and MO1-RR0425 and (UL1TR000124) to the Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center; grant 18018278 from the State of Iowa to the University of Iowa; National Institute of Environmental Health Sciences grant 5 T32 ES 007262–15. This article is based on work presented at the 7th Copenhagen Workshop on Endocrine Disruptors, which was supported by the Danish Ministry of the Environment – Environmental Protection Agency. Publication of this special issue was supported by the Society for Reproduction and Fertility.

Acknowledgements

We gratefully acknowledge the technical assistance of Manori Silva, Jack Reidy, Ella Samandar, and Jim Preau (Centers for Disease Control and Prevention, Atlanta, GA) in measuring the urinary concentrations of phthalate metabolites.

References

- Bammann BL, Coulam CB & Jiang NS 1980 Total and free testosterone during pregnancy. *American Journal of Obstetrics and Gynecology* **137** 293–298.
- Braun JM, Smith KW, Williams PL, Calafat AM, Berry K, Ehrlich S & Hauser R 2012 Variability of urinary phthalate metabolite and bisphenol A concentrations before and during pregnancy. *Environmental Health Perspectives* **120** 739–745. (doi:10.1016/j.toxlet.2011.11.033)
- Davis BJ, Maronpot RR & Heindel JJ 1994 Di-(2-ethylhexyl) phthalate suppresses estradiol and ovulation in cycling rats. *Toxicology and Applied Pharmacology* **128** 216–223. (doi:10.1006/taap.1994.1200)
- Engel SM, Zhu C, Berkowitz GS, Calafat AM, Silva MJ, Miodovnik A & Wolff MS 2009 Prenatal phthalate exposure and performance on the Neonatal Behavioral Assessment Scale in a multiethnic birth cohort. *Neurotoxicology* **30** 522–528. (doi:10.1016/j.neuro.2009.04.001)
- Engel SM, Miodovnik A, Canfield RL, Zhu C, Silva MJ, Calafat AM & Wolff MS 2010 Prenatal phthalate exposure is associated with childhood behavior and executive functioning. *Environmental Health Perspectives* **118** 565–571. (doi:10.1289/ehp.0901470)
- Faupel-Badger JM, Wang Y, Karumanchi SA, Stanczyk F, Pollak M, McElrath T, Hoover RN & Troisi R 2011 Associations of pregnancy characteristics with maternal and cord steroid hormones, angiogenic factors, and insulin-like growth factor axis. *Cancer Causes & Control* **22** 1587–1595. (doi:10.1007/s10552-011-9835-3)
- Fisher JS 2004 Environmental anti-androgens and male reproductive health: focus on phthalates and testicular dysgenesis syndrome. *Reproduction* **127** 305–315. (doi:10.1530/rep.1.00025)
- Foster PM 2005 Mode of action: impaired fetal Leydig cell function – effects on male reproductive development produced by certain phthalate esters. *Critical Reviews in Toxicology* **35** 713–719. (doi:10.1080/10408440591007395)
- Fujii S, Yabe K, Furukawa M, Hirata M, Kiguchi M & Ikka T 2005 A two-generation reproductive toxicity study of diethyl phthalate (DEP) in rats. *Journal of Toxicological Sciences* **30** 97–116. (doi:10.2131/jts.30.S97)
- Glass AR & Klein T 1981 Changes in maternal serum total and free androgen levels in early pregnancy: lack of correlation with fetal sex. *American Journal of Obstetrics and Gynecology* **140** 656–660.
- Gray LE Jr, Ostby J, Furr J, Price M, Veeramachaneni DN & Parks L 2000 Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. *Toxicological Sciences* **58** 350–365. (doi:10.1093/toxsci/58.2.350)
- Gray LE Jr, Wilson VS, Stoker T, Lambright C, Furr J, Noriega N, Howdeshell K, Ankley GT & Guillette L 2006 Adverse effects of

- environmental antiandrogens and androgens on reproductive development in mammals. *International Journal of Andrology* **29** 96–104 discussion 105–108. (doi:10.1111/j.1365-2605.2005.00636.x)
- Hirosawa N, Yano K, Suzuki Y & Sakamoto Y 2006 Endocrine disrupting effect of di-(2-ethylhexyl)phthalate on female rats and proteome analyses of their pituitaries. *Proteomics* **6** 958–971. (doi:10.1002/pmic.200401344)
- Hoppin JA, Brock JW, Davis BJ & Baird DD 2002 Reproducibility of urinary phthalate metabolites in first morning urine samples. *Environmental Health Perspectives* **110** 515–518. (doi:10.1289/ehp.02110515)
- Hornung RW & Reed LD 1990 Estimation of average concentration in the presence of nondetectable values. *Applied Occupational and Environmental Hygiene* **5** 46–51. (doi:10.1080/1047322X.1990.10389587)
- Howdeshell KL, Wilson VS, Furr J, Lambright CR, Rider CV, Blystone CR, Hotchkiss AK & Gray LE Jr 2008 A mixture of five phthalate esters inhibits fetal testicular testosterone production in the Sprague–Dawley rat in a cumulative, dose-additive manner. *Toxicological Sciences* **105** 153–165. (doi:10.1093/toxsci/kfn077)
- Jobling S, Reynolds T, White R, Parker MG & Sumpter JP 1995 A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. *Environmental Health Perspectives* **103** 582–587. (doi:10.1289/ehp.95103582)
- Kato K, Silva MJ, Needham LL & Calafat AM 2005 Determination of 16 phthalate metabolites in urine using automated sample preparation and on-line preconcentration/high-performance liquid chromatography/tandem mass spectrometry. *Analytical Chemistry* **77** 2985–2991. (doi:10.1021/ac0481248)
- Kavlock R, Boekelheide K, Chapin R, Cunningham M, Faustman E, Foster P, Golub M, Henderson R, Hinberg I, Little R *et al.* 2002 NTP Center for the Evaluation of Risks to Human Reproduction: phthalates expert panel report on the reproductive and developmental toxicity of di(2-ethylhexyl) phthalate. *Reproductive Toxicology* **16** 529–653. (doi:10.1016/S0890-6238(02)00032-1)
- Kerlan V, Nahoul K, Le Martelot MT & Bercovici JP 1994 Longitudinal study of maternal plasma bioavailable testosterone and androstenediol glucuronide levels during pregnancy. *Clinical Endocrinology* **40** 263–267. (doi:10.1111/j.1365-2265.1994.tb02478.x)
- Klinga K, Bek E & Runnebaum B 1978 Maternal peripheral testosterone levels during the first half of pregnancy. *American Journal of Obstetrics and Gynecology* **131** 60–62.
- Laskey JW & Berman E 1993 Steroidogenic assessment using ovary culture in cycling rats: effects of bis(2-diethylhexyl)phthalate on ovarian steroid production. *Reproductive Toxicology* **7** 25–33. (doi:10.1016/0890-6238(93)90006-S)
- Lee KY, Shibutani M, Takagi H, Kato N, Takigami S, Uneyama C & Hirose M 2004 Diverse developmental toxicity of di-*n*-butyl phthalate in both sexes of rat offspring after maternal exposure during the period from late gestation through lactation. *Toxicology* **203** 221–238. (doi:10.1016/j.tox.2004.06.013)
- Lin LC, Wang SL, Chang YC, Huang PC, Cheng JT, Su PH & Liao PC 2011 Associations between maternal phthalate exposure and cord sex hormones in human infants. *Chemosphere* **83** 1192–1199. (doi:10.1016/j.chemosphere.2010.12.079)
- Longcope C 1986 Adrenal and gonadal androgen secretion in normal females. *Clinics in Endocrinology and Metabolism* **15** 213–228. (doi:10.1016/S0300-595X(86)80021-4)
- Lopez-Carrillo L, Hernandez-Ramirez RU, Calafat AM, Torres-Sanchez L, Galvan-Portillo M, Needham LL, Ruiz-Ramos R & Cebrian ME 2010 Exposure to phthalates and breast cancer risk in northern Mexico. *Environmental Health Perspectives* **118** 539–544. (doi:10.1289/ehp.0901091)
- Lovekamp-Swan T & Davis BJ 2003 Mechanisms of phthalate ester toxicity in the female reproductive system. *Environmental Health Perspectives* **111** 139–145. (doi:10.1289/ehp.5658)
- Madden JD, Gant NF & MacDonald PC 1978 Study of the kinetics of conversion of maternal plasma dehydroisoandrosterone sulfate to 16 α -hydroxydehydroisoandrosterone sulfate, estradiol, and estriol. *American Journal of Obstetrics and Gynecology* **132** 392–395.
- Melmed S PK, Reed P & Kronenberg HM 2011 *Williams Textbook of Endocrinology: Expert Consult*. 12th Edition. Philadelphia, Pennsylvania: Elsevier (Saunders).
- Mylchreest E, Sar M, Wallace DG & Foster PM 2002 Fetal testosterone insufficiency and abnormal proliferation of Leydig cells and gonocytes in rats exposed to di(*n*-butyl) phthalate. *Reproductive Toxicology* **16** 19–28. (doi:10.1016/S0890-6238(01)00201-5)
- Parks LG, Ostby JS, Lambright CR, Abbott BD, Klinefelter GR, Barlow NJ & Gray LE Jr 2000 The plasticizer diethylhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat. *Toxicological Sciences* **58** 339–349. (doi:10.1093/toxsci/58.2.339)
- Qoubaitary A, Meriggola C, Ng CM, Lumbreras L, Cerpolini S, Pelusi G, Christensen PD, Hull L, Swerdloff RS & Wang C 2006 Pharmacokinetics of testosterone undecanoate injected alone or in combination with norethisterone enanthate in healthy men. *Journal of Andrology* **27** 853–867. (doi:10.2164/jandrol.106.000281)
- Rothman MS, Carlson NE, Xu M, Wang C, Swerdloff R, Lee P, Goh VH, Ridgway EC & Wiernan ME 2011 Reexamination of testosterone, dihydrotestosterone, estradiol and estrone levels across the menstrual cycle and in postmenopausal women measured by liquid chromatography–tandem mass spectrometry. *Steroids* **76** 177–182. (doi:10.1016/j.steroids.2010.10.010)
- Sagiv SK, Thurston SW, Bellinger DC, Altshul LM & Korrick SA 2012 Neuropsychological measures of attention and impulse control among 8-year-old children exposed prenatally to organochlorines. *Environmental Health Perspectives* **120** 904–909. (doi:10.1289/ehp.1104372)
- Shiraishi S, Lee PW, Leung A, Goh VH, Swerdloff RS & Wang C 2008 Simultaneous measurement of serum testosterone and dihydrotestosterone by liquid chromatography–tandem mass spectrometry. *Clinical Chemistry* **54** 1855–1863. (doi:10.1373/clinchem.2008.103846)
- Siiteri PK & MacDonald PC 1966 Placental estrogen biosynthesis during human pregnancy. *Journal of Clinical Endocrinology and Metabolism* **26** 751–761.
- Silva MJ, Slakman AR, Reidy JA, Preau JL Jr, Herbert AR, Samandar E, Needham LL & Calafat AM 2004 Analysis of human urine for fifteen phthalate metabolites using automated solid-phase extraction. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences* **805** 161–167. (doi:10.1016/j.jchromb.2004.02.038)
- Speroff LF & Marc A 2005 *Clinical Gynecologic Endocrinology and Infertility*, 7th edn. Lippincott Williams and Wilkins.
- Strauss JF BR 2009 *Yen and Jaffe's Reproductive Endocrinology: Physiology, Pathophysiology, and Clinical Management*, 6th edn. Philadelphia, PA: Saunders.
- Swan SH, Main KM, Liu F, Stewart SL, Kruse RL, Calafat AM, Mao CS, Redmon JB, Ternand CL, Sullivan S *et al.* 2005 Decrease in anogenital distance among male infants with prenatal phthalate exposure. *Environmental Health Perspectives* **113** 1056–1061. (doi:10.1289/ehp.8100)
- Swan SH 2006 Prenatal phthalate exposure and anogenital distance in male infants. *Environmental Health Perspectives* **114** A88–89.
- Toriola AT, Vaarasmaki M, Lehtinen M, Zeleniuch-Jacquotte A, Lundin E, Rodgers KG, Lakso HA, Chen T, Schock H, Hallmans G *et al.* 2011 Determinants of maternal sex steroids during the first half of pregnancy. *Obstetrics and Gynecology* **118** 1029–1036. (doi:10.1097/AOG.0b013e3182342b7f)
- Tranfo G, Caporossi L, Paci E, Aragona C, Romanzi D, De Carolis C, De Rosa M, Capanna S, Papaleo B & Pera A 2011 Urinary phthalate monoesters concentration in couples with infertility problems. *Toxicology Letters* **213** 15–20. (doi:10.1016/j.toxlet.2011.11.033)
- Tulchinsky D, Hobel CJ, Yeager E & Marshall JR 1972 Plasma estrone, estradiol, estriol, progesterone, and 17-hydroxyprogesterone in human pregnancy. I. Normal pregnancy. *American Journal of Obstetrics and Gynecology* **112** 1095–1100.

Received 30 August 2013

First decision 27 September 2013

Revised manuscript received 24 October 2013

Accepted 6 November 2013