Urinary Phthalates From 168 Girls and Boys Measured Twice a Year During a 5-Year Period: Associations With Adrenal Androgen Levels and Puberty

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Background: Little is known about the possible deleterious effects of phthalate exposure on endogenous sex steroid levels in children.

Objective: Our objective was to investigate whether urinary phthalate metabolite levels are associated with circulating adrenal androgen levels and age at puberty.

Methods: This was a longitudinal study of 168 healthy children (84 girls) examined every 6 months for 5 years. Serum levels of dehydroepiandrostenedione sulfate (DHEAS), Δ4-androstenedione, testosterone, and urinary morning excretion of 14 phthalate metabolites, corresponding to 7 different phthalate diesters were determined. A variation in urinary excretion of phthalates was evident in each child, which made a mean of repetitive samples more representative for long-term excretion than a single determination.

Results: We found that girls with excretion of monobutyl phthalate isomers (MBP) and di(2-ethylhexyl) phthalate metabolites above the geometric group mean (795 and 730 ng/kg, respectively) had lower levels of DHEAS and Δ4-androstenedione, although statistically significant only at 13 years of age. In boys, we found that excretion of monobenzyl phthalate above the geometric group mean (346 ng/kg) was associated with lower levels of DHEAS at 11 years of age but higher levels of testosterone at 13 years of age. The same trend was observed for MBP excretion, albeit not statistically significant. A lower age at pubarche was observed in boys with excretion of MBP above the geometric group mean (11.0 vs 12.3 years, \( P = 0.005 \)).

Conclusion: Our data indicate that exposure to dibutyl phthalate isomers (DBP) (in girls) and butylbenzyl phthalate (in boys) are negatively associated with adrenal androgen levels and in boys positively associated with testosterone level at 13 years of age. High exposure to DBP was associated with earlier age at pubarche in boys. In girls, no associations between phthalate exposure and age at pubertal milestones were observed. (J Clin Endocrinol Metab 98: 3755–3764, 2013)
We hypothesize that an antiandrogenic effect on pubertal timing in humans could accelerate breast development in girls and delay pubic hair development in both girls and boys.

In humans, association between phthalate exposure and breast development in girls has been reported in some studies (17–19), whereas other studies could not confirm this (20, 21). Studies of phthalate exposure in boys are sparse; one study has shown association between phthalate exposure and gynecomastia in pubertal boys (22), although we could not confirm this in another study, which also could not find association between phthalates and testicular growth (23). Interestingly, we recently found a significant trend toward association between high phthalate urinary excretion levels in girls and later age at pubarche (24), as also reported by Wolff et al (25). We have reported results from a longitudinal study of 168 healthy children (84 girls) (5.9–12.8 years at baseline) examined for signs of puberty every 6 months for 5 years including collection of blood for determination of hormones as serum dehydroepiandrostenedione sulfate (DHEAS) and A4-androstenedione (Adione) and morning urine samples for determination of phthalate metabolites.

**Subjects and Methods**

**Subjects**

A total of 208 children from the Copenhagen Puberty Study (5, 8), of which the longitudinal part has earlier been described in detail (27). The study was conducted at 2 schools in the Copenhagen area during the years 2006 through 2010. Participants of non-Caucasian origin (17 girls and 11 boys) or with chronic illness (1 girl) were excluded from the analyses in this study. Only children with a minimum of 2 urine samples, each containing 50 to 750 mL collected in a period of 5 to 15 hours from last voiding, were included in this study, which resulted in 168 children (84 girls) aged 5.9 to 12.8 years at first examination. The children were examined every 6 months. No previous medical history associated with altered pubertal timing or intake of medications was reported in these children. Other aspects from this study have previously been published (23, 24, 27–29).

**Clinical examination**

Pubertal stages were evaluated by clinical examination according to Marshall and Tanner (30, 31). All evaluations of puberty in the girls were done by 2 female pediatricians, and all evaluations in boys were done by 3 male pediatricians. Breast stage (B1–B5) was assessed by palpation, and testicular volume (TV) was measured by palpation to the nearest milliliter using a Prader orchidometer. In case of discrepancy between left and right side, the largest measurement was used for classification. Assessment of pubic hair staging (PH1–PH5 in girls and PH1–PH6 in boys) was done by inspection. Pubertal onset was defined as breast stage at least B2 in girls (pubarche), pubic hair stage at least PH2 (pubarche) in girls and boys, and TV > 3 mL in boys. Gonadarche was defined as secondary sex characteristics due to gonadal activation (ie, B2 [pubarche] in girls and TV > 3 mL [testicular growth] in boys). If breast tissue was palpated at an earlier examination but not present at the subsequent examination, the girl was graded as B1 (no breast development, n = 6). Age at onset of pubic hair (PH2 +), breast tissue (B2 +), or TV > 3 mL was assigned as the mean age between age at first examination in stage 2 and the latest examination in stage 1. In 3 girls and 4 boys, the age at PH2 + was measured with 6 months accuracy because there was a year between examinations. Weight was measured to the nearest 0.1 kg using a digital electronic scale (Seca Delta, model 707; Seca).

**Laboratory analyses**

**Urinary analyses**

Each of the children collected first morning urine samples in a polyethylene container on the day of examination. When received, the samples were immediately weighed, and 15 mL was stored in scintillation vials at −20°C until chemical analysis of 14 metabolites from 7 different phthalate diesters: diethyl phthalate (DEP), di-n-butyl phthalate, di-iso-butyl phthalate, butylbenzyl phthalate, di-n-pentyl phthalate, di-n-octyl-phthalate, di-2-ethylhexyl phthalate (DEHP), and di-iso-nonyl phthalate (DiNP). Urine samples were analyzed for total content (free and glucuronidated) of monoethyl phthalate (MEP), mono-n-butyl phthalate (MnBP), mono-iso-butyl phthalate (MiBP), monobenzyl phthalate (MBzp), monopentyl phthalate (MPP), mono-2-ethylhexyl phthalate (MEHP), mono(2-ethyl-5-hydroxyhexyl) phthalate, mono(2-ethyl-5-oxohexyl) phthalate, mono(2-ethyl-5-carboxypentyl) phthalate, mono-n-octyl-phthalate (MOP), and mono-iso-nonyl phthalate (MiNP), mono-hydroxy-isononyl phthalate, mono-oxo-iso-nonyl phthalate, and mono-carboxyoisoocetyl phthalate by liquid chromatography-mass spectrometry with preceding enzymatic deglucuronidation followed by solid-phase extraction. The method for preparation of standards, standard solutions, and quality controls as well as the instrumental analysis and general method validation has previously been described in detail (21). In the present study, samples were analyzed in 29 batches during a period of 15 months. In short, each batch included standards for calibration curves, about 35 unknown samples, 2 blanks, 2 urine pool controls, and 2 urine pool controls spiked with phthalate standards at low level. The interday variation, expressed as the relative SD was <10% for all analytes except MBzp (13%) and MPP (15%), the recovery of spiked samples was >95% for all analytes except MnBP (89%), MBzp (91%), and MPP (91%). Limit of detection (LOD) was below 1 ng/mL for all analytes except MnBP (1.1 ng/mL), MnBP (1.4 ng/mL), and MBzp (1.1 ng/mL).

**Blood analyses**

Blood samples were drawn from an antecubital vein between 8:00 and 10:00 AM. They were clotted and centrifuged, and serum was stored at −20°C until hormone analyses were performed. Serum FSH and LH were measured by time-resolved immunofluorometric assays (Delfia) with detection limits of 0.06 and 0.05 IU/L for FSH and LH, respectively. Intra- and interassay coefficients of variation (CV) were less than 5% in both gonadotropin assays. Estradiol levels were determined by RIA (Pantex) with detection limit of 18 pmol/L, and the intra- and interassay CV were 7.5% and 12.3%, respectively. Testosterone levels were measured with the DPC Coat-A-Count RIA kit.
Diagnostic Products Corp) with detection limit of 0.23 nmol/L and the intra- and interassay CV were 7.6% and 8.6%, respectively. These assays have been validated in our lab previously. DHEAS and Adione levels were measured by specific solid-phase, competitive chemiluminescent enzyme immunoassays (Immuno 2000; Siemens Healthcare Diagnostics) with detection limits of 0.41 and 1.04 nmol/L, respectively. These assays have been validated in our lab previously.

DHEAS and Adione levels were measured by specific solid-phase, competitive chemiluminescent enzyme immunoassays (Immuno 2000; Siemens Healthcare Diagnostics) with detection limits of 0.41 and 1.04 nmol/L, respectively. The intra- and interassay CV were 6.3% to 7.1%, 7.8% to 10.2%, 7.1% to 10.8%, and 11.0% to 14.9%, respectively (27).

Statistical analysis

To simplify the data analyses, MiBP and MnBP were combined and expressed as the sum of monobutyl phthalate (MBP) isomers ($\Sigma$MBP$_{i+n}$). The molar sum of the DEHP metabolites (MEHP, mono[2-ethyl-5-hydroxyhexyl] phthalate, mono[2-ethyl-5-oxohexyl] phthalate, and mono[2-ethyl-5-carboxypentyl] phthalate) and the molar sum of the DiNP metabolites (MiNP, mono-hydroxy-isononyl phthalate, mono-oxo-isononyl phthalate, and mono-carboxy-isoctyl phthalate) were calculated and expressed as, respectively $\Sigma$DEHPm and $\Sigma$DiNPm in nanograms per milliliter by multiplying by the molecular weight of DEHP and DiNP, respectively. MEP, MOP, and MPP were all highly correlated (Spearman’s $\rho$ correlation coefficient ranging from 0.32–0.52, $P < .001$), and for statistical analysis, we calculated the molar sum of the correlated phthalate metabolites ($\Sigma$corr.phth.m), containing all measured metabolites except MEP, MOP, and MPP. Each phthalate concentration (nanograms per millilitre) was multiplied with the urine volume (milliliters) and divided by the body weight (kilograms) resulting in total amount excreted per kilogram body weight (nanograms per kilogram and nanomoles per kilogram). The phthalate metabolites and the sum of metabolites (nanograms per kilogram or nanomoles per kilogram) were ln transformed, and a mean of the samples for each individual was calculated, resulting in a geometric mean for each child. A group mean of the individual means was calculated in girls and in boys. Similar calculations were performed according concentration (nanograms per milliliter and nanomoles per milliliter) and total amount (nanograms and nanomoles).

Based on each child’s geometric mean excretion (nanograms per kilogram), the girls and the boys were categorized in high or low excretion groups, depending on whether individual mean excretion was above or below the group mean excretion in girls or in boys. Intra-individual as well as the overall variation were estimated by median-centered CV of untransformed data. To compare the girls and boys at an equal age in puberty, the girls and boys were grouped by age and puberty stage (Fig. 1).
were compared at the age of 10 years and the boys at the age of 11 years, because we have earlier reported a median age of 10.07 years for developing breast tissue and a median age of 11.48 years for testicular growth >3 mL in this cohort (27). Girls and boys were compared again in puberty, at 13 years of age, because 80 children (39 girls) reached that age before the end of the study period. Data are presented as medians. Groups were compared by Mann-Whitney U test (nonparametric test). All statistical analyses were carried out using the SPSS software version 19 (SPSS, Inc).

Ethical considerations

The Copenhagen Puberty Study was approved by the local ethical committee (KF01 282214 and V200.1996/90). The study is registered in ClinicalTrials.gov (identifier NCT01411527). All children and their parents gave informed consent.

Results

Phthalate excretion

All phthalate metabolites were detectable in all 788 urinary samples from the 168 children, except MOP (above LOD in only 4 girls and 15 boys), MPP (above LOD in 20 girls and 26 boys) and MiNP (above LOD in 82 girls and 83 boys [514 of 788 samples]). The geometric mean phthalate metabolite levels expressed as concentration (nanograms per milliliter and nmoles per milliliter), as total amount (nanograms and nanomoles), and as amount excreted per bodyweight (nanograms per kilogram and nanomoles per kilogram) are listed in Supplemental Table 1 (published on The Endocrine Society’s Journals Online web site at http://jcem.endojournals.org). Overall, urinary levels of phthalates were higher in boys compared with girls, statistically significant for MBzP, ΣDEHPm, and ΣDiNPm \( P \leq .001 \). ΣMBP\( (i+n) \) and ΣDEHP were the metabolites excreted in overall highest amounts in both girls and boys. The individual total absolute amount of each phthalate metabolite (nanograms) did not change with age. The phthalate concentration (nanograms per milliliter) as well as the total amount per body weight (nanograms per kilogram) decreased slightly with age for all phthalate metabolites in both sexes, except for MEP.

**Figure 2.** Longitudinal urinary levels of phthalate metabolites in high (red dashed lines) and low (black dashed lines) excretion groups of selected phthalate metabolites in boys (A–F) by age. Each line represents a boy. The dots represent the geometric mean of the phthalate metabolite of each boy. The solid black line marks the mean value, separating the boys in the high and low excretion groups.
(Figures 1 and 2). As illustrated by $\Sigma\text{MBP}_{(i+n)}$ in Figure 3, a variation in urinary excretion of phthalates was evident in each child, which made a mean of repetitive samples more representative for long-term excretion than a single determination. The CV within and between individuals are listed in Supplemental Table 1, illustrating that a single determination seemed fairly representative for long-term excretion. The lowest CV was observed for the correlated phthalates; CV within individuals was 36% and 42%, and CV between individuals was 73% and 79% in girls and boys, respectively.

**Associations between phthalate urinary excretion and adrenal androgens**

Hormone levels for girls at 10 years of age and for boys at 11 years of age of the high and the low excretion groups within each phthalate metabolite are compared in Table 1.

In girls, at 10 years of age, there were no significant differences in the levels of DHEAS and Adione between any of the the groups of high and low phthalate excretion calculated as amount per body weight (nanograms per kilogram) or total amount (nanograms). However, comparing groups based on concentrations (nanograms per milliliter), the level of DHEAS was significantly lower in the high $\Sigma\text{MBP}_{(i+n)}$ excretion group. At 13 years of age, the level of DHEAS and Adione in girls was lower in the high excretion groups (except MEP and $\Sigma\text{DiNPm}$), although statistically significant only for DHEAS and Adione versus $\Sigma\text{MBP}_{(i+n)}$ ($P = .008$ and $P = .001$) and Adione versus $\Sigma\text{DEHPm}$ ($P = .018$) (Supplemental Table 2). Furthermore, testosterone was lower in the high $\Sigma\text{MBP}_{(i+n)}$ excretion group ($P = .005$).

In boys, DHEAS at 11 years of age was lower in the high MBzP excretion group ($P = .038$) and also lower in the high MBzP excretion group based on the phthalate concentrations ($P = .045$) (Table 1). A lower level of DHEAS was also observed in the high $\Sigma\text{MBP}_{(i+n)}$ excretion group but not in any of the other high excretion groups. At 13 years of age, nonsignificant lower levels of DHEAS were observed in the high MBzP excretion group and lower testosterone levels were observed in the high $\Sigma\text{DEHPm}$ and $\Sigma\text{DiNPm}$ excretion groups ($P = .049$ and $P = .022$, respectively). However, higher testosterone levels were observed in the high MBzP excretion group ($P = .040$), and the same trend was observed in the high $\Sigma\text{MBP}_{(i+n)}$ excretion group (Supplemental Table 2).

Individual levels of the adrenal androgens in high or low $\Sigma\text{MBP}_{(i+n)}$ excretion groups are illustrated in Figure 4, A–D.

**Pubertal development**

In girls, no significant differences were observed in age at breast or pubic hair development between high and low excretion groups for individual phthalate metabolites. However, there was a tendency toward increasing age at pubarche in high $\Sigma\text{MBP}_{(i+n)}$, $\Sigma\text{DEHPm}$, $\Sigma\text{DiNPm}$, and $\Sigma\text{corr.pht.m}$ excretion groups, respectively.

In boys, the median age at PH2$^+\_i$ was lower in the high $\Sigma\text{MBP}_{(i+n)}$ and $\Sigma\text{corr.pht.m}$ excretion groups ($P = .005$ and $P = .006$). There were no significant differences in age...
Table 1. Median Levels of Anthropometric Measures, Hormones, and Age at Thelarche and Pubarche and TV >3 mL Among Girls (n = 47) and Boys (n = 53) With Low and High Urinary Phthalate Excretion (Based on Geometric Mean of Amount per Body Weight [Nanograms per Kilogram])\textsuperscript{a,b,c,d}

<table>
<thead>
<tr>
<th></th>
<th>Low (n = 24)</th>
<th>Low (n = 24)</th>
<th>High (n = 23)</th>
<th>High (n = 23)</th>
<th>Low (n = 30)</th>
<th>Low (n = 30)</th>
<th>High (n = 23)</th>
<th>High (n = 23)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight, kg</td>
<td>37.7</td>
<td>34.1</td>
<td>36.7 (p &lt; .05)</td>
<td>33.8 (p &lt; .05)</td>
<td>37.4</td>
<td>34.9</td>
<td>38.0 (p &lt; .05)</td>
<td>37.2 (p &lt; .05)</td>
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<tr>
<td>FSH, IU/L</td>
<td>4.3</td>
<td>3.8</td>
<td>2.3 (p &lt; .05)</td>
<td>1.8 (p &lt; .05)</td>
<td>4.3</td>
<td>3.7</td>
<td>4.5 (p &lt; .05)</td>
<td>4.2 (p &lt; .05)</td>
</tr>
<tr>
<td>LH, IU/L</td>
<td>&lt;18</td>
<td>&lt;18</td>
<td>&lt;18 (p &lt; .05)</td>
<td>&lt;18 (p &lt; .05)</td>
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<td>&lt;18 (p &lt; .05)</td>
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<tr>
<td>Estradiol, pmol/L</td>
<td>&lt;0.23</td>
<td>&lt;0.23</td>
<td>&lt;0.23 (p &lt; .05)</td>
<td>&lt;0.23 (p &lt; .05)</td>
<td>&lt;0.23</td>
<td>&lt;0.23</td>
<td>&lt;0.23 (p &lt; .05)</td>
<td>&lt;0.23 (p &lt; .05)</td>
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<tr>
<td>Testosterone, nmo/L</td>
<td>&lt;0.23</td>
<td>&lt;0.23</td>
<td>&lt;0.23 (p &lt; .05)</td>
<td>&lt;0.23 (p &lt; .05)</td>
<td>&lt;0.23</td>
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<td>&lt;0.23 (p &lt; .05)</td>
<td>&lt;0.23 (p &lt; .05)</td>
</tr>
<tr>
<td>IGF-1, ng/mL</td>
<td>242</td>
<td>229</td>
<td>237 (p &lt; .05)</td>
<td>235 (p &lt; .05)</td>
<td>237</td>
<td>235</td>
<td>234 (p &lt; .05)</td>
<td>231 (p &lt; .05)</td>
</tr>
<tr>
<td>DHEAS, nmol/L</td>
<td>0.91</td>
<td>1.25</td>
<td>1.03 (p &lt; .05)</td>
<td>1.03 (p &lt; .05)</td>
<td>1.01</td>
<td>0.96</td>
<td>1.04</td>
<td>1.02</td>
</tr>
<tr>
<td>Adione, nmol/L</td>
<td>1.55</td>
<td>1.86</td>
<td>1.63</td>
<td>1.63</td>
<td>1.63</td>
<td>1.54</td>
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<td>1.54</td>
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<tr>
<td>Age at PH2+, y</td>
<td>10.8</td>
<td>10.8</td>
<td>10.8</td>
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<td>10.8</td>
<td>10.8</td>
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<tr>
<td>Age at TV &gt; 3 mL, y</td>
<td>11.1</td>
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\textsuperscript{a} For MP, SMBP, MBzP, DEHPm, DNPm, and corr. phth.m, the sum of CBzP, MBzP, DEHPm, and DNPm, the molar sum of CBzP, MBzP, DEHPm, and DNPm, respectively.

\textsuperscript{b} Significant differences between high and low phthalate excretion groups (P < .05).

\textsuperscript{c} Significant differences between high and low excretion groups if the grouping was based on concentration (nanograms per milliliter) (P < .05).

\textsuperscript{d} Significant differences between high and low excretion groups if the grouping was based on total amount (nanograms) (P < .05).

at TV > 3 mL between high and low excretion groups for any phthalate metabolite.

As illustrated in Figure 5, a significantly lower age at pubarche was observed in boys in high excretion groups of \(\Sigma MBP_{+n} \) and \(\Sigma corr.\ phth.m\) (P = .005 and P = .006), but not in girls. The opposite pattern was observed in the girls, although not significant. There were no differences between high and low phthalate excretion and age at B2+ in girls or age at TV > 3 mL in boys.

**Discussion**

This is to our knowledge the first longitudinal study on urinary phthalate excretions in relation to serum levels of adrenal steroids in normal prepubertal and pubertal children. The longitudinal design with repetitive sampling revealed that a single morning urine sample was relatively representative of long-term environmental exposure to phthalates, although repetitive measurements gave a better estimate of long-term excretion of these chemicals.

In girls in puberty (13 years of age), we found lower serum levels of the 2 adrenal androgens, DHEAS and Adione, in those who excreted the highest amount of \(\Sigma MBP_{+n} \). The same trend was observed at 10 years of age, albeit not statistically significant. The DBP isomers, together with DEHP, were the phthalates that the girls were most exposed to. A lower level of DHEAS and Adione in the highest exposed girls is in accordance with a possible antiandrogenic effect of DBP, and if so, the effect would be upstream of DHEAS production. We have recently reported an association between high exposure of DBP (and DEHP) and later age at pubarche (21), an association that also has been reported by Wolff et al. (25) and that is in accordance with an inhibition of the adrenal androgen production. However, in the present study, we could not reproduce this finding. This may be explained by differences between the participating girls in the cross-sectional study and the longitudinal study. First, in the longitudinal study, lower exposure levels of phthalates were observed compared with the cross-sectional study and fewer subjects were included. In addition, girls in the longitudinal study were characterized by being leaner than girls in the cross-sectional study, and a positive association between body mass index and phthalates has previously been reported (32). Thus, our longitudinal survey may coincidentally lack inclusion of the most exposed girls, thus diminishing power to detect effects.
In boys, we did not observe a negative association between serum level of adrenal androgens and high excretion of $\Sigma$MBP$_{(i+n)}$. Surprisingly, we found that the boys in the high $\Sigma$MBP$_{(i+n)}$ excretion group developed pubic hair almost a year earlier than the boys in the low $\Sigma$MBP$_{(i+n)}$ excretion group. At 11 years of age, DHEAS was lower in the boys who excreted the highest amount of MBzP. The same trend was seen at 13 years of age, although no longer statistically significant. In contrast, the testosterone level at 13 years of age was higher in the boys who excreted highest amount of MBzP. A lower level of DHEAS combined with a higher level of testosterone may reflect a decreased rate of converting DHEA to DHEAS by inhibition of the sulfotransferase SULT2A1. Inhibition of sulfotransferase SULT2A1 by phthalates has been shown in vitro (33), and such an effect may be most pronounced in boys because they have a higher DHEAS to DHEA ratio, implying a sex difference in enzymatic activity (34). However, lower DHEAS combined with higher testosterone was associated only with MBzP excretion, because high excretion of $\Sigma$DEHPm and DiNPm was associated with lower testosterone levels. Inhibition of steroidogenesis by DEHP and the metabolite MEHP resulting in lower testosterone has also been shown in vitro (35).

Although we found lower levels of DHEAS and androstenedione, both in girls and boys with the highest excretion of some of the phthalates, differences in levels of these hormones between the high and low exposure groups were relatively small.

With regard to associations between phthalate excretion levels and secondary sex characteristics, we found a sex difference, because pubarche was accelerated in boys within the high excretion groups. The sex difference might be explained by the differences in endogenous gonadal hormone levels in boys and girls. Another possibility is that onset of puberty or increasing age is associated with changes in behavior and lifestyle that also changes the phthalate excretion. However, the only phthalate of which excretion seemed to increase with age was DEP (excreted in urine as MEP), possibly reflecting the fact that DEP is widely used in cosmetic products. Also, none of the other milestones in pubertal development like breast development in girls and testicular growth in boys was associated with levels of urinary phthalate excretion. This is

![Figure 4. Longitudinal serum levels of DHEAS (A and B) and Adione (C and D) in high (red dashed lines) and low (black dashed lines) $\Sigma$MBP$_{(i+n)}$ excretion groups in girls (A and C) and boys (B and D) by age. Each line represents a child. Abbreviation: NS, nonsignificant.](https://academic.oup.com/jcem/article-abstract/98/9/3755/2833254)
in line with our recent cross-sectional study of Danish healthy children, where no associations between phthalate excretion and age at breast development in girls (24), age at testicular growth, or development of gynecomastia in boys (23) were observed. Furthermore, Lomenick et al (20) reported no difference in phthalate exposure in girls with and without precocious puberty. In contrast, associations between high phthalate exposure and earlier breast development in girls has been reported in a Puerto Rican study (17), which, however, has been considerably criticized for technical shortcomings in phthalate analysis (36), and in a Taiwan study (18). High phthalate exposure has also been associated with development of gynecomastia in pubertal boys (22). These divergent results of phthalate excretion and puberty might be related to diversity between the levels of phthalates measured in the different studies. In the studies reporting associations between phthalates and early breast development in girls (17, 18) or gynecomastia in boys (22), the children seemed to be much more exposed than the children in our study.

A major challenge in a study of phthalate effects in humans is how to estimate exposure. We investigated the urinary excretion expressed as amount per body weight, as concentration, and as total amount. Because the children were examined at different ages, the negative correlation between collecting period (the period from last night voiding to first morning voiding was longer in young children) and diuresis (more volume in older children) explains why the total amount varied less with age compared with nanograms per kilogram and nanograms per milliliter. Because we compared the mean of urinary phthalate excretions in children examined at different ages, a measurement that does not vary with age is preferable. Because the same absolute amount of phthalate metabolites excreted by a small and a larger child means that the smaller child is relatively more exposed, we have analyzed phthalate metabolite excretion calculated as amount per body weight. However, we also analyzed the phthalate metabolite excretion calculated as concentration and as total amount. Depending on how phthalate metabolite excretion was expressed, the observed associations changed as indicated in Table 1; however, the lower levels of adrenal androgens in high ΣMBP_{1+} excretion groups in girls and high MBzP excretion groups in boys was evident irrespectively. We did not correct phthalate concentrations by dividing with creatinine, because creatinine-adjusted phthalate values will tend to decrease with age and body size (37, 38).

The strength of our study is the longitudinal design, where the collection of multiple urine samples and the conducting of multiple examinations made it possible to calculate a mean phthalate excretion over a period of 5 years in each child. Because phthalate metabolites have
short half-lives, a mean of phthalate measurement in sev-
eral urine samples instead of only 1 sample per child makes
the exposure estimates more reliable. Furthermore, our
design also made it possible to estimate pubic milestones
as PH2+, B2+, and TV >3 mL with an accuracy of 3
months within children of ethnic homogeneity. Limita-
tions of the study are variations in the urinary volumes
collected, variations in the acquisition times, and varia-
tions in the age of the children. Furthermore, associations
in human studies might be hard to find because biological
variations between individuals exist, and further studies
are needed to rule out that some of our results could be
chance findings. Furthermore, the mechanisms of action
of phthalates are complex and may also involve estrogen
action (39) and activation of the nuclear transcription factor
peroxisome proliferator-activated receptor-γ (PPARγ),
which contributes to adipocyte differentiation and insulin
sensitivity (40). Concurrently, several other chemicals
may target similar pathways and give rise to complex biological
effects (26).

In conclusion, we provided evidence that excretion of
some phthalates are negatively associated with adrenal
androgen levels in both girls and boys and also with test-
osterone levels in pubertal boys; observations that are in
line with known effects on steroidogenesis in in vitro stud-
ies. The lower androgen levels were not associated with
later age at pubarche or other pubertal milestones. Sur-
prisingly, boys in puberty with high excretion of MBzP
had higher testosterone level, and the same trend was ob-
served for MBP isomers. Interestingly, boys in the high
excretion groups of MBP isomers and the sum of corre-
lated phthalates also appeared to start pubarche earlier,
which does not reflect an antiandrogenic action.

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