Phthalate exposure and pubertal development in a longitudinal study of US girls

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STUDY QUESTION: Does phthalate exposure during early childhood alter the timing of pubertal development in girls?

SUMMARY ANSWER: Urinary concentrations of high-molecular weight phthalate (high-MWP) metabolites are associated with later pubarche.

WHAT IS KNOWN ALREADY: Phthalates are anti-androgenic environmental agents known to alter early development, with possible effects on pubertal onset.

STUDY DESIGN, SIZE, AND DURATION: This multi-ethnic study included 1239 girls from New York City, greater Cincinnati, and the San Francisco Bay Area who were 6–8 years old at enrollment (2004–2007) and who were followed until 2011.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Phthalate metabolites were measured in urine collected at enrollment from 1170 girls; concentrations ranged from \(1\) to \(10^4\) mg/l. Breast and pubic hair stages and body size were assessed one to two times annually to determine the age at transition from stage 1 to 2 for breast and pubic hair development. Associations between exposures and pubertal ages were estimated using Cox proportional hazard ratios (HR) with 95% confidence intervals (CI) and survival analyses. Associations were examined with respect to age-specific body mass-index percentile, one of the strongest predictors of pubertal onset.

MAIN RESULTS AND THE ROLE OF CHANCE: Urinary concentrations of high-MWP including di(2-ethylhexyl) phthalate (DEHP) metabolites were associated with later pubic hair development during 7 years of observation. The relationship was linear and was stronger among normal-weight girls. Among normal-weight girls, age at pubic hair stage 2 (PH2) was 9.5 months older for girls in the fifth compared with the first quintile of urinary DEHP (medians: 510 and 59 mg/g creatinine, respectively; adjusted HR 0.70, CI 0.53–0.93, P-trend 0.005. Age at first breast development was older for fifth quintile of mono-benzyl phthalate versus first (HR 0.83, CI 0.68–1.02; P-trend 0.018). No associations were observed between low-molecular weight phthalate urinary metabolite concentrations and age at pubertal transition in adjusted analyses.

LIMITATIONS, REASONS FOR CAUTION: While there is evidence that phthalate exposures are fairly consistent over time, the exposure measure in this study may not reflect an earlier, more susceptible window of exposure. We investigated alternative explanations that might arise from exposure misclassification or confounding.

WIDER IMPLICATIONS OF THE FINDINGS: Phthalates are widespread, hormonally active pollutants that may alter pubertal timing. Whether exposures delay or accelerate pubertal development may depend on age at exposure as well as other factors such as obesity and exposures earlier in life. Whether exposures act independently or as part of real life mixtures may also change their effects on maturation from birth through childhood.


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**Introduction**

During the past few decades, the age at which girls begin puberty has declined, including age at breast and pubic hair onset and age at menarche. Much interest has arisen about the possible role of environmental agents in relation to pubertal timing, with the expectation that development would be accelerated by estrogenic chemicals in females and by androgenic agents in males. Phthalate diesters are added to various products to improve performance of building materials, medical devices and personal products. Widespread exposure occurs from use of consumer products such as perfume or plastic tubing, from air volatization and from the diet. Phthalates are best known for their anti-androgenic potential that does not involve the androgen receptor. This research has found parallels in experimental and human research for impeding genital development and spermatogenesis (Hauser, 2008; Swan, 2008). In female rats, phthalates exhibit both agonist and antagonist effects, suggesting that pubertal development might be accelerated or delayed depending on timing, dose and other factors (Grande et al., 2006).

Developmental effects in humans that might arise from exposure to phthalates are of concern, as exposures to phthalates are universal, and the upper ranges of measured urinary biomarkers exceed micromolar concentrations, comparable to those that cause developmental delay in animals. Two reports have found later pubertal development with phthalate exposures among girls, in cross-sectional study designs including loge-creatinine in models using continuous log-biomarker variables and from the diet. Phthalates are best known for their anti-androgenic potential that does not involve the androgen receptor. This research has found parallels in experimental and human research for impeding genital development and spermatogenesis (Hauser, 2008; Swan, 2008). In female rats, phthalates exhibit both agonist and antagonist effects, suggesting that pubertal development might be accelerated or delayed depending on timing, dose and other factors (Grande et al., 2006).

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Methods

Subjects and outcomes

The Breast Cancer and Environment Research Program (BCERP) epidemiology project is a longitudinal study of girls enrolled at 6–8 years of age who are being followed through puberty. Enrollment occurred at three sites from 2004 to 2007. Mount Sinai School of Medicine (MSSM) recruited in East Harlem in New York City; Cincinnati Children’s Hospital (Cincinnati) recruited in the greater Cincinnati metropolitan area and through the Breast Cancer Registry of Greater Cincinnati and Kaiser Permanente Northern California (KPNC) recruited members of the KPNC Health Plan in the San Francisco Bay Area. Eligibility included age, female sex, no underlying endocrine medical conditions and at MSSM eligibility required black or Hispanic race/ethnicity. Protocols and urinary phthalate metabolite concentrations have been described previously (Wolff et al., 2010).

Ethical approval

All sites obtained informed consent from parent or guardian, approved by the Institutional Review Boards at each institution and at the Centers for Disease Control and Prevention (CDC).

Data collection

A questionnaire was completed by the girl’s caregiver (usually the mother) that included medical history, environmental exposures, demographic variables and race/ethnicity (Black, White, Asian and Hispanic or non-Hispanic). Weight, height, breast and pubic hair stages 1–5 (PH1–5) were measured at each visit using standardized protocols previously described (Biro et al., 2010), and urine was collected at baseline. Visits for MSSM and KPNC were annual and for Cincinnati every 6 months through 2010, once in 2011. The main goal of BCERP was to examine determinants for age at first pubertal development (stage 2 versus stage 1 which is no development). Pubertal stages for breast (B1–B5) and PH1–5 were determined by observation and palpation. A standardized written protocol with photographs of each stage was used to train and test examiners (van Wieringen et al., 1985; Biro et al., 2010). Inter-rater assessments were performed at each site by one pediatrician who found ‘substantial agreement’ (kappa 0.67) and concordance (87%; 117/127) among 39 examiners (Biro et al., 2010). Age- and sex-specific body mass-index percentiles (BMI%) were calculated based on CDC growth charts (CDC, 2000).

Urinary biomarker measurements

Samples (n = 1170), collected at baseline, were analyzed at the National Center for Environmental Health laboratones at CDC for the nine phthalate metabolites most frequently detected in the US general population. These are monoethyl phthalate (MEP), mono-n-butyl phthalate (MBP), mono-isobutyl phthalate (MiBP), mono-benzyl phthalate (MBzP), mono-3-carboxypropyl phthalate (MCPP), mono(2-ethyl-5-carboxypentyl) phthalate (MECTP), mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono(2-ethyl-5-oxohexyl) phthalate (MEOHHP) and mono(2-ethylhexyl) phthalate (MEHP) (CDC, 2009). Analytic and quality control procedures have been reported (Wolff et al., 2010). We imputed values below the limit of detection (LOD) as LOD/√2. For the majority of phthalate biomarkers in our study, measurements were above the LOD (>98%), except for MEHP (80% > LOD; Wolff et al., 2010), and thus imputation is unlikely to alter our findings (Lubin et al., 2004). However, as methods of handling imputation have evolved over recent years, we examined results using MEHP values imputed with Helsel’s robust probability plotting method for LODs (Helsel, 1990). Results were almost identical to those reported in the tables for MEHP and molar sum of low-molecular weight phthalate metabolites (low-MWP). Normalization for urine dilution was accomplished by including loge-creatinine in models using continuous log-biomarker variables (loge-µg/l); for quintiles we used creatinine-corrected concentrations (µg/g creatinine) to create cut points. To ascertain whether results were affected by the method of adjusting for dilution, we determined that extreme metabolite concentrations were not due to creatinine concentration alone and that low creatinine values were distributed across the range of biomarker concentrations. Results reported in the tables using creatinine-corrected or -adjusted urinary concentrations were not attenuated if low...
creatinines were removed. Models using concentrations not creatinine-corrected or adjusted were very similar to those reported if low creatinines were excluded (<50 mg/dl). Further, we assessed concordance of quintile assignments for ΣDEHP, by comparing the frequency distribution of observations by μg/l cut points versus those by μg/g creatinine (kappa = 0.36; for creatinine >50 mg/dl, kappa = 0.48). As described in previous papers (Wolff et al., 2010), we also combined the phthalate metabolites into groups that represent similar sources and similar biologic activity, low- (<250 dalton, low-MWP) and high-molecular weight (>250 dalton, molar sum of high-molecular weight phthalate metabolites, high-MWP) phthalate metabolites. High-MWP include the 4 di(2-ethylhexyl) phthalate (DEHP) metabolites (ΣDEHP; including MECPP, MEHHP, MEOPHP and MEHP), MBzP and MCPP. Low-MWP include MEP, MBP and MIBP. We expressed low-MWP molar sum as MEP (molecular weight 194) and high-MWP (micromoles/l) as MEHP (molecular weight 278) so that units were the same as the single analytes (μg/l or μg/g creatinine).

Statistical analyses

We analyzed ages at breast and pubic hair stages, assessed at entry and in as many as 6 years of follow-up, in relation to urinary concentrations of phthalate metabolites and potential confounders to determine the relative risk of advancing from stage 1 to 2. Phthalate exposure biomarkers were analyzed as continuous variables and in addition as quintiles of concentration to evaluate the shape of the dose–response. To examine intercorrelations of the phthalate urinary metabolites, we computed the rank correlation coefficient (Spearman ρ) between the individual and total concentrations. Analyses were conducted using SAS (version 9.2; SAS Institute, Inc.). We performed multivariable analyses using Cox proportional hazards models with Proc PHREG to compute hazard ratios (HR) and 95% confidence intervals (CIs) for age at development (breast stage 2 = B2 or pubic hair stage 2 = PH2) by exposure. HRs < 1 were interpreted as reduced risk of pubertal development or delayed pubertal onset, while HRs greater than one were interpreted as increased risk of pubertal development. For most girls, event age was the midpoint between the last stage 1 visit (with no earlier stage 2 exam) and the first stage 2 or higher visit (with no successive stage 1 visits; n = 826 for B2, n = 793 for PH2). Girls who were B2 (n = 175) or PH2 (n = 138) at entry were assigned event age as 6 months before enrollment; girls who did not achieve stage 2 (n = 169 B1, n = 234 PH1) were treated in the model as censored at the time of their last visit. Adjusted survival curves and PH2 median ages were predicted from the baseline survivor function of adjusted Cox models; quintiles of the phthalate biomarker concentrations were included in the strata statement (Allison, 2010).

We evaluated potential confounders (those in Table I as well as height and season of urine collection (which might reflect different dietary intake patterns and indoor air concentrations) related to pubertal development and urinary biomarker concentrations using a conceptual model based on their biologic plausibility and correlations within the pathway. BMI% at the last pubertal stage 1 visit (e.g. B1-BMI%) was chosen as a covariate in order to control for adiposity at a consistent time point within the cohort, because baseline was not equidistant from stages 2 for all participants. For girls who were B2 or PH2 at entry, baseline BMI% was used. We justified use of baseline BMI% by finding in a subset of girls that BMI% at the last B1-visit and the B2-visit were almost identical (Spearman ρ = 0.94, n = 598). In the same subset, B1-BMI% was imputed from B2-BMI% in models predicting B2-height [r² = 0.94] or B2-weight [r² = 0.92] adjusted for site and race; it was also very close to actual B1-BMI%. Variables that did not improve precision or alter the biomarker estimate by more than 10% were eliminated in a manual backward stepwise manner from the models; they were re-entered individually into the final model to verify model fit. Final models included child race/ethnicity, caregiver education, BMI% and site. We also used quintiles of biomarkers to elucidate dose–response and influence of BMI% on associations of phthalate biomarkers with pubertal onset. To evaluate linearity, we checked that estimates for continuous loge-biomarker variables were consistent with those using quintiles. We computed the P-trend of HRs across biomarker quintiles by substituting median biomarker concentrations of the quintiles (loge, μg/g creatinine) as a continuous variable in the quintile models. We did not observe any trends for quintile models suggestive of a threshold response.

For comparison, we examined results from Weibull accelerated failure models appropriate for interval censored longitudinal data using PROC LIFEREG; results were almost identical to Cox models. Because adiposity is a strong endogenous hormonal risk factor for pubertal development (Kaplowitz, 2008), we examined possible exposure modification by BMI% (BMI dichotomized at the 85th- age-specific percentile: normal and overweight). To test interactions, we used the product of dichotomized BMI% times the biomarker variable (continuous or ordinal quintile values). We conducted other sensitivity analyses to understand potential confounders better.

<table>
<thead>
<tr>
<th>Table I Geometric means (95% CI) of phthalate urinary biomarker concentrations at baseline examination in relation to covariates.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N</strong></td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td><strong>Age at baseline urine donation (years)</strong></td>
</tr>
<tr>
<td>6.0–6.9</td>
</tr>
<tr>
<td>7.0–7.9</td>
</tr>
<tr>
<td>≥8.0</td>
</tr>
<tr>
<td><strong>Race/ethnicity</strong></td>
</tr>
<tr>
<td>White</td>
</tr>
<tr>
<td>Asian</td>
</tr>
<tr>
<td>Hispanic</td>
</tr>
<tr>
<td>Black</td>
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<tr>
<td><strong>BMI percentile at last B1-visit (age-specific)</strong></td>
</tr>
<tr>
<td>&lt;50th</td>
</tr>
<tr>
<td>50–85th</td>
</tr>
<tr>
<td>≥85th</td>
</tr>
<tr>
<td><strong>BMI percentile at last PH1 visit age-specific</strong></td>
</tr>
<tr>
<td>&lt;50th</td>
</tr>
<tr>
<td>50–85th</td>
</tr>
<tr>
<td>≥85th</td>
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<tr>
<td><strong>Caregiver education</strong></td>
</tr>
<tr>
<td>High school</td>
</tr>
<tr>
<td>&gt;High School</td>
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<tr>
<td><strong>Study site</strong></td>
</tr>
<tr>
<td>New York</td>
</tr>
<tr>
<td>Ohio</td>
</tr>
<tr>
<td>California</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

N (total) = 1170 girls with at least one biomarker (5 missing age at PH2, 1 missing PH1 BMI percentile (BMI%), 1 missing B1 BMI%, 28 missing education). All distributions differed by characteristics (P < 0.001) except for high-MWP with BMI%. Adjusted geometric means differed by race/ethnicity, site.

Low/high-MWP, low/high-molecular weight phthalate metabolites; mg/gCr, mg/g creatinine.

Wolff et al.
Results

Characteristics of subjects and exposures

Girls were 6–8 years old at enrollment, and the proportions of Black, Hispanic, White race/ethnicities were similar, with few Asians (Table I). Approximately 30% of girls were overweight or obese at baseline or at the last pubertal stage 1 visit (Table I). Among 1170 girls with urinary biomarker results, the Kaplan–Meier estimate for median age at B2 was 112.5 months (CI 111.5–113) or 9.4 years; for median age at PH2 it was 119 months (CI 117.5–121) or 9.9 years (not shown). The minimum ages for both B2 and PH2 were 67 months; the maxima were 156 months, which occurred for age at B2 and PH2 in the same girl (not shown). Phthalate urinary biomarker means varied by study characteristics as reported earlier (Wolff et al., 2010), but when mutually adjusted for these factors, the means differed only by race/ethnicity and site (Table I). In addition, low-MWP varied by season of urine collection [not shown, as it was not a confounder in final models; shown in Wolff et al. (2010)]. Phthalate urinary biomarker sums spanned a wide range of concentrations. Concentrations of the four DEHP metabolites were highly intercorrelated ($r_s$ 0.7–0.99, not shown). MBzP and MCPP had lower correlations with each other and with the other high-MWP biomarkers ($r_s$ 0.2–0.4). Low-MWP metabolites had correlations of 0.3–0.6 with each other. Correlations ($r_s$) of low-MWP with high-MWP or $\Sigma$DEHP were 0.3. These relationships are consistent with the phthalate exposures having come from similar environmental sources. Low-MWP and high-MWP concentrations were $>150 \mu g/l$ (geometric means, Table I). The 90th percentiles exceeded 2 micromolar (2.7 $\mu M/g$ creatinine for low-MWP, 2.2 $\mu M/g$ creatinine for high-MWP, respectively; not shown). Medians of high-MWP and $\Sigma$DEHP in the fifth and first quintiles approximated the 90th and 10th percentiles, respectively.

Associations of high-MWP exposures with age at puberty

High-MWP exposures including $\Sigma$DEHP and the constituent high-MWP metabolites were associated with later age at PH2 (Table II). Risk estimates were similar for high-MWP and $\Sigma$DEHP. Reduced risk for PH2 was $\sim$8–9% per loge-unit change (2.7-fold) in urinary metabolite concentrations. For the fifth versus first quintiles of $\Sigma$DEHP risk for PH2 was 20–30% lower, and the adjusted median age at PH2 was 8.5 months older (Table III).

$\Sigma$DEHP–body size interactions and age at PH2

Later ages at PH2 appeared to be more linear across biomarker quintiles among normal-weight than overweight girls. For example, the risk (HRs) decreased with quintiles of $\Sigma$DEHP and older predicted ages at PH2 monotonically increased for normal-weight girls, $P$-trend 0.005 (Table III). As seen in Fig. 1, the association of $\Sigma$DEHP with adjusted age at PH2 was better in the normal-weight stratum than overweight. However, interaction terms for BMI% $\times$ $\Sigma$DEHP were not significant. Age at PH2 was 9.5 months later in the fifth than first quintile of $\Sigma$DEHP for normal-weight girls (adjusted); the difference was smaller for overweight girls (6.5 months; Fig. 1, as estimated from data in Table III). Comparing normal-weight with overweight girls within the same quintiles of $\Sigma$DEHP, age at PH2 was 8–12 months later, representing the BMI-effect (Table III and Fig. I).

High-MWP was not associated with age at B2. MBzP, a high-MWP constituent, was modestly associated with later age at B2 ($P$-trend = 0.082 for continuous log$_e$ MBzP, Table II); for the fifth versus first quintile of the biomarker the HR was 0.83 (CI 0.68–1.02; $P$-trend 0.042, not shown). However, a similar linear association for MBzP with age at PH2 ($P = 0.022$ for continuous log$_e$-biomarker, Table II) was attenuated using quintiles of exposure [HR 0.85 (CI 0.69–1.04), $P$-trend 0.125, fifth versus first quintile, not shown].

Associations between low-MWP exposures and age at puberty

Low-MWP was not associated with PH2- or B2-age after adjusting for covariates (Table II). MBP (a low-MWP metabolite) showed a weak inverse association with age at PH2 as a continuous variable [Table II, HR 0.92 (CI 0.85–1.00)], but there was no association with age at PH2 in models using MBP quintiles; estimates and CIs all included the null, $P$-trend = 0.107 (not shown). The range of concentrations for MBP was also narrower than for the molar sums, e.g. low-MWP, possibly limiting statistical power to detect an association.

Alternative explanations

The phthalate–PH2 associations were robust in various sensitivity analyses that attempted to examine alternative explanations and potential residual confounding. Excluding BMI%, which may be on the path between phthalate exposure and pubertal onset, changed neither estimates nor precision very much. As an example, for log$_e$-high-MWP with age at PH2 risk was lower (HR 0.91, CI 0.84–0.99, Table II); the HR was 0.92 (CI 0.84–0.99) when BMI% was removed from the model. For low-MWP with age at PH2 the association was the same with or without BMI% [fully adjusted HR 0.97 (CI 0.90–1.05, Table II); without BMI% HR 0.99 (CI 0.91–1.07, not shown)]. We were concerned that inclusion of site as a covariate in the models might over-adjust the estimates; the variability due to differences across study site populations is desirable, and in addition site was collinear with other covariates. In our results, excluding site only slightly altered either estimates or precision. For example, using the joint $\Sigma$DEHP tertile-site variable models for age-at-PH2 and rotating the reference group so that it was the first tertile for each site, we observed HRs that were 0.74 (CI 0.54–1.01) for NYC, 0.82 (CI 0.63–1.07) for KPNC and 0.89 (CI 0.65–1.22) for Cincinnati (third versus first tertile of $\Sigma$DEHP; joint tertile-site variable, $P = 0.028$, not shown). Site-stratified models were similar but less

as there were collinearity and unbalanced distributions among biomarker concentrations and covariates. To clarify associations within and between sites, we used a joint exposure-site variable combining three sites with tertiles of the molar sum of DEHP metabolite ($\Sigma$DEHP) concentrations (nine-level variable). The model was run three times in order to compare high versus low exposure estimates within a particular site, while retaining data from other sites, by changing the reference group to be the category, first $\Sigma$DEHP tertile-site. We also examined results stratified by site or race/ethnicity and by including height; models that included only girls with temporally consistent stages (e.g. stage B1 followed by B2, no successive stage B1, no girls without a B1-visit); with three or more visits. These analyses did not alter our findings.
precise (not shown). The P-interaction for ΣDEHP × site was 0.73. Models stratified by other factors did not materially alter the findings.

In a previous report, similar to the findings we report now, we observed reduced risk of PH2 at the second annual visit with high-MWP [prevalence ratio (PR) 0.94 (CI 0.88–1.00) for fifth versus first quintile, P-trend 0.04; Wolff et al., 2010). In the same report, earlier B2 and PH2 were seen with increasing low-MWP, though these positive associations were weak (PRs, 1.10, P-trend, 0.09).

**Discussion**

Our main finding is that in these girls, 10-fold higher exposures to high-MWP including DEHP, as estimated from the urinary concentrations of their metabolites, was associated with the transition from PH1 to PH2 being later by ~8 months. In hazards models, this represented 20–30% decreased risk over a 10-fold range of exposure. Ages for both PH2 and B2 were younger with increasing low-MWP in unadjusted associations, but there were no effects on pubertal age in models controlled for confounders. Unadjusted associations of low-MWP with pubertal ages may be due to its strong correlation with BMI%; the correlation was poor for high-MWP (not shown). Although removing BMI% from adjusted models of low-MWP did not change the null associations, other covariates were correlated with BMI and might have served as its surrogate. Our hypothesis was that exposure effects should be more apparent in low- than high-BMI girls. Actual estimates of age at PH2 were not very different by BMI% strata for high-MWP and ΣDEHP metabolites, but the dose–response relationships were more apparent in normal-weight girls. Reasons could be biological and....

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Age at first breast stage 2</th>
<th>N = 1170</th>
<th>Adjusted models</th>
<th>N = 1141</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unadjusted models HR (95% CI)</td>
<td>P</td>
<td>Adjusted models HR (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>High-MWP</td>
<td>0.99 (0.91–1.07)</td>
<td>0.764</td>
<td>0.99 (0.91–1.08)</td>
<td>0.834</td>
</tr>
<tr>
<td>ΣDEHP</td>
<td>0.99 (0.92–1.07)</td>
<td>0.881</td>
<td>1.01 (0.94–1.09)</td>
<td>0.797</td>
</tr>
<tr>
<td>MECPP</td>
<td>0.98 (0.91–1.05)</td>
<td>0.510</td>
<td>1.01 (0.94–1.09)</td>
<td>0.776</td>
</tr>
<tr>
<td>MEHHP</td>
<td>1.02 (0.95–1.09)</td>
<td>0.627</td>
<td>1.02 (0.95–1.1)</td>
<td>0.574</td>
</tr>
<tr>
<td>MEHP</td>
<td>0.99 (0.93–1.05)</td>
<td>0.632</td>
<td>1.01 (0.95–1.07)</td>
<td>0.853</td>
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<td>MEOHP</td>
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<td>0.806</td>
<td>1.01 (0.94–1.09)</td>
<td>0.724</td>
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<td>MBzP</td>
<td>0.99 (0.93–1.05)</td>
<td>0.729</td>
<td>0.95 (0.89–1.01)</td>
<td>0.082</td>
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<tr>
<td>MCPP</td>
<td>0.88 (0.81–0.96)</td>
<td>0.003</td>
<td>1.04 (0.95–1.13)</td>
<td>0.390</td>
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<td>Low-MWP</td>
<td>1.11 (1.03–1.19)</td>
<td>0.003</td>
<td>1.02 (0.95–1.11)</td>
<td>0.538</td>
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<td>MEP</td>
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<td>1.03 (0.96–1.09)</td>
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<td>MiBP</td>
<td>1.01 (0.94–1.08)</td>
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<td>0.97 (0.9–1.04)</td>
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<tr>
<td>MBP</td>
<td>1.02 (0.95–1.11)</td>
<td>0.553</td>
<td>0.99 (0.92–1.08)</td>
<td>0.917</td>
</tr>
</tbody>
</table>

**Table II** Associations (HR, with 95% CI) of total and individual phthalate urinary biomarker concentrations (log_{10}μg/l) with age at breast (B2) or PH2, BCERP cohort 2004–2011.

Cox proportional hazard models included log_{10} urinary creatinine. Adjusted models further added child race, caregiver education, child BMI% and study site. N’s differ because 5 girls had no pubic staging and/or missing covariates. Unadjusted models were similar when restricted to this number. Adjusted models excluding site, BMI%, or including height% had similar estimates and CI. High-MWP included ΣDEHP plus MCPP and MBzP.

Low-/High-MWP, Low-/High-molecular weight metabolites; ΣDEHP, molar sum of di(2-ethylhexyl) phthalate metabolites; MECPP, mono-2-ethyl-5-carboxypentyl phthalate; MEHHP, mono(2-ethyl-5-hydroxyhexyl) phthalate; MEHP, mono(2-ethylhexyl) phthalate; MEOHP, mono(2-ethyl-5-oxohexyl) phthalate; MBzP, mono-benzyl phthalate; MCPP, mono3-carboxypropyl phthalate; MEP, monoethyl phthalate; MiBP, mono-isobutyl phthalate; MBP, mono-n-butyl phthalate.

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inter-examiner error was not greater for overweight girls (Biro et al., 2010). Levels of exposure biomarkers, for a number of reasons. In our study, particularly early breast stages, and could lead to different overweight children. Overweight may compromise assessments of puberty (pubarche) but not B2-age, which may be consistent with an anti-androgen hypothesis, as androgens are more closely tied to pubic hair development (stage 2) and relation to overweight. MEP (not significant) and significant correlations of MEP with ages at PH2 may be attributed to several factors. First, high-MWP exposures may be less variable over time, due to sources in the indoor environment and the diet, for example (Adibi et al., 2008), than exposures to low-MWP which may change with habits that accompany age and weight-gain, reasons that were noted by others (Frederiksen et al., 2012). High- and low-MWP also have different relationships with BMI in our data and in a previous report (Teitelbaum et al., 2013a). A few other reports of phthalates and earlier puberty were flawed because they used a blood matrix to assess exposure which likely reflects contamination of blood specimens by exogenous phthalates [see references in Wolff and Swan (2013); Engel and Wolff (2013)].

That high-MWP but not low-MWP shows a relationship with age at PH2 may be attributed to several factors. First, high-MWP exposures are the prototypical anti-androgen, and MEP in toxicologic studies is inactive (NSF, 2008).

Context of other research in children
Two studies since 2010 have had similar findings with regard to phthalate exposure and puberty in girls. In addition to this report and our earlier cross-sectional report of this cohort (Wolff et al., 2010), a European cross-sectional study with high-MWP metabolite concentrations similar to those in our study detected delayed PH2 (4.8 months for ΣDEHP fourth versus first quartile, unadjusted; Frederiksen et al., 2012). As in our findings, this study found earlier B2 and PH2 with MEP (not significant) and significant correlations of MEP with ages at stages 1–4 for B2 and PH2 (highest MEP in stage 4 but lowest high-MWP in stage 4; unadjusted). A study of 84 girls by the European group found inverse relationships between urinary phthalate biomarkers and adrenal androgens, but no associations with pubertal timing possibly because of sample size (Mouritsen et al., 2013b). A few other reports of phthalates and earlier puberty were flawed because they used a blood matrix to assess exposure which likely reflects contamination of blood specimens by exogenous phthalates [see references in Wolff and Swan (2010); Calafat et al. (2013); Engel and Wolff (2013)]. The magnitude of the delay in age at PH2 of 8.5 months in our findings is similar to several reports that examined hormonally active exposures in

### Table III

<table>
<thead>
<tr>
<th>Quintile: median</th>
<th>Adjusted ages at median survival, months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1: 59 µg/gC</td>
<td>107 113 103.5 229</td>
</tr>
<tr>
<td>Q2: 93</td>
<td>113.5 116.5 108 228</td>
</tr>
<tr>
<td>Q3: 142</td>
<td>114 119 111.5 227</td>
</tr>
<tr>
<td>Q4: 233</td>
<td>115 121 111.5 225</td>
</tr>
<tr>
<td>Q5: 510</td>
<td>115.5 122.5 110 227</td>
</tr>
</tbody>
</table>

P for Q5 versus Q1: 0.0373 0.013 0.617

P-trend         0.018 0.005 0.630

P-class         0.062 0.090 0.093

HRs were computed using Cox proportional hazard models that included quintiles based on µg/g creatinine cutpoints. Adjusted models further added child race, caregiver education, child BMI percentile and study site. Interactions were assessed using BMI percentile (<85 or ≥85%) times the exposure variable (loge-µg/l or quintiles). Median ages by quintiles were determined from the survivor function of the adjusted model, evaluated at reference values of covariates.

**Context of other research in children**
Two studies since 2010 have had similar findings with regard to phthalate exposure and puberty in girls. In addition to this report and our earlier cross-sectional report of this cohort (Wolff et al., 2010), a European cross-sectional study with high-MWP metabolite concentrations similar to those in our study detected delayed PH2 (4.8 months for ΣDEHP fourth versus first quartile, unadjusted; Frederiksen et al., 2012). As in our findings, this study found earlier B2 and PH2 with MEP (not significant) and significant correlations of MEP with ages at stages 1–4 for B2 and PH2 (highest MEP in stage 4 but lowest high-MWP in stage 4; unadjusted). A study of 84 girls by the European group found inverse relationships between urinary phthalate biomarkers and adrenal androgens, but no associations with pubertal timing possibly because of sample size (Mouritsen et al., 2013b). A few other reports of phthalates and earlier puberty were flawed because they used a blood matrix to assess exposure which likely reflects contamination of blood specimens by exogenous phthalates [see references in Wolff and Swan (2010); Calafat et al. (2013); Engel and Wolff (2013)]. The magnitude of the delay in age at PH2 of 8.5 months in our findings is similar to several reports that examined hormonally active exposures in...
children, but 8.5 months is less than reported differences in pubertal ages with regard to BMI and race. For example, in our study group whites are older than blacks in age at B2 by ~11 months, and obese girls are younger at B2 by 19 months than normal-weight girls (1.6 year, 95th versus 50th%; Biro et al., 2013). The age at menarche difference for blacks versus whites has been ~6 months for several decades [e.g. 12 versus 12.5 year; (Biro and Wolff, 2011)]. Various other environmental exposures have also been associated with later puberty, including perfluorochemicals and phytosterogens; however, puberty was earlier in relation to polybrominated biphenyl (PBB) exposure measured perinatally using biomarkers (see references in Biro and Wolff, 2011; Lopez-Espinosa et al., 2011; Mervish et al., 2013). Following environmental exposure to lead (Pb), boys were older at puberty by 6–8 months (Hauser et al., 2008), and girls with higher Pb exposure were ~4 months older for B2 and PH2 (Selevan et al., 2003). Like our study, investigations of Pb in boys and PBBs in girls were longitudinal so that exposure was measured well before pubertal development (Blank et al., 2000; Hauser et al., 2008). Simultaneous modeling of six environmental biomarkers in relation to age at menarche, had expected associations for estrogenic PCBs and lead (earlier and later age, respectively) in a small cross-sectional study (Denham et al., 2005). Yet effects were similar to the six single-chemical models. In an evaluation of phthalate toxicity, a scientific panel recommended that various phthalate exposures should be combined (NSF, 2008), as we attempted to do in our summed concentrations, but combinations also should include other chemicals with similar activity, such as anti-androgenic agents not necessarily environmental in origin. Therefore, appropriate methods to incorporate phthalates into multiple exposure models is a needed next step.

Of note, many of the findings listed above for hormonally active chemicals reveal delayed, rather than earlier, onset of puberty. Perhaps detection of earlier puberty caused by hormonally active chemicals is masked by the stronger influence of BMI at earlier than at later ages of puberty. If so, would such agents more likely act as estrogen agonists than antagonists? Associations of perinatal PBB and soy exposures with earlier puberty in girls may have been detectable because exposure was measured in a susceptible window or because it was measured before the initial rise in childhood BMI that precedes pubertal onset in girls (Karaolis-Danckert et al., 2009; Adgent et al., 2012). Therefore, an important future question is to explain whether pubertal timing can be accelerated or delayed due to environmental exposures at earlier or later ages and whether childhood exposure increments perinatal exposure effects.

Laboratory research in rodents is consistent with our findings (Salazar et al., 2004; Grande et al., 2006), but it also supports both later and earlier female puberty following phthalate treatment (Ashby et al., 1997; Ma et al., 2006), inconsistencies that may reflect dosage amount, route and timing. The strongest biologic evidence regarding phthalate toxicity is for anti-androgenicity in male rodents, but delayed vaginal opening and other signs of reproductive dysfunction have been seen in female rats for several exposure windows; for example, later vaginal opening followed prepubertal administration of DEHP (Grande et al., 2006). Notably, nipple inversion has been observed in rats exposed prenatally, both male and female, a phenomenon characterized as ‘feminization’ (Foster, 2006; McKee et al., 2006). Experimentally, phthalate monoester metabolites disrupt androgen action by non-androgen receptor mechanisms, which include the peroxisome proliferator-activated receptor family for which they may be agonists or antagonists. These pathways may reduce or increase obesogenesis through complex mechanisms that control lipid and steroid metabolism as well as neuroendocrine function (Grun and Blumberg, 2009).

Limitations and strengths of this research

Strengths of this investigation include a longitudinal design, wide ranges of both exposures and child characteristics, including three geographical sites and a large sample size for this kind of study. A limitation is that environmental biomarkers measured in this study represent only a single point during the peripubertal window. Earlier exposures may be more influential because younger children are more susceptible or because the mechanisms governing development operate at younger ages (e.g. prenatal imprinting). There is also concern that a single biomarker measurement is not a reliable indicator of the critical window of exposure. However, our criteria for selecting biomarkers for this research project included having an adequate range of detectable biomarker concentrations (Wolff et al., 2007) and reliability over a reasonable period of time, e.g. 6–12 months’ exposure in children this age or pregnant women (Teitelbaum et al., 2008; Braun et al., 2012). Additional directions of interest are to evaluate multiple exposures, including incorporating weighting factors for relative toxicity and other developmental end-points including menarche.

We considered various confounding factors in detail, because of collinearity of BMI and the demographic covariates, but none of these factors materially altered our findings. It is of interest that two cross-sectional studies, including our own earlier analysis of this sample at baseline, found similar associations. There are some concerns about using

**Figure 1** Survival analysis of age at PH2 jointly by BMI% (<85% normal, ≥85% overweight + obese) and the molar sum of the concentrations of d(2-ethylhexyl) phthalate urinary metabolites (ΣDEHP) expressed as quintiles (fifth quintile median = 510 μg/g creatinine and first = 59 μg/g creatinine) based on Cox proportional hazards model, calculated for the average or reference values of the covariates in the study. Predicted median ages between the fifth and first quintiles differed by 9.5 months for normal weight (P = 0.013) and 6.5 months for overweight girls (P = 0.617; adjusted for covariates, see Table III).
Phthalate exposure and puberty in girls

creatinine-corrected biomarker concentrations to normalize urine concentration. However, in the models for continuous variables, we adjusted separately for creatinine by including it in the model with the uncorrected value (μg/l), and the model estimates were quite consistent with those from quintiles based on creatinine-corrected values (μg/g creatinine), both showing a strong linear trend. Creatinine correction did not create any outliers for the distribution of quintiles, for example, compared with the uncorrected values. We did not measure specific gravity in our samples, an alternate way of dilution normalization (Hauser et al., 2004); yet specific gravity and creatinine are closely correlated in many reports. There is likely some error in the pubertal stage assessment, but inter-rater agreement was satisfactory in this study. The exposure measure (urinary metabolites) also has some misclassification, for example, due to temporality because it is a one-time measure within <5 years of pubertal onset, but errors in exposure and outcome are likely to be non-differential. There is also the possibility that some associations are due to chance; in particular, the results for MBzP and age at B2 differ from results for other phthalate metabolites. Yet an argument can be made that MBzP has certain enhanced biologic potential, as seen in other studies (Just et al., 2012).

Summary
Phthalate exposures are highly prevalent in the environment, and children's absorbed levels are higher than adults (CDC, 2009). More than 10% of the girls in our study had urinary biomarker levels totaling >2 μM, for either low- or high-MWP, a biologically relevant exposure level. Understanding the impact of phthalates on development has public health significance, and there is a need to integrate lifetime exposures, especially prenatal through infancy where susceptibility to exogenous influences may be greatest. Key questions are whether childhood exposure adds to earlier life insults or acts alone, as well as how variable phthalate exposures might be over early life. There is a need to move beyond the examination of single phthalate exposures or even groupings of similar phthalates (as we have done in this analysis) toward considering combinations of chemicals where a composite mixture may act or compete in the hormonal trajectory of maturation. Relevant neuroendocrine and hormonal mechanisms also remain to be elucidated, especially with a view to how effects vary with age at exposure. Methodologic questions include whether environmental agents are more likely to be detectable for delayed rather than early puberty; also whether the magnitude of such effects are likely to be small, requiring adequate statistical power and analytic models. This research adds to the growing knowledge that phthalates may interfere with development, including genital and neurologic in addition to pubertal maturation.

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Authors’ roles
M.S.W. and S.L.T. both participated in the conception, design and planning of the study, implementation of research, data analysis, interpretation of the results and manuscript writing. K.M. contributed to the statistical analysis, interpretation and preparation of the manuscript. S.M.P., G.W. and M.G. took part in the study planning, data collection and management and manuscript review. A.M.C. participated in the conception and implementation of exposure measurement methodology and in preparation of the manuscript. F.M.B. and L.H.K. participated in the conception, design and planning of the study, implementation of research, data analysis, interpretation of the results. All authors have reviewed and approved the final version of the manuscript.

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Conflict of interest
L.H.K. is employed by Kaiser Permanente. The remaining authors declare they have no actual or potential competing financial interests.

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