Journal of Andrology, Vol. 30, No. 3, May/June 2009

Urinary Metabolites of Di(2-ethylhexyl) Phthalate Are Associated With Decreased Steroid Hormone Levels in Adult Men

JOHN D. MEEKER,* ANTONIA M. CALAFAT,† AND RUSS HAUSER‡§

*From the *Department of Environmental Health Sciences, University of Michigan, Ann Arbor, Michigan; the †Centers for Disease and Control and Prevention, Atlanta, Georgia; the ‡Department of Environmental Health, Harvard School of Public Health, Boston, Massachusetts; and the §Vincent Memorial Obstetrics and Gynecology Service, Andrology Laboratory and In Vitro Fertilization Unit, Massachusetts General Hospital, Boston, Massachusetts.*

ABSTRACT: Experimental animal studies have demonstrated that exposure to some phthalates may be associated with altered endocrine function and adverse effects on male reproductive development and function, but human studies are limited. In the present study, urine and serum samples were collected from 425 men recruited through a US infertility clinic. Urinary concentrations of mono(2-ethylhexyl) phthalate (MEHP), the hydrolytic metabolite of di(2-ethylhexyl) phthalate (DEHP), and other phthalate monoester metabolites were measured, along with serum levels of testosterone, estradiol, sex hormone–binding globulin (SHBG), follicle-stimulating hormone, luteinizing hormone, inhibin B, and prolactin. Two oxidized urinary metabolites of DEHP were also measured in urine from 221 of the men. In multiple regression models adjusted for potential confounders, MEHP was inversely associated with testosterone, estradiol, and free androgen index (FAI). An interquartile range increase in MEHP was associated with 3.7% (95% confidence interval [CI], −6.8% to −0.5%) and 6.8% (95% CI, −11.2% to −2.4%) declines in testosterone and estradiol, respectively, relative to the population median hormone levels. There was limited evidence for effect modification of the inverse association between MEHP and FAI by the proportion of DEHP metabolites in the urine measured as MEHP (MEHP%), which is considered a phenotypic marker of less efficient metabolism of DEHP to its oxidized metabolites. Finally, the ratio of testosterone to estradiol was positively associated with MEHP ($P = .07$) and MEHP% ($P = .007$), suggesting potential relationships with aromatase suppression. In conclusion, these results suggest that urinary metabolites of DEHP are inversely associated with circulating steroid hormone levels in adult men. However, additional research is needed to confirm these findings.

Key words: Androgen, biomarker, endocrine, environment, human, estrogen, male reproduction, testosterone.

J Androl 2009;30:287–297

There is concern for adverse human health risks resulting from exposure to environmental endocrine-disrupting compounds (EDCs). A number of recent studies from several countries have suggested secular declining trends in testosterone levels among males (Andersson et al, 2007; Travison et al, 2007) that may coincide with increased use and human exposure to EDCs. In men, altered hormone levels from environmental or occupational exposures may be associated with or lead to declined reproductive capacity or possibly increased risk of testicular or prostate cancer (Fleming et al, 1999; Pflieger-Bruss et al, 2004; Toft et al, 2004). Certain environmental chemicals may cause altered hormone levels through a number of biological mechanisms alone or in combination, ranging from effects on hormone receptors to effects on hormone synthesis, secretion, or metabolism. Although the health impacts of subclinical alterations in circulating hormone levels remain unclear, a limited but growing body of evidence exists for these changes to be associated with environmental and occupational exposure to commonly used chemicals.

Phthalates have a wide range of industrial and commercial uses, resulting in widespread human exposure through various pathways. High-molecular-weight phthalates (eg, di(2-ethylhexyl) phthalate [DEHP], diisononyl phthalate, and di(n-octyl) phthalate) are used primarily as plasticizers in the manufacture of flexible vinyl, which, in turn, is used in consumer products, flooring and wall coverings, food contact applications, and medical devices (David et al, 2001; ATSDR 2002). Low-molecular-weight phthalates (eg, diethyl phthalate [DEP] and dibutyl phthalate [DBP]) can be used in

Supported by grants ES009718 and ES00002 from the National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health (NIH).

Correspondence to: Dr John Meeker, Department of Environmental Health Sciences, University of Michigan School of Public Health, 6635 SPH Tower, 109 S Observatory St, Ann Arbor, MI 48109 (e-mail: meekerj@umich.edu).

Received for publication July 25, 2008; accepted for publication December 1, 2008.

Disclaimer: The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the CDC.

DOI: 10.2164/jandrol.108.006403

personal-care products (eg, perfumes, lotions, cosmetics), as solvents and plasticizers for cellulose acetate, and in making lacquers, varnishes, and coatings, including those used to provide timed releases in some pharmaceuticals (ATSDR, 1995, 2001; David et al, 2001). The Centers for Disease Control and Prevention's (CDC's) Third National Report on Human Exposure to Environmental Chemicals showed that the majority of males in the United States have detectable concentrations of several phthalate monoesters in urine (monoethyl phthalate [MEP], mono(2-ethylhexyl) phthalate [MEHP], monobutyl phthalate [MBP], and monobenzyl phthalate [MBzP]), demonstrating that exposure to the parent diester compounds is common among the general population (CDC, 2005). Two oxidized metabolites of DEHP, mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) and mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), were present in most subjects at urinary concentrations higher than those of MEHP, the hydrolytic metabolite of DEHP (CDC, 2005).

Although experimental studies have demonstrated antiandrogenic activity and male reproductive toxicity in relation to several phthalates (Foster, 2006; Ge et al, 2007), only limited human studies have investigated associations between exposure to phthalates and circulating hormone levels. A relationship between phthalates and hormone levels in infants has been reported. Within a Danish/Finnish cohort on cryptorchidism, Main et al (2006) analyzed breast milk samples for phthalate metabolites and measured reproductive serum hormone levels in 3-month-old breastfeeding boys. The authors reported positive associations of MEP, monomethyl phthalate, and MBP with luteinizing hormone (LH):free testosterone ratio, which is a measure of Leydig cell function. There were also positive associations among MEP, MBP, and sex hormone–binding globulin (SHBG) and between monoisononyl phthalate and LH, and an inverse association between MBP and free testosterone. One US study among male infants found inverse associations between urinary MBP, MBzP, MEP, and monoisobutyl phthalate and anogenital distance, which is thought to be a sensitive marker for androgen activity (Swan et al, 2005).

Among a group of adult Chinese workers producing PVC flooring with high exposure to DEHP and DBP, urinary concentrations of metabolites of these phthalates were inversely associated with free testosterone levels (Pan et al, 2006). In a study of 295 men from a Massachusetts infertility clinic, we previously reported a suggestive (though not statistically significant) inverse association between urinary MEHP and testosterone, along with a statistically significant positive association between urinary MBP (a urinary metabolite of DBP) and inhibin B ($P < .05$), and a statistically significant inverse association between urinary MBzP and follicle-stimulating hormone (FSH) (Duty et al, 2005). However, the significant results for MBP and MBzP and hormone levels were in patterns inconsistent with our study hypotheses. Finally, a study of 234 young Swedish men found an inverse association between urinary MEP (the main DEP metabolite) and LH but no association between MEP, MEHP, or other phthalate metabolites in urine and FSH, testosterone, estradiol, or inhibin B (Jonsson et al, 2005).

The present study extends our previous analysis (Duty et al, 2005) by including a much larger sample size, by expanding the range of hormones measured to include estradiol, prolactin, and testosterone:LH ratio (a measure of Leydig cell function), and by expanding the phthalate metabolites that were measured by also including 2 oxidized DEHP metabolites: MEHHP and MEOHP.

Materials and Methods

Subjects were recruited from an ongoing study on the relationship between environmental agents and male reproductive health. They were men who were partners in subfertile couples seeking treatment from the Vincent Burnham Andrology Laboratory at Massachusetts General Hospital in Boston between January 2000 and May 2004. The study was approved by the Human Studies Institutional Review Boards of Massachusetts General Hospital, the Harvard School of Public Health, the CDC, and the University of Michigan. After the study procedures were explained and all questions answered, subjects signed an informed consent. Men between the ages of 18 to 55 years without postvasectomy status who presented to the andrology laboratory were eligible to participate. Of those approached, approximately 65% consented. Most men who declined to participate in the study cited lack of time on the day of their clinic visit as the reason for not participating.

Phthalate Metabolites in Urine

A single spot urine sample was collected from each subject on the day of their clinic visit in a sterile specimen cup prescreened for phthalate metabolites. Phthalate metabolites were measured in urine because of potential sample contamination from the parent diester and because the metabolites, as opposed to the parent diesters, are believed to be the active toxicants (Li et al, 1998; Peck and Albro 1982). The analytical approach for the analysis of the urinary phthalate monoester metabolites (ie, MEHP, MBP, MBzP, and MEP) and 2 oxidized metabolites of DEHP (ie, MEHHP and MEOHP) involved enzymatic deconjugation of the metabolites from their glucuronidated form, solid-phase extraction, separation with high-performance liquid chromatography, and detection by isotope-dilution tandem mass spectrometry (Blount et al, 2000; Silva et al, 2003, 2004a). Detection limits were in the low nanogram per milliliter range and varied slightly depending on the

analytical method used (Blount et al, 2000; Silva et al, 2003, 2004a) for each phthalate metabolite (MEP, 1.00 to 1.21 ng/ml; MBP, 0.60 to 1.07 ng/ml; MBzP, 0.47 to 1.0 ng/ml; MEHP, 0.87 to 1.20 ng/ml; MEHHP, 0.95 to 1.60 ng/ml; MEOHP, 1.07 to 1.20 ng/ml). Isotopically labeled internal standards and conjugated internal standards were used to increase precision of measurements. Along with the unknown samples, each analytical run included calibration standards, reagent blanks, and quality control materials of high and low concentration to monitor for accuracy and precision. Analysts at the CDC, Atlanta, Georgia, were blind to all information concerning subjects. Urinary phthalate metabolite concentrations were adjusted for urine dilution by specific gravity (SG) using the following formula: $P_c = P[(1.024 - 1)/(SG - 1)]$, where P_c is the SG-adjusted phthalate metabolite concentration (ng/ml), P is the observed phthalate metabolite concentration, and SG is the SG of the urine sample. SG was measured using a handheld refractometer (National Instrument Company Inc, Baltimore, Maryland).

We calculated (in nanomoles per milliliter) the sum of DEHP metabolites that were measured (ie, MEHP, MEHHP, and MEOHP). These values were also used to calculate the percentage of these DEHP metabolites excreted as MEHP. We refer to this as MEHP% and consider it a phenotypic marker of the proportion of DEHP metabolized to and excreted in the urine as MEHP (Hauser et al, 2006, 2007; Meeker et al, 2007). The greater the MEHP%, the larger the percentage of DEHP excreted as MEHP relative to the excretion of the 2 oxidized metabolites. To calculate MEHP%, we converted MEHP, MEHHP, and MEOHP concentrations to nanomoles per milliliter, divided MEHP concentrations by the sum of concentrations of MEOHP, MEHHP, and MEHP, and multiplied by 100.

Serum Hormones

One nonfasting blood sample was drawn between the hours of 9 AM and 4 PM on the same day that the semen sample was collected. Semen analysis procedures and relationships between semen parameters and phthalate metabolites were described in a previous report (Hauser et al, 2006). Blood samples were centrifuged and the resulting serum was stored at −80°C until analysis. Testosterone was measured directly using the Coat-A-Count RIA kit (Diagnostics Products, Los Angeles, California), which has interassay and intra-assay coefficients of variation (CVs) of 12% and 10%, respectively, with a sensitivity of 4 ng/dL (0.139 nmol/L). The free androgen index (FAI) was calculated as the molar ratio of total testosterone to SHBG. SHBG was measured using a fully automated system (Immulite; DPC Inc, Los Angeles, California) that uses a solid-phase 2-site chemiluminescent enzyme immunometric assay and has an interassay CV of less than 8%. Inhibin B was measured using a commercially available, double antibody, enzyme-linked immunosorbent assay (Oxford Bioinnovation, Oxford, United Kingdom) with interassay and intra-assay CVs of 20% and 8%, respectively, limit of detection (LOD) of 15.6 pg/mL and a functional sensitivity (20% CV) of 50 pg/mL. Serum LH, FSH, estradiol, and prolactin concentrations were determined by microparticle enzyme immunoassay using an automated Abbott AxSYM system (Abbott Laboratories, Chicago, Illinois). The Second International Reference Preparation (WHO 71/223) was used as the reference standard. The assay sensitivities for LH and FSH were 1.2 IU/L and 1.1 IU/L, respectively. The intra-assay CVs for LH and FSH were less than 5% and less than 3%, respectively, with interassay CVs for both hormones of less than 9%. The testosterone:LH ratio, a measure of Leydig cell function, was calculated by dividing testosterone (nmol/L) by LH (IU/L). The assay sensitivities for estradiol and prolactin were 20 pg/mL and 0.6 ng/mL, respectively. For estradiol the within-run CV was between 3% and 11%, and the total CV was between 5% and 15%. For prolactin the within-run CV was ≤3% and the total CV was ≤6%.

Statistical Analysis

Data analysis was performed using SAS version 9.1 (SAS Institute Inc, Cary, North Carolina). Descriptive statistics on subject demographics were tabulated, along with the distributions of phthalate metabolite concentrations and reproductive hormones. For phthalate metabolite concentrations or hormone levels below the LOD, an imputed value equal to one-half the LOD was used. In preliminary data analysis, hormone and phthalate metabolite concentrations were stratified by demographic categories to investigate the potential for confounding. Multivariable linear regression was used to explore relationships between urinary phthalate metabolite and hormone concentrations. Concentrations of inhibin B, testosterone, and estradiol closely approximated normality and were used in statistical models untransformed, whereas the distributions of FSH, LH, SHBG, FAI, T:LH ratio, and prolactin were skewed left and transformed by the natural logarithm for statistical analyses. SG-adjusted phthalate metabolite concentrations were also ln-transformed. Inclusion of covariates was based on statistical and biologic considerations (Kleinbaum et al, 1998). Age and body mass index (BMI) were modeled as a continuous variable, smoking status was dichotomized by current smoker vs never smoked or former smoker, and race was categorized into 4 groups: white, African American, Hispanic, and other. Previous exam for infertility (yes or no), prior ability to impregnate a partner (yes or no), and timing of blood/urine samples by season (winter vs spring, summer, or fall) and time of day (9:00 AM–12:59 PM vs 1:00 PM–4:00 PM) were considered for inclusion in the models as dichotomous variables. To improve interpretability, the regression coefficients were back transformed and expressed as a change in the dependent variable (ie, hormone levels) for an interquartile range (IQR) increase in phthalate metabolite concentrations.

For MEHP, we also included MEHHP, MEOHP, or MEHP% in the models to explore evidence of whether individual differences in DEHP metabolism alter susceptibility to MEHP. We also explored the possibility of effect modification of MEHP-hormone associations by MEHP%. Our hypothesis is that the concentrations of MEHHP (or MEOHP) and/or MEHP% may represent phenotypic markers for efficient or inefficient metabolism of DEHP to its oxidized metabolites (Hauser et al, 2007; Meeker et al, 2007). In secondary sensitivity analyses, the multivariable models were

Table 1. *Distribution of SG-adjusted phthalate metabolites in urine (ng/mL)*

Phthalate Metabolite	n	Geometric Mean	Selected Percentiles						
			10th	25th	50th	75th	90th	95th	Maximum
MEP	425	179	30.2	59.9	153	518	1376	2269	11 370
MBP	425	17.1	5.06	10.6	17.7	32.7	50.8	69.9	14 460
MBzP	425	7.73	2.27	4.20	8.20	15.9	24.9	40.6	540
MEHP	425	8.22	1.04	3.18	7.89	20.7	64.3	122	876
MEHHP	221	55.6	13.2	23.1	47.0	105	272	784	4806
MEOHP	221	36.2	8.35	15.4	32.2	61.9	193	446	3063
Sum DEHP (nmol/mL)	221	0.36	0.09	0.16	0.31	0.67	2.09	4.53	29.4
MEHP% (%)	221	9.39	3.46	5.76	10.3	16.8	24.3	30.7	61.5

Abbreviations: DEHP, di(2-ethylhexyl) phthalate; MBP, monobutyl phthalate; MBzP, monobenzyl phthalate; MEHP, mono(2-ethylhexyl) phthalate; MEHP%, proportion of DEHP metabolites in the urine measured as MEHP; MEHHP, mono(2-ethyl-5-hydroxyhexyl) phthalate; MEOHP, mono(2-ethyl-5-oxohexyl) phthalate; MEP, monoethyl phthalate; SG, specific gravity.

rerun after excluding men with highly concentrated or highly dilute urine samples (SG above 1.03 or below 1.01) (Teass et al, 1998), and rerun among the strata of men with sperm concentration, motility, and morphology all above reference values (Hauser et al, 2006). Finally, we assessed nonlinear relationships between phthalate metabolite concentrations and hormones by regressing the hormones on tertiles or quintiles of phthalate metabolites. We also assessed whether phthalate metabolite tertiles or quintiles predicted low steroid hormone levels, where the odds of being in the lowest quartile for testosterone, estradiol, or FAI were calculated using multiple logistic regression.

Results

A total of 484 men had phthalate metabolite concentrations measured in urine, and 439 of these men also had hormone levels measured in serum. An additional 14 subjects taking hormone medications (eg, Propecia, finasteride, cabergoline, Clomid, gonadotropin-releasing hormone, testosterone, or prednisone taper) were excluded from the present analysis. Among the remaining subjects (n = 425), most were white (85%) and had never smoked (72%). The mean (SD) age and BMI were 36 (5.3) years and 28 (4.5), respectively. Distributions of SG-adjusted urinary phthalate metabolite concentrations are presented in Table 1 and serum hormone levels in Table 2. Among the 425 urine samples, MEP was detected in 100% of the samples, and MBP and MBzP were detected in over 97% and 94% of the samples, respectively. Eighty-three percent of samples had detectable concentrations of MEHP. The sample size for MEOHP and MEHHP was 221 because analytical methods for the quantification of these analytes were not available at the onset of the study. Over 95% of these samples had detectable concentrations of MEHHP and MEOHP. Spearman correlations between MEHP and MEHHP or MEOHP concentrations were 0.75 and 0.71, respectively.

FAI and estradiol were both inversely associated with age (Spearman correlation coefficients = −0.2 and −0.1, respectively; both $P < .05$), but positively associated with BMI (Spearman coefficients = 0.2 and 0.1, respectively; $P < .05$). BMI was inversely associated with inhibin B, testosterone, and SHBG, but positively associated with MBzP and MEOHP (all $P < .05$). Current smokers had lower median prolactin levels (9.2 ng/mL) than never smokers (11.7 ng/mL). Current smokers also had higher median concentrations of MEP but lower concentrations of MEHHP and MEOHP compared with never smokers. Median inhibin B was higher in samples collected in the morning compared with samples collected in the afternoon (166 vs 155 pg/mL), but morning samples had lower median estradiol (29 vs 30 pg/mL) and prolactin (9.9 vs 12.7 ng/mL) compared with afternoon samples. As previously observed (Silva et al, 2004b), median MEHP concentration was also higher among men whose urine sample was collected in the afternoon (9.0 ng/mL) vs men providing the urine sample in the morning (6.9 ng/mL). Finally, urine samples collected in the winter had median inhibin B concentrations that were lower than those collected in spring, summer, or fall (143 vs 168 pg/mL), but a median FAI that was slightly higher (0.55 vs 0.52). Samples collected in the winter also had slightly higher concentrations of MEHHP and MEOHP, but also a higher MEHP%, than samples collected in spring, summer, or fall.

All linear regression results in Table 3 were adjusted for age, BMI, smoking, and time of day blood/urine samples were collected. Crude regression results (not shown) were similar to the adjusted results presented in Table 3, though the relationship between MEHP and FAI became somewhat less statistically significant in the adjusted model ($P < .05$ in the crude model, .1 in the adjusted model). In adjusted models (Table 3), there was an inverse association between SG-adjusted urinary MEHP concentration and serum testosterone levels,

Table 2. *Distribution of reproductive hormones in serum (n = 425)*

Hormone	Geometric Mean	Selected Percentiles						
		5th	10th	25th	50th	75th	90th	95th
FSH (IU/L)	8.03	3.67	4.33	5.67	7.51	10.5	15.7	22.7
LH (IU/L)	10.1	4.90	5.76	7.34	9.90	13.6	17.2	20.4
Inhibin B (pg/mL)	166[a]	61.6	81.6	118	160	198	262	283
Testosterone (ng/dL)	420[a]	223	254	324	408	492	609	665
T:LH ratio	39.4	17.0	22.0	30.5	40.9	54.5	70.0	78.8
SHBG (nmol/mL)	25.8	12.5	15.3	10.1	26.0	33.7	44.0	49.3
Free androgen index	0.53	0.32	0.35	0.43	0.52	0.67	0.84	0.95
Estradiol (pg/mL)	29.2[a]	<10	<10	23.0	30.0	36.0	45.0	49.0
Prolactin (ng/mL)	11.6	5.67	6.53	8.30	11.5	15.8	21.4	25.6

Abbreviations: FSH, follicle-stimulating hormone; LH, luteinizing hormone; SHBG, sex hormone–binding globulin; T:LH ratio, ratio of testosterone to luteinizing hormone.

[a] Arithmetic mean.

where an IQR increase in MEHP was associated with a 14.9 ng/dL decrease in testosterone (95% confidence interval [CI], −27.5 to −2.30 ng/dL; $P = .02$). For the median level of testosterone (408 ng/dL), this represents a 3.7% (−6.8% to −0.5%) decrease in testosterone for an IQR increase in MEHP (IQR, 3.18 to 20.7 ng/dL). SG-adjusted MEHP was also inversely associated with estradiol, where an IQR increase in MEHP was associated with a 6.8% decline (95% CI, −11.2% to −2.4%) in estradiol relative to the population median estradiol level (30 pg/mL).

In sensitivity analyses, effect estimates from the multivariable models were similar when only men with SG between 1.01 and 1.03 were included (n = 343, results not shown). The effect estimates were also similar when limiting the analyses to men with above reference (''normal'') semen quality measures (data not shown). This provided limited evidence that the relationships between MEHP and reproductive hormones among men with semen parameters above reference values are similar to those among men with below-reference semen parameters. This suggests that the associations reported may be generalizable to men with normal fertility, recognizing, however, that even men with above reference semen parameters may be infertile.

When the analysis was limited to the subset of men with oxidized DEHP metabolite measures (n = 221), there was no evidence of strengthened associations between MEHP and hormone levels when additionally adjusting for MEHHP, MEOHP, or MEHP%, as we previously reported for sperm DNA damage and thyroid hormone levels (Hauser et al, 2007; Meeker et al, 2007). However, both MEHHP and MEOHP were inversely associated with FAI when modeled without MEHP. For both metabolites an IQR increase was associated with a 5% decline in FAI (Table 3). Likewise, the sum of measured DEHP metabolites was associated with a statistically significant 4% decline in FAI. There was also an inverse association between MEHP% and estradiol. Because MEHP% was associated with estradiol but not testosterone, an additional secondary analysis involved exploring associations with the ratio of testosterone to estradiol ($T:E_2$; a measure of aromatase activity). In this analysis MEHP% was positively associated with $T:E_2$ ratio; an IQR increase in MEHP% was associated with a 14% increase in $T:E_2$ (95% CI, 4% to 25%; $P = .007$). There was also a suggestive positive association between MEHP and $T:E_2$ in the full dataset (n = 425), where an IQR increase in MEHP was associated with a 6% increase in $T:E_2$ (95% CI, −0.4% to 12%; $P = .07$).

To assess the robustness of the associations between phthalate metabolite concentrations and steroid hormones, and potential nonlinear relationships, we divided SG-adjusted phthalate concentrations into categories. In quintile analysis among the full cohort (n = 425) there were significant but nonmonotonic trends in the associations between MEHP and testosterone and between MEHP and estradiol (Figure 1a and 1b). There was a sharp decline in the regression coefficient for change in testosterone among the highest MEHP quintile, whereas the regression coefficients for declined estradiol among increasing quintiles of MEHP appeared to plateau at quintile 4. The lack of a monotonic trend could perhaps be a result of unequal bin widths stemming from a skewed-right distribution, where the occurrence of exposure misclassification may be more likely in the lower quintiles where cutoff values are closer together. Conversely, there was no trend in FAI in relation to MEHP quintiles (results not shown; P for trend = .33). However, in multiple logistic regression for low FAI, there was a significant increasing trend in the adjusted odds of being in the lowest FAI quartile among increasing MEHP quintiles (P for trend = .01; Figure 2a). When limiting this analysis to the 221

Table 3. *Adjusted[a] regression coefficients (95% confidence intervals) for change in hormones associated with an interquartile range (IQR) increase in SG-adjusted urinary phthalate metabolite concentrations (n = 425)[b]*

Phthalate Metabolite[c]	FSH[d,e]	LH[d,e]	Inhibin B[f]	Testosterone[f]	T:LH ratio[d,e]	SHBG[d,e]	FAI[d,e]	Estradiol[f]	T:E_2 ratio[d,e]	Prolactin[d,e]
MEP	0.98 (0.91, 1.06)	0.98 (0.91, 1.04)	0.73 (−9.99, 11.4)	8.87 (−7.18, 24.9)	1.03 (0.95, 1.11)	0.97 (0.91, 1.02)	1.04 (0.99, 1.09)	0.71 (−0.97, 2.40)	1.00 (0.92, 1.07)	0.97 (0.91, 1.03)
MBP	1.02 (0.97, 1.08)	1.01 (0.97, 1.06)	1.34 (−5.98, 8.66)	−4.65 (−15.7, 6.33)	0.99 (0.94, 1.04)	1.02 (0.98, 1.06)	0.98 (0.94, 1.01)	−0.47 (−1.62, 0.68)	1.01 (0.96, 1.07)	1.00 (0.96, 1.04)
MBzP	0.98 (0.92, 1.04)	1.00 (0.95, 1.05)	1.81 (−6.54, 10.2)	4.58 (−7.91, 17.0)	1.02 (0.97, 1.09)	1.00 (0.95, 1.04)	1.03 (0.99, 1.07)	−0.21 (−1.53, 1.09)	1.04 (0.98, 1.11)	1.01 (0.96, 1.06)
MEHP	1.03 (0.97, 1.10)	0.97 (0.92, 1.02)	−1.32 (−9.82, 7.18)	−14.9 (−27.5, −2.30)[g]	0.99 (0.93, 1.05)	0.99 (0.94, 1.03)	0.98 (0.96, 1.00)[h]	−2.04 (−3.37, −0.73)[g]	1.06 (1.00, 1.12)[h]	1.00 (0.95, 1.05)
MEHHP[b]	0.97 (0.89, 1.05)	0.98 (0.91, 1.06)	−2.45 (−15.9, 11.0)	−9.33 (−25.3, 6.60)	1.01 (0.93, 1.08)	1.04 (0.98, 1.11)	0.95 (0.90, 1.00)[g]	−1.32 (−2.98, 0.33)	1.03 (0.95, 1.13)	1.01 (0.94, 1.08)
MEOHP[b]	0.96 (0.90, 1.04)	0.99 (0.93, 1.06)	−1.22 (−13.4, 10.9)	−8.88 (−23.4, 5.59)	1.00 (0.94, 1.07)	1.05 (0.99, 1.11)[h]	0.95 (0.91, 0.99)[g]	−1.09 (−2.60, 0.42)	1.02 (0.95, 1.11)	1.01 (0.95, 1.08)
Sum DEHP[b]	0.98 (0.92, 1.04)	0.99 (0.94, 1.04)	−1.53 (−11.2, 8.07)	−6.35 (−17.8, 5.10)	1.00 (0.95, 1.06)	1.03 (0.99, 1.08)	0.96 (0.93, 1.00)[g]	−1.03 (−1.15, 0.16)[h]	1.03 (0.97, 1.10)	1.03 (0.97, 1.10)
MEHP%[b]	1.01 (0.92, 1.11)	0.98 (0.90, 1.06)	6.88 (−7.78, 21.5)	7.13 (−10.3, 24.6)	1.00 (0.99, 1.01)	0.96 (0.90, 1.02)	1.05 (0.99, 1.11)[h]	−2.01 (−3.81, −0.21)[g]	1.14 (1.04, 1.25)[g]	0.95 (0.88, 1.03)

Abbreviations: BMI, body mass index; DEHP, di(2-ethylhexyl) phthalate; FAI, free androgen index; FSH, follicle-stimulating hormone; LH, luteinizing hormone; MBP, monobutyl phthalate; MBzP, monobenzyl phthalate; MEHHP, mono(2-ethyl-5-hydroxyhexyl) phthalate; MEHP, mono(2-ethylhexyl) phthalate; MEHP%, proportion of DEHP metabolites in the urine measured as MEHP; MEOHP, mono(2-ethyl-5-oxohexyl) phthalate; MEP, monoethyl phthalate; SG, specific gravity; SHBG, sex hormone–binding globulin; T:E_2 ratio, ratio of testosterone to estradiol; T:LH ratio, ratio of testosterone to luteinizing hormone.

[a] Adjusted for age, BMI, current smoking, season, and time of day blood sample was collected. Testosterone and estradiol were additionally adjusted for SHBG.

[b] n = 221 for MEHHP, MEOHP, sum of DEHP metabolites, and MEHP%.

[c] In all models ln transformations of urinary phthalate metabolite concentrations were used.

[d] For FSH, LH, SHBG, FAI, T:LH, T:E_2, and prolactin, ln transformations of were used. Inhibin B, testosterone, and estradiol were modeled untransformed.

[e] Coefficient represents a multiplicative change in hormone level for an IQR change in phthalate metabolite concentration after back transformation of both hormone and phthalate metabolite concentrations. For an IQR change in phthalate metabolite concentration, a coefficient equal to 1.0 indicates no change in hormone level, a coefficient <1.0 indicates a multiplicative decrease in hormone level, and a coefficient >1.0 indicates a multiplicative increase in hormone level.

[f] Coefficient represents the change in hormone level for an IQR change in phthalate metabolite concentration after back transformation of the phthalate metabolite concentrations. For an IQR change in phthalate metabolite concentration, a coefficient equal to 0 indicates no change in hormone level, a coefficient <0 indicates a decrease in hormone level, and a coefficient >0 indicates an increase in hormone level.

[g] $P \leq .05$.

[h] $P < .1$.

men with oxidized metabolite measures the trend became weaker (P for trend = .13 among MEHP tertiles; tertiles were used instead of quintiles because of the smaller sample size). However, there was evidence of effect modification by MEHP% in this relationship, as an MEHP-FAI trend was present among men with MEHP% above the median but not among men with MEHP% below the median (Figure 2b). The odds ratio for low FAI among men in the highest MEHP tertile and with high MEHP% was 11.2 (95% CI, 1.3 to 97.1). There was no clear evidence of effect modification by MEHP% in the other MEHP-hormone relationships, but the number of men with DEHP oxidized metabolite measures (n = 221), and thus a calculable MEHP%, limited statistical power for detecting interactions. Among the full 425 men there were also increased adjusted odds for low (lowest quartile) testosterone and estradiol among increasing MEHP quintiles (results not shown; P for trend = .04 and .02, respectively).

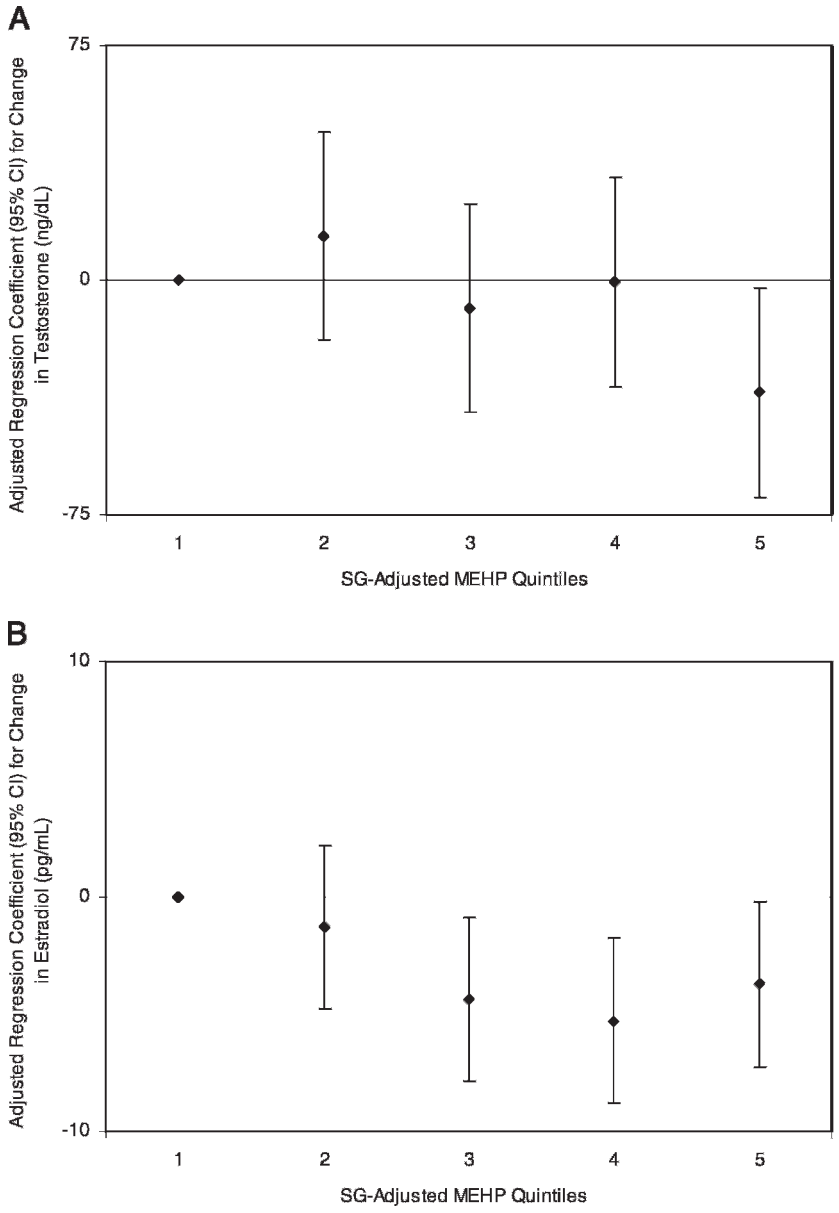

Figure 1. Adjusted (for age, body mass index, smoking, season, and time of day) regression coefficients for a change in hormone level associated with increasing quintiles of urinary specific gravity (SG)–adjusted mono(2-ethylhexyl) phthalate (MEHP) (n = 425). **(A)** Testosterone. *P* for trend = .03. **(B)** Estradiol. *P* for trend = .004.

Discussion

In the present study, urinary MEHP concentrations were inversely associated with circulating testosterone and estradiol levels in adult men recruited through an infertility clinic. We also found evidence for inverse associations of MEHP, MEHHP, MEOHP, and sum of measured DEHP metabolites with FAI, and a positive association between MEHP% and T:E_2 ratio. We previously reported associations between MBP and inhibin B, and between MBzP and FSH, among a smaller and overlapping group of men in our study (Duty et al, 2005). However, in the present analysis we observed no relationships among MEP, MBP, or MBzP with any of the measured hormones.

Only a limited number of studies have investigated the relationship between human phthalate exposure and circulating reproductive hormone levels or hormone indicators (Jonsson et al, 2005; Swan et al, 2005; Main et al, 2006; Pan et al, 2006), and only 2 other previous studies have been conducted among men (Jonsson et al, 2005; Pan et al, 2006). Pan et al (2006) studied 74

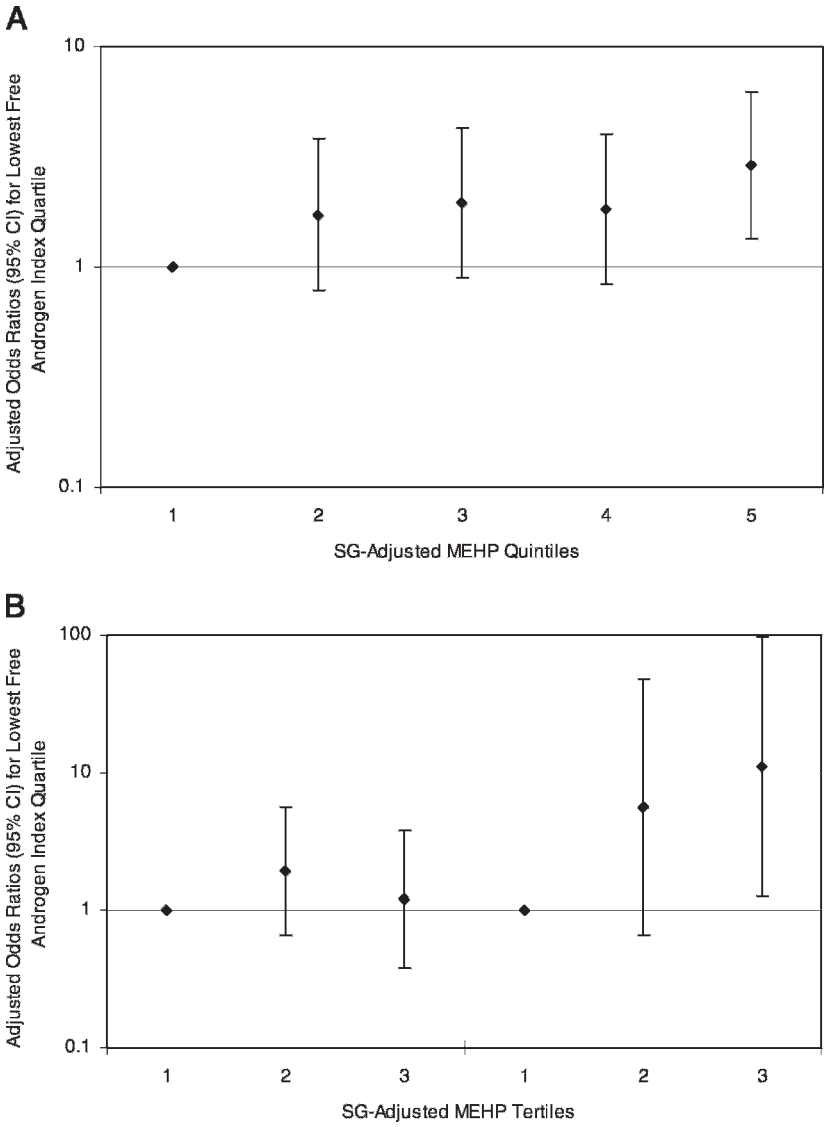

Figure 2. Adjusted (for age, body mass index, smoking, season, and time of day) odds ratios for having free androgen index level in the lowest quartile with increasing urinary specific gravity (SG)–adjusted mono(2-ethylhexyl) phthalate (MEHP) categories. **(A)** MEHP quintile analysis among all men (n = 425). *P* for trend = .01. **(B)** MEHP tertile analysis when stratifying by proportion of DEHP metabolites in the urine measured as MEHP (MEHP%). This analysis could be conducted only among men with oxidized metabolite measures (n = 221). *P* for trends = .6 and .01, respectively.

workers in a Chinese factory exposed to high levels of DEHP and DBP in the production of unfoamed PVC and 64 unexposed workers. The authors reported inverse associations between urinary MBP and MEHP concentrations and free testosterone among the workers, but no relationship between the phthalate metabolites and estradiol. Interestingly, although MEHP concentrations in the present study among non–occupationally exposed men were several orders of magnitude lower than those measured in the Chinese workers with occupational exposure (Pan et al, 2006), the evidence for decreased testosterone in relation to DEHP and/or MEHP was consistent between the 2 studies.

Conversely, the findings in the present study were inconsistent with those reported in the other study of non–occupationally exposed Swedish men by Jonsson and colleagues (2005). In that study no associations between urinary MEHP and testosterone or estradiol were observed. It is possible that the discrepancies in findings between studies may be explained by differences in study design or laboratory methods. For example, there were large differences in the ages of the study populations and the methods of recruitment. The Swedish study population consisted of young men (median age 18 years; range, 18–21 years) undergoing a medical examination before military service. Because

approximately 95% of young men in Sweden undergo the conscript examination, these young men reflected the general population of young Swedish males. In contrast, in the present study the median age was 35.5 years, and ages ranged from 22 to 54 years. However, it is unclear whether men presenting to an infertility clinic are more susceptible to exposure to reproductive toxicants than men from the general population or whether older men are more susceptible to reproductive toxicants because of an age-related response. Other differences between the 2 studies include major differences in participation rates (14% in the Swedish study and 65% in the US study) and differences in the analytical methods used to measure urinary phthalate metabolites, where the method in the present study was more sensitive than that used in the Swedish study.

Consistent with our findings, animal and in vitro studies have demonstrated that several phthalates, including DEHP or its metabolites, are endocrine-disrupting chemicals that possess antiandrogenic activity and reduce testosterone and estradiol levels (ATSDR, 2002). The mechanisms by which these alterations are occurring are not fully established. Recent evidence suggests that phthalates may inhibit expression of genes or proteins related to steroidogenesis, such as steroidogenic acute regulated protein, peripheral benzodiazepine receptor, and P450 side chain cleavage in Leydig cells (Borch et al, 2006). However, this and most other studies of DEHP or MEHP antiandrogenic activity that have been conducted to date were performed in rat fetal testis and thus may not reflect what occurs in adult humans. Additional research is needed on reproductive health effects from phthalate exposures in adulthood, and the involvement of alternative mechanisms, such as hepatic SHBG transcription activity and altered pituitary function, should be considered.

We found limited evidence for interaction by MEHP% of the inverse association between MEHP and FAI. In multiple logistic models for having FAI in the lowest quartile, the association was much stronger among men with high MEHP%. This was consistent with our a priori hypothesis that a high percentage of MEHP, a bioactive hydrolytic metabolite of DEHP, in the sum of all measured DEHP metabolites reflects increased susceptibility to DEHP exposure because of less efficient oxidation and excretion of DEHP and/or MEHP. However, we did not find evidence for modification of the other observed associations by MEHP%, though this may be because of the small number of men with oxidized DEHP metabolites measured (n = 221). The possibility of effect modification by MEHP% should be tested in future epidemiological studies with larger sample sizes.

Another secondary finding from the present study was a suggestive positive association between $T:E_2$ ratio and MEHP, and a strong positive association between $T:E_2$ ratio and MEHP%. A larger $T:E_2$ ratio is a marker for reduced aromatase activity. Consistent with these findings, animal and in vitro studies have demonstrated that DEHP and/or MEHP can suppress aromatase in a human adrenocortical carcinoma cell line, in cultured rat ovarian granulosa cells, and in the brain and testes of young male rats (Davis et al, 1994; Lovekamp and Davis, 2001; Kim et al, 2003; Andrade et al, 2006; Noda et al, 2007). Although suggested as a potential treatment for certain cases of male infertility (Liu and Handelsman, 2003; Schiff et al, 2007), aromatase suppression could be associated with adverse effects on male reproduction (Carreau et al, 2006). A potential alternative explanation for an increased $T:E_2$ ratio in relation to DEHP and/or its metabolites in the present study could be through an increase in estradiol metabolism through PPARα-dependent mechanisms (Corton et al, 1997).

Recent experimental evidence has suggested that estradiol plays an important role in spermatogenesis and male reproduction, and that estradiol is a potent inhibitor of male germ cell death (Hess et al, 1997; Pentikainen et al, 2000). We also recently reported strong inverse associations between estradiol levels and human sperm DNA damage (Meeker et al, 2008). Thus, it is possible that a decrease in estradiol levels and/or aromatase activity may explain our previous observation of a significant increase in sperm DNA damage associated with increased urinary MEHP when accounting for oxidized DEHP metabolites (Hauser et al, 2007). Alternatively, the significant positive association between MEHP% and $T:E_2$ ratio may reflect relationships between aromatase and enzymes involved in efficient DEHP or MEHP metabolism. Future research is needed to help explain these findings.

A potential limitation of the present study is the measurement of urinary phthalate metabolites and serum hormones at a single point in time. Phthalates are rapidly metabolized and excreted, and metabolite concentrations in urine reflect exposure in only the preceding 1 or 2 days. Several studies have explored temporal variability of urinary phthalate metabolites, where high within-individual variability has been reported over the course of several days to months (Hoppin et al, 2002; Fromme et al, 2007; Teitelbaum et al, 2008). Nevertheless, we demonstrated that a single sample may adequately predict average monoester concentrations over a 3-month period in adult men (Hauser et al, 2004). Serum hormone levels may also vary within an individual over time, but a single measure has been shown to provide a reliable measure in

population studies (Bain et al, 1988; Vermeulen and Verdonck, 1992; Schrader et al, 1993; Bjornerem et al, 2006). In addition, requiring multiple blood samples from participants may result in a reduced participation rate and lower statistical power.

Concentrations of (unadjusted) urinary phthalate metabolites measured in the present study were compared with those among US males measured as part of the 2003–2004 National Health and Nutrition Examination Survey (NHANES) (CDC, 2008). Metabolite distributions were generally similar between the present study and the national data, although in the present study concentrations of MEP, MBP, and MBzP were slightly lower, and concentrations of MEHP, MEHHP, and MEOHP were somewhat higher. For example, the median and 95th percentile values for MEHP (unadjusted for SG) in the present study were 6.0 and 112 ng/ml, respectively, compared with 2.2 and 33.3 ng/ml in males from NHANES 2003–2004.

In conclusion, the present study found that urinary DEHP metabolites, at levels that are representative of those found among the general US population, may be associated with altered steroid hormone levels and perhaps aromatase activity. Additional work is needed to confirm these findings and determine clinical implications of subclinical alterations in hormone levels in adult men following exposure to environmental EDCs.

Acknowledgments

The authors gratefully acknowledge research nurses Jennifer Ford and Myra Keller at MGH, the technical assistance of Manori Silva, Jack Reidy, Ella Samandar, and Jim Preau (CDC, Atlanta, Georgia) in measuring the urinary concentrations of phthalate metabolites, and Janna Frelich in data management.

References

Andersson AM, Jensen TK, Juul A, Petersen JH, Jorgensen T, Skakkebaek NE. Secular decline in male testosterone and sex hormone binding globulin serum levels in Danish population surveys. *J Clin Endocrinol Metab*. 2007;92(12):4696–4705.

Andrade AJ, Grande SW, Talsness CE, Grote K, Chahoud I. A dose-response study following in utero and lactational exposure to di-(2-ethylhexyl)-phthalate (DEHP): non-monotonic dose-response and low dose effects on rat brain aromatase activity. *Toxicology*. 2006;227(3):185–192.

ATSDR. *Toxicological Profile for Diethyl Phthalate (DEP)*. Atlanta, GA: Agency for Toxic Substances and Disease Registry; 1995.

ATSDR. *Toxicological Profile for Di-n-butyl Phthalate (DBP)*. Atlanta, GA: Agency for Toxic Substances and Disease Registry; 2001.

ATSDR. *Toxicological Profile for Di(2-ethylhexyl)phthalate (DEHP)*. Atlanta, GA: Agency for Toxic Substances and Disease Registry; 2002.

Bain J, Langevin R, D'Costa M, Sanders RM, Hucker S. Serum pituitary and steroid hormone levels in the adult male: one value is as good as the mean of three. *Fertil Steril*. 1988;49(1):123–126.

Bjornerem A, Straume B, Oian P, Berntsen GK. Seasonal variation of estradiol, follicle stimulating hormone, and dehydroepiandrosterone sulfate in women and men. *J Clin Endocrinol Metab*. 2006;91(10):3798–3802.

Blount BC, Milgram KE, Silva MJ, Malek NA, Reidy JA, Needham LL, Brock JW. Quantitative detection of eight phthalate metabolites in human urine using HPLC-APCI-MS/MS. *Anal Chem*. 2000;72(17):4127–4134.

Borch J, Metzdorff SB, Vinggaard AM, Brokken L, Dalgaard M. Mechanisms underlying the anti-androgenic effects of diethylhexyl phthalate in fetal rat testis. *Toxicology*. 2006;223(1–2):144–155.

Carreau S, Delalande C, Silandre D, Bourguiba S, Lambard S. Aromatase and estrogen receptors in male reproduction. *Mol Cell Endocrinol*. 2006;246(1–2):65–68.

CDC. *Third National Report on Human Exposure to Environmental Chemicals*. Washington, DC: Centers for Disease Control and Prevention; 2005.

CDC. NHANES 2003–2004 laboratory files, lab 24: urinary phthalates. Atlanta, GA: US Centers for Disease Control and Prevention; 2008 http://www.cdc.gov/nchs/about/major/nhanes/nhanes2003-2004/lab03_04.htm. Accessed June 27, 2008.

Corton JC, Bocos C, Moreno ES, Merritt A, Cattley RC, Gustafsson JA. Peroxisome proliferators alter the expression of estrogen-metabolizing enzymes. *Biochimie*. 1997;79(2–3):151–162.

David RM, McKee RH, Butala JH, Barter RA, Kayser M. Esters of aromatic mono-, di-, and tricarboxylic acids, aromatic diacids, and di-, tri-, or polyalcohols. In: Bingham E, Cohrssen B, Powell CH eds. *Patty's Toxicology*. New York, NY: John Wiley and Sons; 2001:635–932.

Davis BJ, Weaver R, Gaines LJ, Heindel JJ. Mono-(2-ethylhexyl) phthalate suppresses estradiol production independent of FSH-cAMP stimulation in rat granulosa cells. *Toxicol Appl Pharmacol*. 1994;128(2):224–228.

Duty SM, Calafat AM, Silva MJ, Ryan L, Hauser R. Phthalate exposure and reproductive hormones in adult men. *Hum Reprod*. 2005;20(3):604–610.

Fleming LE, Bean JA, Rudolph M, Hamilton K. Cancer incidence in a cohort of licensed pesticide applicators in Florida. *J Occup Environ Med*. 1999;41(4):279–288.

Foster PM. Disruption of reproductive development in male rat offspring following in utero exposure to phthalate esters. *Int J Androl*. 2006;29(1):140–147; discussion 181–185.

Fromme H, Bolte G, Koch HM, Angerer J, Boehmer S, Drexler H, Mayer R, Liebl B. Occurrence and daily variation of phthalate metabolites in the urine of an adult population. *Int J Hyg Environ Health*. 2007;210(1):21–33.

Ge RS, Chen GR, Dong Q, Akingbemi B, Sottas CM, Santos M, Sealfon SC, Bernard DJ, Hardy MP. Biphasic effects of postnatal exposure to diethylhexylphthalate on the timing of puberty in male rats. *J Androl*. 2007;28(4):513–520.

Hauser R, Meeker JD, Duty S, Silva MJ, Calafat AM. Altered semen quality in relation to urinary concentrations of phthalate monoester and oxidative metabolites. *Epidemiology*. 2006;17(6):682–691.

Hauser R, Meeker JD, Park S, Silva MJ, Calafat AM. Temporal variability of urinary phthalate metabolite levels in men of reproductive age. *Environ Health Perspect*. 2004;112(17):1734–1740.

Hauser R, Meeker JD, Singh NP, Silva MJ, Ryan L, Duty S, Calafat AM. DNA damage in human sperm is related to urinary levels of phthalate monoester and oxidative metabolites. *Hum Reprod*. 2007;22(3):688–695.

Hess RA, Bunick D, Lee KH, Bahr J, Taylor JA, Korach KS, Lubahn DB. A role for oestrogens in the male reproductive system. *Nature*. 1997;390(6659):509–512.

Hoppin JA, Brock JW, Davis BJ, Baird DD. Reproducibility of urinary phthalate metabolites in first morning urine samples. *Environ Health Perspect*. 2002;110(5):515–518.

Jonsson BA, Richthoff J, Rylander L, Giwercman A, Hagmar L. Urinary phthalate metabolites and biomarkers of reproductive function in young men. *Epidemiology*. 2005;16(4):487–493.

Kim HS, Saito K, Ishizuka M, Kazusaka A, Fujita S. Short period exposure to di-(2-ethylhexyl) phthalate regulates testosterone metabolism in testis of prepubertal rats. *Arch Toxicol*. 2003;77(8):446–451.

Kleinbaum DG, Kupper LL, Muller KE, Nizam A. *Selecting the Best Regression Equation. Applied Regression Analysis and Other Multivariate Methods.* 3rd ed. Pacific Grove, CA: Brooks/Cole Publishing Company; 1998.

Li LH, Jester WF Jr, Orth JM. Effects of relatively low levels of mono-(2-ethylhexyl) phthalate on cocultured Sertoli cells and gonocytes from neonatal rats. *Toxicol Appl Pharmacol*. 1998;153(2):258–265.

Liu PY, Handelsman DJ. The present and future state of hormonal treatment for male infertility. *Hum Reprod Update*. 2003;9(1):9–23.

Lovekamp TN, Davis BJ. Mono-(2-ethylhexyl) phthalate suppresses aromatase transcript levels and estradiol production in cultured rat granulosa cells. *Toxicol Appl Pharmacol*. 2001;172(3):217–224.

Main KM, Mortensen GK, Kaleva MM, Boisen KA, Damgaard IN, Chellakooty M, Schmidt IM, Suomi AM, Virtanen HE, Petersen DV, Andersson AM, Toppari J, Skakkebaek NE. Human breast milk contamination with phthalates and alterations of endogenous reproductive hormones in infants three months of age. *Environ Health Perspect*. 2006;114(2):270–276.

Meeker JD, Calafat AM, Hauser R. Di(2-ethylhexyl) phthalate metabolites may alter thyroid hormone levels in men. *Environ Health Perspect*. 2007;115(7):1029–1034.

Meeker JD, Singh NP, Hauser R. Serum concentrations of estradiol and free T4 are inversely correlated with sperm DNA damage in men from an infertility clinic. *J Androl*. 2008;29(4):379–388.

Noda M, Ohno S, Nakajin S. Mono-(2-ethylhexyl) phthalate (MEHP) induces nuclear receptor 4A subfamily in NCI-H295R cells: a possible mechanism of aromatase suppression by MEHP. *Mol Cell Endocrinol*. 2007;274(1–2):8–18.

Pan G, Hanaoka T, Yoshimura M, Zhang S, Wang P, Tsukino H, Inoue K, Nakazawa H, Tsugane S, Takahashi K. Decreased serum free testosterone in workers exposed to high levels of di-n-butyl phthalate (DBP) and di-2-ethylhexyl phthalate (DEHP): a cross-sectional study in China. *Environ Health Perspect*. 2006; 114(11):1643–1648.

Peck CC, Albro PW. Toxic potential of the plasticizer Di(2-ethylhexyl) phthalate in the context of its disposition and metabolism in primates and man. *Environ Health Perspect*. 1982;45:11–17.

Pentikainen V, Erkkila K, Suomalainen L, Parvinen M, Dunkel L. Estradiol acts as a germ cell survival factor in the human testis in vitro. *J Clin Endocrinol Metab*. 2000;85(5):2057–2067.

Pflieger-Bruss S, Schuppe HC, Schill WB. The male reproductive system and its susceptibility to endocrine disrupting chemicals. *Andrologia*. 2004;36(6):337–345.

Schiff JD, Ramirez ML, Bar-Chama N. Medical and surgical management male infertility. *Endocrinol Metab Clin North Am*. 2007;36(2):313–331.

Schrader SM, Turner TW, Breitenstein MJ, Simon SD. Measuring male reproductive hormones for occupational field studies. *J Occup Med*. 1993;35(6):574–576.

Silva MJ, Barr DB, Reidy JA, Malek NA, Hodge CC, Caudill SP, Brock JW, Needham LL, Calafat AM. Urinary levels of seven phthalate metabolites in the US population from the National Health and Nutrition Examination Survey (NHANES) 1999–2000. *Environ Health Perspect*. 2004b;112(3):331–338.

Silva MJ, Malek NA, Hodge CC, Reidy JA, Kato K, Barr DB, Needham LL, Brock JW. Improved quantitative detection of 11 urinary phthalate metabolites in humans using liquid chromatography-atmospheric pressure chemical ionization tandem mass spectrometry. *J Chromatogr B*. 2003;789(2):393–404.

Silva MJ, Slakman AR, Reidy JA, Preau JL Jr, Herbert AR, Samandar E, Needham LL, Calafat AM. Analysis of human urine for fifteen phthalate metabolites using automated solid-phase extraction. *J Chromatogr B*. 2004a;805(1):161–167.

Swan SH, Main KM, Liu F, Stewart SL, Kruse RL, Calafat AM, Mao CS, Redmon JB, Ternand CL, Sullivan S, Teague JL. Decrease in anogenital distance among male infants with prenatal phthalate exposure. *Environ Health Perspect*. 2005;113(8):1056–1061.

Teass AW, Biagini RE, DeBord DG, Hull RD. Application of biological monitoring methods. In: Eller PM, ed. *NIOSH Manual of Analytical Methods*. Cincinnati, OH: National Institute for Occupational Safety and Health; 1998;52–62.

Teitelbaum SL, Britton JA, Calafat AM, Ye X, Silva MJ, Reidy JA, Galvez MP, Brenner BL, Wolff MS. Temporal variability in urinary concentrations of phthalate metabolites, phytoestrogens and phenols among minority children in the United States. *Environ Res*. 2008;106(2):257–269.

Toft G, Hagmar L, Giwercman A, Bonde JP. Epidemiological evidence on reproductive effects of persistent organochlorines in humans. *Reprod Toxicol*. 2004;19(1):5–26.

Travison TG, Araujo AB, O'Donnell AB, Kupelian V, McKinlay JB. A population-level decline in serum testosterone levels in American men. *J Clin Endocrinol Metab*. 2007;92(1):196–202.

Vermeulen A, Verdonck G. Representativeness of a single point plasma testosterone level for the long term hormonal milieu in men. *J Clin Endocrinol Metab*. 1992;74(4):939–942.