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Abbreviations with definitions

AGD (anogenital distance), AGI (anogenital index), ASD (anoscrotal distance), BzBP (benzyl butyl phthalate), CDC (Centers for Disease Control and Prevention), CL (confidence limit), CV (coefficient of variation), DBP (dibutyl phthalate), DEP (diethyl phthalate), DEHP (di -2-ethylhexyl phthalate), DINP (di-iso-nonyl phthalate), FSH (follicular stimulating hormone), LOD (limits of detection), MBP (mono-n-butyl phthalate), MBzP (mono-benzyl phthalate), MCPP (mono-3-carboxypropyl phthalate), MEHHP (mono-2-ethyl-5-hydroxyhexyl phthalate), MEHP (mono-2-ethylhexyl phthalate), MEOHP (mono-2-ethyl-5-oxohexyl phthalate), MEP (mono-ethyl phthalate), MiBP (mono-isobutyl phthalate), MMP (mono-methyl phthalate), ng/mL (nanogram per milliliter), QC (quality control), SFF (Study for Future Families).

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Figure 1

Abstract

Prenatal phthalate exposure impairs testicular function and shortens anogenital distance (AGD) in male rodents. We present data from the first study to examine AGD and other genital measurements in relation to prenatal phthalate exposure in humans. A standardized measure of AGD was obtained in 134 boys 2-30 months of age. AGD was significantly correlated with penile volume ($R^2 = 0.24$, $p = 0.005$) and the proportion of boys with incomplete testicular descent ($R^2 = 0.23$, $p = 0.007$). We defined the anogenital index (AGI) as AGD divided by weight at examination (AGI = AGD [mm]/weight [kg]) and calculated the age-adjusted AGI by regression analysies. Nine phthalate monoester metabolites, measured in prenatal urine samples were examined as predictors of age-adjusted AGI in regression and categorical analyses that included all participants with urine samples (N=85). Urinary concentrations of four phthalate metabolites (mono-ethyl phthalate [MEP], mono-n-butyl phthalate [MBP], mono-benzyl phthalate [MBzP], and mono-isobutyl phthalate [MiBP]) were inversely related to AGI. After adjusting for age at examination, p-values for regression coefficients ranged from 0.012 (for MEP) to 0.055 (for MBzP). Comparing boys with prenatal MBP concentration in the highest quartile to those in the lowest quartile, the odds ratio for a shorter than expected AGI was 10.2 (95% confidence limits [CL] = 2.5–42.2). The corresponding odds ratios for MEP, MBzP and MiBP were 4.7, 3.8, and 7.3, respectively (all p-values<0.05). We defined a summary phthalate score to quantify joint exposure to these four phthalate metabolites. The age-adjusted AGI decreased significantly with increasing phthalate score (p-value for slope = 0.012). The associations between male genital development and phthalate exposure seen here are consistent with the phthalate-related syndrome of incomplete virilization that has been reported in prenatally exposed rodents. The median concentrations of phthalate metabolites that are associated with short AGI and incomplete testicular descent are below those found in one-quarter of the female population of the United States, based on a nation-wide sample. These data support the hypothesis that prenatal phthalate exposure at environmental levels can adversely affect male reproductive development in humans.

Introduction

Diesters of phthalic acid, commonly referred to as phthalates, are widely used in industry and commerce; they are used in personal care products (e.g. makeup, shampoo and soaps), plastics, paints and some pesticide formulations. Consistent toxicologic evidence indicates as association between several of these phthalate esters and reproductive effects. In particular, dibutyl phthalate (DBP), benzylbutyl phthalate (BzBP), di-2-ethylhexyl phthalate (DEHP) and di-isononyl phthalate (DINP) have been shown to disrupt reproductive tract development in male rodents in an anti-androgenic manner (Parks et al. 2000). Recent studies have reported significant reductions in anogenital distance (AGD) in Sprague-Dawley rats after prenatal exposure at high doses to BzBP (Nagao et al. 2000; Tyl et al. 2004), DBP (Barlow and Foster 2003; Foster et al. 2000) and DEHP (Gray, Jr. et al. 2000; Parks et al. 2000).

Despite the growing body of literature on phthalate reproductive toxicity and data demonstrating extensive human exposure (Silva et al. 2004a), few studies have examined the effects of these chemicals on human reproductive development. In 2000 Colon (Colon et al. 2000) reported elevated levels of several phthalates (including DEP, DBP and DEHP) in serum samples from young girls with premature breast development. However, the timing of exposure was unknown and high exposure levels may have reflected phthalate contamination of serum samples (McKee and Toxicology Research Task Group 2004). Until recently, the only study of humans to evaluate phthalate exposure and male reproductive toxicity measured phthalate diesters in semen. As with the Colon study, contamination from diesters in laboratory equipment could not be excluded (Murature et al. 1987).

More recent studies have examined phthalate monoester metabolites in urine. Because urinary metabolites are not likely to be present as the result of contamination, these studies avoid this potential source of measurement error. Duty et al. reported dose-response relationships between tertiles of mono-butyl phthalate and sperm motility and sperm concentration, and between tertiles of mono-benzyl phthalate (MBzP) and sperm concentration (Duty et al. 2003a). They also reported inverse dose-response relationships between mono-ethyl phthalate (MEP) and sperm DNA damage measured using the neutral single-cell gel electrophoresis (Comet) assay (Duty et al. 2003b). In this population of men attending an infertility clinic, increased urinary concentration of MBzP was also associated with decreased follicle stimulating hormone

(FSH), while increases in mono-butyl phthalate were marginally associated with increased inhibin-B (Duty et al. 2005).

Newborn male rodents have no scrotum and the external genitalia are undeveloped; only a genital tubercle is apparent for both. The distance from the anus to the insertion of this tubercle, the AGD, is androgen-dependent and about twice as long in males as females. The AGD has been shown to be a sensitive measure of prenatal anti-androgen exposure (Rhees et al. 1997). Recently, Salazar-Martinez (Salazar-Martinez et al. 2004) studied AGD in 45 male and 42 female infants. They measured the distance from the anus to the base of the scrotum in males and from the anus to the base of the genitals (the fourchette) in females. By these measures, AGD was sexually dimorphic and about twice as long in males as females. No other studies have examined AGD among human males, although two other studies have evaluated AGD in female infants (Callegari et al. 1987; Phillip et al. 1996).

Materials and Methods

Study Participants

Women included in our study were originally recruited into the first phase of Study for Future Families (SFFI), a multi-center pregnancy cohort study, at prenatal clinics in Los Angeles, CA (Harbor-UCLA and Cedars-Sinai), Minneapolis, MN (University of Minnesota Health Center) and Columbia, MO (University Physicians), from September 1999 through August 2002. Data collection is still ongoing in IA, where a center was added late in SFFI, so Iowa City, IA participants are not included in this analysis. Methods are described in detail elsewhere (Swan et al. 2003). 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, most gave blood samples and, after urine collection was added midway through the study, most also gave a urine sample.

Eight-five percent of SFFI participants agreed to be recontacted and we invited these mothers to take part in our follow-up study. The family was eligible for the follow-up study (SFFII) if the pregnancy ended in a live birth, the baby was 2-36 months of age, lived within 50 miles of the clinic, and could attend at least one study visit. Here we report on results from the first study visit only. Human Subject Committees at all

participating institutions approved SFFI and SFFII and all participants signed informed consents for each study.

Physical Examination

After obtaining standard anthropometric measurements (height, weight, head circumference and skin-fold thickness), a detailed examination of the breast and genitals was conducted under the supervision of pediatric physicians who were trained in its administration. Every attempt was made to standardize the examination, which was developed specifically for this study. These methods included training sessions before and during the study and the use of standardized equipment. Neither the pediatric physicians nor the support staff had any knowledge of the mother's phthalate concentrations.

Boys' genital examinations included a description of the testes and scrotum, location and size of each testicle, and measurement of the penis. The placement of each testicle was initially coded in six categories; in the current analysis boys are dichotomized into those with normal (placement of both testes coded as normal or normal retractile) or those with incomplete testicular descent (all other cases). The scrotum was categorized as distinct from surrounding tissue or not and by size (small or not). Penis width and (stretched) length were recorded and penile volume (proportional to $[\text{penile width}/2]^2 \times \text{penile length}$) was calculated. We recorded the anogenital distance (AGD), measured from the center of the anus to the anterior base of the penis. We also recorded the anoscrotal distance (ASD), measured from the center of the anus to the posterior base of the scrotum. This latter measurement was used by Salazar and colleagues (Salazar-Martinez et al. 2004) who refer to it as anogenital distance.

Phthalate Metabolite Analysis

Urinary phthalate metabolite analyses were carried out by the Division of Laboratory Sciences, National Center for Environmental Health (NCEH), Centers for Disease Control and Prevention (CDC), which had no access to participant data. The analytical approach for the analysis of urinary phthalate metabolites (Silva et al. 2004b) is a modification of previously published methods (Silva et al. 2003). The analysis involves the enzymatic deconjugation of the phthalate metabolites from their glucuronidated form, automated on-line solid-phase extraction, separation with high performance liquid chromatography, and detection by isotope-

dilution tandem mass spectrometry. This high throughput method allows for the simultaneous quantification in human urine of the nine phthalate metabolites reported in this work. Limits of detection (LOD) are in the low nanogram per milliliter (ng/mL) range. Isotopically labeled internal standards were used along with conjugated internal standards to increase precision and accuracy of the measurements. The method is accurate (spiked recoveries are near 100%), and precise with between-day relative standard deviations of less than 10%. Quality control (QC) samples and laboratory blanks were analyzed along with unknown samples to monitor performance of the method. The metabolite concentrations reported here are from 85 prenatal maternal urine samples of a total of 214 that also included post-natal maternal and baby samples from the same mothers and their children. The 214 samples were analyzed for phthalate metabolites in six batches, none of which had to be re-extracted for QC failures. Of the 214 samples, seven were re-extracted using less than 1 milliliter of urine because concentrations of MEP calculated using 1 mL were above the linear range of the method.

Statistical Analysis

After examining descriptive and summary statistics for all study variables, we explored models for AGD. We fit several alternative measures of body size (weight, height and body mass index) and both additive and multiplicative functions of these. We defined the anogenital index (AGI = AGD [mm] /weight [kg]) as a weight-normalized index of AGD.

AGD and AGI were modeled as both linear and quadratic functions of age. For babies born at less than 38 weeks, age at examination in the first year was calculated from the estimated date of conception instead of the birth date. Once the best fitting model was identified, we plotted the expected AGI and its 25th and 75th percentiles as a function of age. We categorized boys in two ways; we dichotomized boys into those with AGI smaller than, or at least as large as, expected. We also used the difference between observed and expected AGI to define three groups of boys; short AGI (AGI <25th percentile for age), intermediate (25th percentile $\leq$ AGI <75th percentile), and long (AGI $\geq$ 75th percentile for age). We also calculated the proportion of boys in these three groups with normal testicular descent (both testes normal or normal retractile) and normal scrotal (scrotum of normal size and distinct from surrounding tissue). We calculated the correlations between AGD and AGI and penile volume, testicular placement and scrotal

parameters (size and distinctness from surrounding tissue). Our decision to use AGI as the measure of genital development was made, and cut points for categorical analyses of outcomes were selected, prior to obtaining phthalate metabolite values.

We used General Linear Models to explore the relationships between phthalate metabolite concentration (unadjusted for urine concentration) and genital parameters. Most metabolite concentrations were above the LOD; those below the LOD were assigned the value LOD/square root (2), which has been recommended when the data are not highly skewed, as was the case here (Hornung and Reed 1990). Metabolite concentrations were logarithmically transformed to normalize distributions. We examined several potentially confounding factors including mother's ethnicity and smoking status, time of day and season in which the urine sample was collected, gestational age at sample collection and baby's weight at examination.

We also categorized metabolite concentrations into low (<25th percentile), intermediate (between the 25th and 75th percentiles) and high (≥75th percentile) and examined the odds ratio for smaller than expected AGI for babies with high compared to low exposure. On the basis of these regression and categorical analyses we identified the phthalate metabolites most strongly associated with AGI. We refer to these as AGI-associated phthalates.

Because phthalate metabolite concentrations are highly correlated, and because our limited sample size prohibited us from examining multi-way interactions, we constructed a summary phthalate score to examine the effect of joint exposure to more than one AGI-associated phthalate. For this purpose, we used quartiles of metabolite concentration; values in the lowest quartile did not contribute to the sum, while higher values increased the sum one unit per quartile. We divided this sum into three categories; low (reflecting little or no exposure to AGI-associated phthalates), intermediate, and high (high exposure to all, or almost all, AGI-associated phthalates). We examined the magnitude of the residual (observed–expected) AGI as a function of this summary phthalate score.

Results

The population for the current analysis was identified from families recruited in CA, MN or MO for whom data entry was complete by December 17, 2004, the cutoff date for the current analysis. At that time, 654 participants from these three centers had completed SFFI and given permission to be recontacted. Of these,

477 (72.9%) were eligible for SFFII and 346 (72.5%) participated (Table 1). SFFII participants were demographically similar to non-participants except that non-participants were more likely to be Hispanic because of a lower eligibility rate (60%) in CA, where the majority of participants were Hispanic. Of the 172 boys born to these mothers, five boys in twin births were excluded, leaving 156 boys with data from the first examination. Of these, AGI was not recorded for 23; two mothers declined the genital exam and the remainder were older boys (mean age 19.6 months) for whom the study examiner felt the measurement was not reliable, usually because of the boys' activity level. The remaining 134 boys comprise the sample used for the analysis of AGD and other genital measurements.

Among the 134 boys for whom we have genital measurements, no frank genital malformations or disease were detected and no parameters appeared grossly abnormal. The mean age at first examination was 15.9 months and mean weight was 10.5 kg (Table 2). Mean AGD was 69.9 mm (standard deviation 11.7 mm) with a distribution that was well approximated by a normal curve. Overall, 86.6% of boys had both testes classified as normal or normal-retractile.

A prenatal urine sample was assayed for phthalate metabolites for mothers of 85 of these boys. These mother-son pairs comprise the data set for the analysis of AGD and phthalate metabolite concentration. Since urine collection began midway through SFFI, mothers with a stored urine sample were recruited later in the study, and their sons tended to be younger at examination; mean age 12.6 months (interquartile range 5-16 months). Summary statistics for all boys included in the analysis of physical measurements and the subset of boys for whom mothers' prenatal phthalate concentrations were also available are shown separately in Table 2.

All phthalate metabolites tested were above the LOD in ≥ 49% of women, and most were above the LOD in > 90% of samples (Table 3). Concentrations spanned four orders of magnitude, from below the LOD (estimated value = 0.71 ng/mL) to 13,700 ng/mL for MEP. Means ranged from 2.68 for mono-3-carboxypropyl phthalate (MCPP) to 629.8 for MEP. All metabolites were highly correlated. In particular, the pair-wise correlations for MBP, MBzP, and MiBP were between 0.64 and 0.73 (all p-values <0.0001).

Regression Analyses

We initially modeled AGD as a linear function of age and weight, but this model fit poorly (adjusted R^2 =

22%). We found that using anogenital index (AGI = AGD [mm] /weight [kg]) as a function of age provided the best fit, as has been shown in rodent models (Vandenbergh and Huggett 1995). The best fitting model for AGI includes linear and quadratic terms for age and is given by: AGI = 10.9335 – 0.3927 (age) + 0.0072 (age^2) (adjusted $R^2 = 0.60$). Using this model, we calculated mean AGI and its 5th, 25th, 75th and 95th percentiles (Figure 1).

We then examined models that included individual phthalate metabolites. Other than age and age^2, no covariates altered regression coefficients for the phthalate metabolites by more than 15% and none were included in final models. All regression coefficients for individual metabolites (logarithmically transformed to normalize distributions) were negative (Table 4). MEP, MBP, MBzP and MiBP were (inversely) related to AGI; p-values for regression coefficients were between 0.012 and 0.055. We also measured three metabolites of DEHP. While the hydrolytic monoester metabolite, mono-2-ethylhexyl phthalate, (MEHP) was unrelated to AGI (regression coefficient -0.09, 95% CL [–0.60, 0.42]), regression coefficients for the oxidative monoester metabolites of DEHP, mono-2-ethyl-5-oxohexyl phthalate (MEOHP) and mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP) were of magnitude comparable to those for MEP and MBzP, and of borderline statistical significance (p = 0.08 and 0.10 for MEOHP and MEHHP, respectively). AGI appeared to be independent of the concentrations of mono-methyl phthalate (MMP) and mono-3-carboxypropyl phthalate (MCPP), metabolites of di-methyl phthalate and di-n-octyl phthalate, respectively.

Categorical Analyses

The twenty-four boys with AGI below the 25th percentile for age were classified as having a short AGI. This group had an AGI that was, on average, 19% (range 9% - 54%) shorter than expected based on the final regression model. Boys with AGI $\geq$75th percentile of expected were classified as having a long AGI, while those with AGI between the 25th and 75th percentile of expected, were considered intermediate. Boys' weight and age did not differ appreciably among these groups.

Table 5 shows mean and median values for the AGI-associated metabolites for boys in the short, intermediate, and long categories of AGI. We calculated the odds ratios for short AGI for each monoester metabolite (Table 6). For high compared to low concentration of MBP, the odds ratios for a short AGI was 10.2 (95% CL [2.5, 42.2]), while for medium concentration compared to low it was 3.8 (95% CL [1.2 –

12.3]). The corresponding OR for high compared to low concentration of MEP, MBzP and MiBP were 4.7, 3.8 and 7.3, respectively (all p-values <0.05).

Other Genital Parameters

Testicular descent was associated with AGI. The proportions of boys with one or both testicles incompletely descended were 20.8%, 8.9% and 6.7% for boys classified as having short, intermediate, and long AGI (p-value for short AGI compared to all other boys <0.001). The proportion of boys with a scrotum categorized as small and/or "not distinct from surrounding tissue" was also elevated for boys with short AGI ($p < 0.001$). AGD was significantly associated with penile volume; $R^2 = 0.24$ ($p = 0.006$) and penile volume divided by weight was correlated with AGI ($R^2 = 0.41$, $p = 0.001$). Testicular volume, which was measured by orchidometer, is not shown here since participating physicians considered the measurement to be unreliable – a decision made prior to analyses of phthalate exposure.

Anoscrotal distance (ASD) was on average 55% as long as AGD, and these two measurements were correlated ($R^2 = 0.34$, $p < 0.0001$). However, the model predicting ASD as a function of baby's age and weight fit poorly (adjusted $R^2 = 0.09$). The model using ASD/weight as a function of age and age-squared was better (adjusted $R^2 = 0.45$), but did not fit as well as the model using AGI ($R^2 = 0.60$). ASD/weight was associated with MEP concentration (regression coefficient = –0.357, 95% CL [–0.664, –0.049]). For the other phthalate metabolites, regression coefficients were only 25-50% as large as those for AGI and none approached statistical significance (all p-values between 0.21 and 0.48).

Summary Phthalate Score

We used the summary phthalate score (see definition in *Statistical Analysis*) to study the effect of joint exposure to more than one AGI-associated phthalate. The summary phthalate score was inversely related to the proportion of boys with short AGI ($p = 0.012$). Of the 10 boys whose phthalate scores were high (score = 11-12), all but one had a short AGI. Conversely, of the 11 boys whose score were low (score = 0 or 1) only one had a short AGI. The odds ratios for having a short AGI for high summary phthalate score compared to low (OR = 90.0, 95% CL [4.88 – 1659]), and high compared to medium (32.1, 95% CL [3.75, 276]) were large and significant, although the confidence intervals were very wide. These data are shown graphically in

Figure 1, where boys with a high summary phthalate score are shown in red, those with a low score in blue, and the remainder in gray.

Discussion

In the recent National Health and Nutrition Examination Survey (NHANES 1999–2000), the majority of the general population in the United States had measurable exposure to multiple phthalates (Centers for Disease Control and Prevention 2003; Silva et al. 2004a). The samples in the present study and in NHANES were both analyzed using comparable methods and standards by the same laboratory, although the specific metabolites that were measured in the two studies differed somewhat. We compared the medians and 75th percentiles of the AGI-associated phthalate metabolite concentrations among two groups of mothers in our study; those whose boys fell in the short AGI group and all others, to females in the NHANES sample (Table 7). In the analysis of the NHANES samples, mono-butyl phthalate (MBP) includes both mono-n-butyl and mono-isobutyl phthalates, which were measured separately in our study. Metabolite concentrations for mothers of boys with short AGI were consistently higher than those of other mothers. Compared to women in the NHANES sample, MEP was slightly higher in our population, while concentrations of other metabolites were somewhat lower. Our population cannot be directly compared to NHANES; the proportion of pregnant women in the NHANES sample is unknown and age distributions differ. Nonetheless, these data demonstrate that the four AGI-associated phthalate metabolites are prevalent in the US female population, and levels were not unusually high among mothers whose sons had short AGI.

Thought not identical, AGD in pups is most similar to AGD as we defined it in this study. In rodents, AGD has been shown to be one of the most sensitive endpoints for phthalates, such as DBP (Mylchreest et al. 2000) and other anti-androgens such as flutamide (McIntyre et al. 2001; Barlow and Foster 2003) and finasteride (Bowman et al. 2003). It is difficult to compare the dose to humans from low level, ongoing, environmental exposure to that delivered to rodents experimentally in a narrow window of gestation. Nonetheless, it is likely that the doses to which our participants were exposed are lower than those used in toxicologic settings, suggesting that humans may be more sensitive to prenatal phthalate exposure than rodents. This greater sensitivity in humans has been observed for other toxicants. For example, humans are more sensitive to trenbolone by an order of magnitude (Neumann 1976). This greater sensitivity is thought to

be a result of rodents' higher metabolic rate and more rapid inactivation of toxicants, both of which have been shown to be inversely related to body size (White and Seymour 2005).

In light of the toxicologic literature for MBP, MBzP, and MiBP (Ema et al. 2003; Foster et al. 1980; Foster et al. 1981; Gray, Jr. et al. 2000; Nakahara et al. 2003), our data suggest that the endpoints affected by these phthalates are quite consistent across species. A boy with short AGI has, on average, an AGI that is 19% shorter than expected based on his age and weight as well as an increased likelihood of testicular maldescent, small and indistinct scrotum and smaller penile size. These changes in AGD and testicular descent are consistent with those reported in rodent studies following high dose phthalate exposure (Ema et al. 2003; Gray, Jr. et al. 2000; Mylchreest et al. 2000). The lack of association for MCPP and MMP, which have not been widely studied, is not inconsistent with the toxicologic literature.

With respect to DEP and its metabolite, MEP, we note that there are three other human studies suggesting reproductive toxicity (Duty et al. 2003b; Colon et al. 2000), (Main, unpublished data). It is, uncertain, therefore, whether the absence of data in rodents showing reproductive toxicity is the result of failure to detect it, unmeasured confounding in human studies, or interspecies differences in response to these compounds.

DEHP has been shown to shorten AGD (Gray, Jr. et al. 2000) and reduce testosterone (Parks et al. 2000). While MEHP was not associated with AGD in our data, the associations for the oxidative metabolites of DEHP (MEOHP and MEHHP) were of comparable magnitude to those for metabolites of DBP and BzBP, and of borderline statistical significance ($p = 0.08$ for MEOHP; $p = 0.10$ for MEHHP). Thus, it is unclear whether MEOHP and MEHHP are (inversely) associated with AGI, but associations are of borderline statistical significance because of our sample size, or whether human and rodent responses to this phthalate and its metabolites differ.

Masculinization of external male genitalia, represented by longer AGD, is controlled by dihydrotestosterone (Clark et al. 1990). Ema and colleagues demonstrated that this metabolite of testosterone is markedly decreased by prenatal administration of MBP, suggesting that MBP acts as an anti-androgen (Ema and Miyawaki 2001). AGD in male rodents is associated with other adverse developmental effects (Foster and McIntyre 2002) and some phthalate-induced changes have been shown to be permanent. For example, Barlow and colleagues (Barlow et al. 2004) report that prenatal exposure to 500 mg/kg/day of DBP

resulted in permanently decreased AGD and testicular dysgenesis. They also report that in utero DBP exposure induced proliferative Leydig Cell lesions. Follow-up of exposed children until adulthood will be required to determine whether long-term effects, including testicular dysgenesis, are seen in humans following prenatal phthalate exposure.

Two recent studies of the variability of phthalate monoester concentration in human samples suggest that phthalate concentration in humans is fairly stable, perhaps reflecting habitual use of phthalate-containing household and consumer products (Colon et al. 2000; Hauser et al. 2004; Hoppin et al. 2002). These studies lend support to the use of a single sample for exposure assessment. We obtained only a single prenatal urine sample from each woman, and most samples were obtained quite late in pregnancy (mean = 28.3 weeks). Therefore, the measured phthalate metabolite levels may not reflect exposure during the most sensitive developmental window, resulting in some degree of exposure misclassification. However, unless this misclassification varied systematically with outcome, such errors would bias the effect estimate towards the null. In fact, the categorical analysis, which should be less sensitive to such misclassification, showed stronger associations than the continuous analysis.

Our analysis is based on a single measure of AGD, and the reliability of this measurement in humans has not been established. During two training sessions, three study physicians each measured AGD in four male infants (mean age 8.1 months). The mean AGD for these measurements was 58.6 mm, standard deviation (within infant) 4.2 mm and coefficient of variation (CV) of 7.2%, suggesting that AGD can be measured reliably. Use of this measurement in larger studies in a range of diverse populations, with many more such training sessions, will be needed to obtain normative data.

While it might have been ideal to examine babies shortly after birth, the timing of grant funding did not allow this. Babies were born to SFFI mothers as early as January 2000 and the first baby visits did not occur until April 2002. To maximize the number of children participating we allowed recruitment over a range of ages. On the other hand, since the use of AGD in humans is new, the optimal timing for this measurement is not known. Our data suggest that measurements are reliable and informative in young children at least until 18 months, when AGD becomes more difficult to obtain reliably. Its value in adolescents and adults has yet to be determined.

We note that phthalate metabolite levels were highly correlated and the majority of women were exposed to all metabolites at detectable levels. Gray and colleagues suggested that risk assessments for phthalate-induced reproductive toxicity should consider phthalates as a group and include exposures from multiple sources (Gray, Jr. et al. 2000). The score we use reflects joint exposure to the four AGI-associated phthalates and our results suggest that joint exposure may convey greater than additive risk, but larger sample sizes are needed to confirm this.

Gray and Foster refer to a "phthalate syndrome" characterized by testicular, epididymal, and gubernacular cord agenesis as well as decreased AGD, and stress the importance of evaluating all components of a syndrome so that affected animals are not misidentified (Gray, Jr. and Foster 2003). It has recently been suggested (Fisher 2004) that this "phthalate syndrome" shares many features with the human Testicular Dysgenesis Syndrome (TDS) proposed by Skakkebaek to follow chemically-induced disruption of embryonic programming and gonadal development during fetal life (Skakkebaek et al. 2001). The current findings, though based on small numbers, provide the first data in humans linking measured levels of prenatal phthalates to outcomes that are consistent with this proposed syndrome.

This is the first study to look at subtle patterns of genital morphology in humans in relation to any prenatal exposure. It was motivated by toxicologic studies showing that genital morphology is altered by anti-androgens, including some phthalates. We report that AGI, the most sensitive marker of anti-androgen action in toxicologic studies, is shortened and testicular descent impaired, in boys whose mothers had elevated prenatal phthalate exposure. These changes in male infants, associated with prenatal exposure to some of the same phthalate metabolites that cause similar alterations in male rodents, suggest that commonly used phthalates may undervirilize humans as well as rodents.

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Table 1. Participants included in current analysis

	Number	% Potential participants	% Male babies
All pregnancy outcomes (CA, MN, and MO)			
Potential participants [(a)]	654	100%	-------
Eligible for SFFII	477	72.9%	-------
SFFII participant	346	72.5%	-------
Male babies only (CA, MN and MO)			
SFFII participant	176	------	100%
With AGD, age and weight [(b)]	134	------	78%
Prenatal urine sample[(c)]	85	------	49%

[(a)] A Potential Participant is an SFFI participant from CA, MO or MN who gave permission to be recontacted for future studies and for whom all study data were entered by December 17, 2004.

[(b)] Boys in twin births and boys with missing data or AGD measurements considered unreliable by pediatricians excluded.

[(c)] Urine collection began mid-way through SFFI.

Table 2. Characteristics of boys with complete physical examination

All boys (N=134)			Percentile		
	Mean	SD	25th	50th	75th
Age (months)	15.9	8.6	11.0	15.0	23.0
Height (cm)	79.1	10.6	72.6	80.0	87.2
Weight (kg)	10.5	2.7	8.7	10.7	12.3
Anogenital Distance (mm) [(a)]	69.9	11.7	63.8	70.2	76.6
Anogenital Index (mm/kg) [(b)]	7.0	1.9	5.7	6.7	7.8
Anoscrotal Distance (mm) [(c)]	37.6	7.8	31.4	36.9	43.5
Characteristics of boys whose mother's prenatal urine was assayed for phthalate metabolites (N=85)					
Age (months)	12.6	6.9	5.0	14.0	16.0
Height (cm)	75.6	9.5	66.5	77.6	82.0
Weight (kg)	9.7	2.4	8.4	10.0	11.1
Anogenital distance (mm) [(a)]	67.3	10.8	61.0	66.6	74.4
Anogenital index (mm/kg) [(b)]	7.3	1.9	6.1	7.0	8.2
Anoscrotal distance (mm) [(c)]	36.2	7.6	30.5	36.1	41.6

[(a)]Anogenital distance = Center of the anus to anterior base of the penis

[(b)]Anogenital index = Anogenital Distance/weight.

[(c)]Anoscrotal distance = Center of the anus to posterior base of the scrotum

Table 3. Percentiles of phthalate monoester metabolites.

	Percentile (ng/mL)			
Phthalate monoester metabolite[(a)]	25th	50th	75th	Percent > LOD[(b)]
MBP	7.2	13.5	30.9	96.5
MBzP	3.5	8.3	23.5	94.1
MCPP	0.7	2.1	3.6	69.4
MEP	53.3	128.4	436.9	97.6
MiBP	0.7	2.5	5.1	74.1
MMP	0.7	0.7	3.2	49.4
Metabolites of DEHP				
MEHHP	6.0	11.4	20.1	97.6
MEHP	1.3	3.3	9.0	77.6
MEOHP	5.1	11.1	19.0	94.1

[(a)] Abbreviations: MBP = Mono-n-butyl phthalate. MBzP = Mono-benzyl phthalate. MCPP = Mono-3-carboxypropyl phthalate. MEHHP = Mono-2-ethyl-5-hydroxyhexyl phthalate. MEHP = Mono-2-ethylhexyl phthalate. MEOHP = Mono-2-ethyl-5-oxohexy1 phthalate. MEP = Mono-ethyl phthalate. MiBP = Mono-isobutyl phthalate. MMP = Mono-methyl phthalate.

[(b)] Limit of detection (LOD) for all metabolites was between 0.95 and 1.07 ng/mL.

Table 4. Regression analyses of anogenital index on monoester metabolite concentration controlling for age and age-square

Monoester metabolite [(a)]	Log_{10} monoester metabolite concentration		
	Coefficient (SD)	P-value	95% Confidence limits
MBP	–0.657 (0.284)	0.023	(–1.222, –0.092)
MBzP	–0.475 (0.244)	0.05	(–0.962, 0.011)
MEHHP	–0.472 (0.285)	0.102	(–1.039, 0.096)
MEHP	–0.091 (0.255)	0.772	(–0.598, 0.416)
MEOHP	–0.477 (0.272)	0.084	(–1.019, 0.065)
MEP	–0.444 (0.173)	0.012	(–0.788, –0.100)
MiBP	–0.737 (0.292)	0.014	(–0.318, –0.156)
MMP	–0.513 (0.338)	0.133	(–1.187, 0.160)

[(a)] Abbreviations: MBP = Mono-n-butyl phthalate. MBzP = Mono-benzyl phthalate. .MCPP = Mono-3-carboxypropyl phthalate. MEHHP = Mono-2-ethyl-5-hydroxyhexyl phthalate. MEHP = Mono-2-ethylhexyl phthalate. MEOHP = Mono-2-ethyl-5-oxohexy1 phthalate. MEP = Mono-ethyl phthalate. MiBP = Mono-isobutyl phthalate. MMP = Mono-methyl phthalate.

Table 5. Mean (*median*) phthalate monoester metabolite levels by anogenital index category.

	Anogenital index category		
Monoester metabolite [a]	Long [b] (n = 15) Mean (*median*) (ng/mL)	Intermediate [c] (n = 46) Mean (*median*) (ng/mL)	Short [d] (n = 24) Mean *(median)* (ng/mL)
MBP	14.0 *(11.5)*	21.3 *(13.0)*	39.6 *(22.3)*
MBzP	11.6 *(8.3)*	13.6 *(6.8)*	28.2 *(13.7)*
MEP	139 *(83.6)*	537 *(115)*	1152 *(251)*
MiBP	2.5 *(2.2)*	3.5 *(2.1)*	7.4 *(4.4)*

[a] Abbreviations: MBP = Mono-n-butyl phthalate. MBzP = Mono-benzyl phthalate. MEP = Mono-ethyl phthalate. MiBP = Mono-isobutyl phthalate.

[b] Long AGI: AGI $\geq$75th percentile of expected AGI.

[c] Intermediate AGI: 25th percentile$\leq$ AGI < 75th percentile of expected AGI.

[d] Short AGI: AGI < 25th percentile of expected AGI.

Table 6. Odds ratio (OR) and 95% confidence limits (CL) for AGI less than expected from regression model by monoester metabolite level.

Monoester metabolite	Level	AGI < expected	AGI ≥ expected	OR	95% CL
MBP	Low (<25th)	5	15		Referent
	Med (≥25th and <75th)	24	19	3.8	(1.2, 12.3)
	High (≥75th)	17	5	10.2	(2.5, 42.2)
MBzP	Low (<25th)	6	13		Referent
	Med (≥25th and <75th)	26	18	3.1	(1.001, 9.8)
	High (≥75th)	14	8	3.8	(1.03, 13.9)
MEP	Low (<25th)	7	14		Referent
	Med (≥25th and <75th)	25	19	2.6	(0.9, 7.8)
	High (≥75th)	14	6	4.7	(1.2, 17.4)
MiBP	Low (<25th)	7	15		Referent
	Med (≥25th and <75th)	21	19	2.5	(0.8, 7.4)
	High (≥75th)	17	5	7.3	(1.9, 27.9)

[(a)] Abbreviations: MBP = Mono-n-butyl phthalate. MBzP = Mono-benzyl phthalate. MEP = Mono-ethyl phthalate. MiBP = Mono-isobutyl phthalate.

Table 7. Concentrations of four phthalate metabolites in three groups of women (ng/mL)

Metabolite [a]	Percentile	This Study		NHANES [b]
		Short AGI	Others	
MBP	50th	22.3	12.6	30.0
	75th	47.3	28.3	59.5
MBzP	50th	13.7	7.7	16.0
	75th	36.7	19.1	35.8
MEP	50th	251	93	174
	75th	769	276	425
MiBP	50th	4.4	2.1	[c]
	75th	11.8	4.3	[c]

[a] Abbreviations: MBP = Mono-n-butyl phthalate. MBzP = Mono-benzyl phthalate. MEP = Mono-ethyl phthalate. MiBP = Mono-isobutyl phthalate.

[b] Females only (Centers for disease Control and Prevention, 2003)

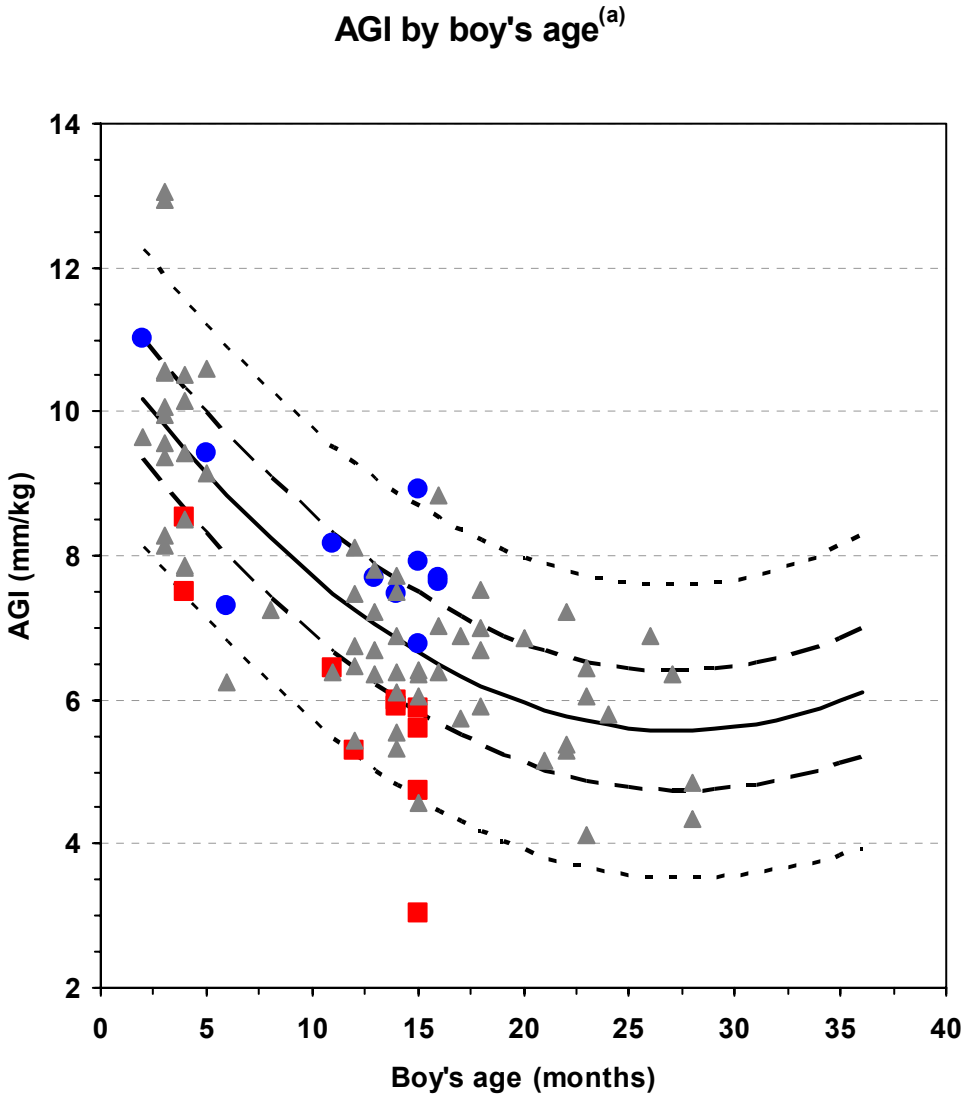
[c] MBP in the NHANES analysis includes both mono-n-butyl and mono-isobutyl phthalates; in this study these metabolites were measured separately.

Figure 1 Legend

Title: Mean Anogenital index (mm/kg) in relation to boys' age at examination (months)

Legend:

- Blue circle: Summary phthalate score 0-1
- Grey triangle: Summary phthalate score 2-10
- Red square: Summary phthalate score 11-12



(a)AGI = distance from anus to base of penis (mm) / weight (kg)

- Summary phthalate score 0-1
- Summary phthalate score 2-10
- Summary phthalate score 11-12