Title: Children’s urinary phthalate metabolites and fractional exhaled nitric oxide in an urban cohort

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At a Glance Commentary

Scientific Knowledge on the Subject

Phthalates are used widely in consumer products, including plastics, vinyl flooring and personal care products such that exposure is ubiquitous. Exposure has been linked to adverse neurobehavioral and reproductive effects in children, and there is emerging evidence of associations between the phthalates commonly measured in indoor air and asthma, including associations with respiratory symptoms and decreased lung function.

What this Study Adds to the Field

This is the first study to demonstrate an association between current phthalate exposure and fractional exhaled nitric oxide (FeNO), a marker of airway inflammation. This association was observed among a well-characterized population-based study of inner-city children. The association was greater among children with recent wheeze, who may be most susceptible to changes in and consequences of airway inflammation, supporting a relevance of these exposures to asthma morbidity.

Online supplement: This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org
Abstract

Rationale: Phthalates are used widely in consumer products. Exposure to several phthalates has been associated with respiratory symptoms and decreased lung function. Associations between children’s phthalate exposures and fractional exhaled nitric oxide (FeNO), a biomarker of airway inflammation, have not been examined.

Objectives: We hypothesized that urinary concentrations of four phthalate metabolites would be positively associated with FeNO, and these associations would be stronger among children with seroatopy or wheeze.

Methods: In an urban ongoing birth cohort, 244 children had phthalate metabolites determined in urine collected on the same day as FeNO measurement. Repeated sampling gathered \( n=313 \) observations between ages 4.9-9.1 years. Seroatopy was assessed by specific IgE. Wheeze in the past year was assessed by validated questionnaire. Regression models used generalized estimating equations.

Measurements and Main Results: Log-unit increases in urinary concentrations of metabolites of diethyl phthalate (DEP) and butylbenzyl phthalate (BBzP) were associated with a 6.6% (95% confidence interval [CI] 0.5%, 13.1%) and 8.7% (95% CI 1.9%, 16.0%) increase in FeNO, respectively, adjusting for other phthalate metabolites and potential covariates/confounders. There was no association between concentrations of metabolites of di(2-ethylhexyl) phthalate or di-\( n \)-butyl phthalate and FeNO. There was no significant interaction by seroatopy. The BBzP metabolite association was significantly stronger among children who wheeze (\( p=0.016 \)).

Conclusions: Independent associations between exposures to DEP and BBzP and FeNO in a cohort of inner-city children were observed. These results suggest that these two ubiquitous
phthalates, previously shown to have substantial contributions from inhalation, are positively associated with airway inflammation in children.

**Number of words in abstract: 250**

**Key words:** airway inflammation, asthma, diethyl phthalate (DEP), butylbenzyl phthalate (BBzP), FeNO

**Introduction**

Phthalates are a group of high-production volume compounds added to plastics to confer flexibility (e.g., vinyl flooring) and used in personal care and other consumer products (1). Several phthalates can act as endocrine disruptors, and early life exposure has been associated with adverse neurobehavioral and reproductive effects in children (2-6). Numerous studies monitoring phthalate metabolites in urine have shown widespread exposure, including among inner-city populations, with higher concentrations observed among children than adults (7-12). Although phthalates are rapidly metabolized, the ubiquity of their metabolites in urine suggests that exposure occurs nearly constantly with contributions from ingestion, inhalation, and dermal absorption (13). Personal air concentrations of two phthalates, diethyl phthalate (DEP), which is more volatile than most other phthalates, and butylbenzyl phthalate (BBzP), which is found on respirable particles, were correlated with maternal urinary concentrations of the corresponding metabolites suggesting inhalation as an important route of exposure (8, 11).

In a cross-sectional case-control analysis of Swedish children ages 3-8 years, di(2-ethylhexyl) phthalate (DEHP) and BBzP in bedroom dust were associated with physician-
confirmed asthma and rhinitis, respectively (14). In a cross-sectional analysis of adults from the National Health and Nutrition Examination Survey (NHANES), urinary concentrations of monoethyl phthalate (MEP), the metabolite of DEP, and metabolites of di-n-butyl phthalate (DnBP) and di-isobutyl phthalate were associated with decreases in forced expiratory volume in 1 sec (FEV$_1$) (15). While these findings with asthma, rhinitis and airflow obstruction suggest the potential for increased airway inflammation with phthalate exposure, it has not been demonstrated.

The measurement of fractional exhaled nitric oxide (FeNO), a biomarker of subclinical airway inflammation, offers a method to test for associations between phthalate exposure and airway inflammation (16). Prospective studies in children reported associations of higher FeNO with subsequent exacerbations in asymptomatic asthmatic children after withdrawal of inhaled corticosteroids and increased risk of new-onset asthma among non-asthmatic children (17-19). FeNO is responsive to environmental pollutants that contribute to respiratory health problems. Exposure to inhalant air pollutants such as respirable particulate matter (PM$_{2.5}$), black carbon, and formaldehyde, has been associated positively with FeNO (20-23). We are unaware of any population-based studies examining the association between phthalates and FeNO. Airway inflammation and FeNO are increased in both seroatopic and asthmatic children; thus these children may be more responsive to exposures that affect airway inflammation.

Based on the prior human observational studies, we hypothesized that higher urinary concentrations of four phthalate metabolites: MEP, mono-$n$-butyl phthalate (MnBP; the metabolite of DnBP), monobenzyl phthalate (MBzP; the major metabolite of BBzP), and mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP; a metabolite of DEHP) would be associated with higher concurrent FeNO in children. We also hypothesized that children with seroatopy and
wheeze, as surrogate measures of allergy and the hyperresponsive airways of asthma, would be more susceptible to airway inflammation triggered by these environmental exposures.

**Methods**

Participants were selected based on available samples from the Columbia Center for Children’s Environmental Health (CCCEH) study, a longitudinal birth cohort that enrolled 727 nonsmoking Dominican and African American pregnant women free of hypertension, diabetes, and known HIV living in Northern Manhattan and the South Bronx (24). While expectant mothers were recruited from neighborhoods that also had high asthma prevalence (25), women were not recruited based on reported history of asthma or allergy. Children (ages 4.9-9.1 years) included in these analyses provided at least one spot urine sample during an office visit when FeNO was measured between 2006 and 2010. This study was approved by the institutional review boards of Columbia University and the Centers for Disease Control and Prevention (CDC).

Phthalate metabolites in urine collected between mid-morning and early evening were measured at the CDC as described (8, 10). We used urinary concentrations of MEHHP as the proxy for exposure to DEHP (justification in online supplement). Correction factors of 0.66 and 0.72 were applied to the MEP and MBzP concentrations, respectively, to adjust for previous overestimations of the analytic standards (further details in online supplement) (12).

*FeNO collection*
FeNO was collected with a modified offline device along with ambient NO as described previously and in the online supplement (26-28). FeNO data were excluded if the child reported having a cold at the time of the measurement.

**Seroatopy measurement**

Seroatopy was defined as specific IgE to dust mite, cockroach, or mouse allergens (≥0.35 IU/ml) by ImmunoCap (Phadia, Uppsala, Sweden) in serum collected within one year of the FeNO measure (29).

**ISAAC wheeze by questionnaire**

Mothers were asked whether their child had wheezed in the past 12 months at age 5, 6, 7 and 9 years using the International Study of Allergy and Asthma in Children (ISAAC) questionnaire (30). Children with a report of wheeze on the questionnaire administered on the day of FeNO collection or at the next study visit (within one year) were classified as having wheezed.

**Modeled probability of wheeze**

Because reports on single questionnaires may not characterize the episodic and variable timing of wheeze in childhood, a larger set of repeated questionnaires also was used to model the probability of wheezing in the last three months as a continuous measure at each FeNO collection for all \( n=313 \) observations. Four childhood wheeze phenotypes (patterns) were identified using latent class growth analysis, and the posterior probabilities of phenotype membership as well as the probabilities of wheezing in each phenotype were estimated for each child in the study at the age of FeNO measurement (31).

**Statistical analysis**
Individual children had 1-3 observations with phthalate and FeNO collections; therefore, repeated measures were modeled using generalized estimating equations (GEE) with robust standard errors. Models included specific gravity to adjust for urinary dilution (32), as well as potential confounders; age, sex, race/ethnicity, time of day of FeNO collection and ambient NO. Phthalate metabolite, FeNO, and ambient NO variables were log transformed due to the right skew and high variance at the upper range of their distributions. Analyses were conducted using R version 2.13.1 (33-35).

Results

The 244 participants included 125 girls and 119 boys (Table 1). Of the 244 children enrolled who provided at least one measure for both phthalates and FeNO, 65 had two measures, and two children had three, resulting in a total of n=313 paired observations of phthalate metabolites and FeNO. The demographics of the children included in the analyses were similar to those who were enrolled, but excluded from these analyses, except a slightly higher frequency of first-born children (Table E1 in online supplement). Exposures to the phthalates examined were widespread as suggested by the detection of the metabolites of all four phthalates in 100% of urine samples (Table E2). The concentrations of the four phthalate metabolites within each sample were positively correlated with a range of pair-wise Spearman’s correlations from rho = 0.21-0.68 (See Online Table E3).

To characterize predictors of children’s phthalate metabolites urinary concentrations, GEE models with age, sex, and race/ethnicity as predictors were fit for each of the four log-transformed metabolite concentrations also including specific gravity as a covariate. The urinary
concentrations of three of the metabolites were higher in girls than in boys: MEP (41% higher, 95% CI, 8.0% to 83%, p=0.011), MnBP (32% higher, 95% CI 9.0% to 60%, p=0.005), and MEHHP (45% higher, 95% CI 16% to 81%, p=0.001). There was no significant difference by age or race/ethnicity for the urinary concentrations of any of the metabolites.

Phthalate metabolites and FeNO

FeNO measures ranged from 2.3 to 71.6 ppb with a median of 7.9 ppb and an interquartile range from 5.7 to 12.3 ppb and were approximately log-normally distributed. In a GEE model of FeNO with age, sex, race/ethnicity, time of day and ambient NO concentration as predictors, only ambient NO concentration was significantly positively associated with FeNO. In four separate adjusted models, FeNO was associated with urinary concentrations of MEP, MnBP, and MBzP but not MEHHP, after controlling for specific gravity, age, sex, race/ethnicity, time of day of FeNO collection and ambient NO concentration (Table 2). The effect size for MnBP was slightly larger than for MEP and MBzP although all three were very similar and were consistent with linearity in adjusted models using quintiles of urinary concentrations (Figure 1). For example, there was a 6.8% increase in FeNO (95% CI 1.1% to 12.9%, p=0.019) for each log-unit increase in MBzP concentrations, adjusting for covariates. Similarly, a log-unit increase in MEP concentration was associated with a 6.5% increase in FeNO (95% CI 1.0% to 12.4%, p=0.021) adjusted for covariates, while a log unit change in MnBP concentration was associated with a 8.5% increase in FeNO (95% CI 0.2% to 17.6%, p=0.045) adjusting for covariates. Effect estimates of the metabolites on FeNO were altered less than 10% in adjusted analyses including potential confounding variables individually: oral/inhaled corticosteroid use, older siblings, number of people living in the home, type of heating, having a smoker in the home, and
indicators of socioeconomic status, maternal education and material hardship (data not shown) (36). There was no significant interaction by child sex (data not shown).

Seroatopy measured within one year was available for 88% of observations \(n=274/313\) from 217 children with 31% of measures classified as seroatopic at their FeNO observation \(n=86/274\). Adjusting for seroatopy did not substantially alter the estimates for the individual phthalate metabolites (Table 2). In sensitivity analyses similar results were seen when restricting to seroatopy measured on the day of FeNO collection \(n=160\), online supplement Table E4).

In an adjusted multi-pollutant model of FeNO including concentrations of all four metabolites (MEP, MnBP, MBzP, and MEHHP) that was also adjusted for seroatopy, MEP and MBzP remained statistically significant independent predictors of FeNO with similar effect size estimates to those in the single metabolite models as shown in Table 2. While MEHHP concentrations remained non-significant as in the single metabolite models, the single-metabolite beta estimate for MnBP decreased from 0.080 in the seroatopy adjusted model to a beta of -0.016 in the multi-pollutant model and was no longer statistically significantly associated with FeNO after adjusting for the other phthalate metabolite concentrations \(p=0.77\). Therefore, the MnBP association with FeNO seen in the single phthalate model may be due to the high correlation between MnBP and MBzP (Spearman’s correlation = 0.68). In the adjusted four-pollutant model, a log-unit higher concentration of MEP and MBzP was associated with a 6.6% increase (95% CI 0.5% to 13.1%, \(p=0.034\)) and a 8.7% increase (95% CI 1.9% to 16.0%, \(p=0.011\)) in FeNO respectively after adjusting for all four metabolite concentrations, seroatopy, specific gravity, age, sex, race/ethnicity, time of day of FeNO collection and ambient NO.
There was no direct association between the urinary concentrations of any of the four metabolites with incident seroatopy or reported wheeze (see online supplement). As seen in Figure 2, FeNO was higher among children with seroatopy (geometric mean (GM) 11.6 ppb, $n=86$) than those without seroatopy (GM 7.5 ppb, $n=188$; p<0.001). Although the slopes of the MEP or MBzP and FeNO associations were slightly larger among those with seroatopy than those without seroatopy (Figure 2); testing interaction terms in the GEE models showed that the differences in the slopes were not statistically significant (p=0.51 and p=0.60).

We also assessed the interaction between phthalate concentrations and wheeze using data from the ISAAC questionnaire responses to test whether children who wheeze are more susceptible to airway inflammation associated with phthalate exposure. Overall, 23% of children had a report of wheezing in the past 12 months at their FeNO observation or their next questionnaire collected within 12 months (available for $n=284$ observations). There was no significant interaction between concentrations of MEP, MnBP, or MEHHP and report of wheeze in the past year in the association with FeNO (p=0.96, 0.17, and 0.52, respectively). There was a significant positive interaction of the MBzP and FeNO association by ISAAC wheeze (Figure 3; p=0.016, $n=284$). In sensitivity analyses, this interaction was stronger and remained significant when restricted to ISAAC wheeze questionnaires asked on the same day as FeNO collection (p=0.011, $n=187$).

As a further exploration of the interaction between phthalates and wheeze, a latent class growth analysis was used to model the probability of wheezing in the past three months. This LCGA included 7048 questionnaires on report of wheeze in the past 3 months from 15 timepoints between 3 months and 9 years of age in a larger subset ($n=689$) of the children in the CCCEH study (31). The resulting predicted probability of wheezing was available for all $n=313$
observations and ranged from 0.01 to 0.66 although they were highly skewed with a median of 0.04, a 75th percentile of 0.08, and a 95th percentile of 0.44. There was no evidence of interaction between concentrations of MEP, MnBP, or MEHHP and LCGA predicted probability of wheeze on FeNO. However, as in the analysis using a dichotomous ISAAC wheeze question, the interaction between MBzP urinary concentrations and the LCGA probability of wheezing on FeNO was positive and highly significant (p=0.006). The association between concentrations of MBzP and FeNO was of a larger magnitude among those with a higher probability of wheezing (Figure 4).

**Discussion**

In this observational study of children, urinary concentrations of three phthalate metabolites (MEP, MnBP, and MBzP) were associated positively with FeNO measured on the same day in separate adjusted models. This is the first population-based study to show an association between phthalate exposure and this measure of airway inflammation. All three associations remained largely unchanged after adjusting for seroatopy. When all four metabolites (MEP, MnBP, MBzP, MEHHP) were included in the same adjusted model, MEP and MBzP remained independent predictors of higher FeNO, while concentrations of MnBP, which are highly correlated with concentrations of MBzP, were no longer associated with FeNO. These results suggest that urinary biomarkers of exposure to two phthalates, believed to have substantial inhalational exposure, are associated with a measure of subclinical airway inflammation in children.
This observational study of children is the first to report a positive association between biomarkers of exposure to phthalates and FeNO. The methodology for measuring FeNO allows for non-invasive collection and measurement of NO produced by resident airway cells in response to inflammatory cytokines and mediators (37). While its diagnostic utility in children remains debated (37, 38), FeNO has been established as a biomarker of airway inflammation in response to air pollutants with more than a decade of epidemiology studies (20, 22, 23, 39, 40). In population-based studies, the majority of asthmatics have mild disease with infrequent exacerbations, making temporal associations between environmental exposures and exacerbations difficult to establish. Elevated FeNO reflects eosinophilic airway inflammation in response to known asthma triggers and has been associated both with increased symptoms among asthmatics and with the development of asthma among non-asthmatic children (20, 41). As such, it offers an objective biomarker to detect subclinical variations in airway inflammation reflecting increased risk for exacerbation. Our findings of associations with phthalates and FeNO contrast with those from two small controlled chamber studies of adults exposed to polyvinyl chloride surfaces in which there was no increase in post- versus pre-challenge FeNO (42, 43).

We hypothesized that children with report of wheeze in the last 12 months would be more susceptible to airway inflammation triggered by phthalate exposures than the non-wheezing children in our cohort. We observed a positive interaction between MBzP urinary concentrations and wheeze. There was also a positive interaction between urinary concentrations of MBzP and the probability of wheezing predicted from a latent class growth analysis of questionnaire data, suggesting that the MBzP and FeNO association is stronger among a subset of children who wheeze. Although wheeze itself is episodic, the LCGA-based prediction was based on the pattern of wheezing at up to 15 time points between 3 months and 9 years of age.
and may offer a more stable indication of children with airway disease than a single questionnaire response. An additional advantage of the LCGA-based wheeze prediction is that it could be computed for all observations and derived a continuous variable and therefore also may be more informative than a dichotomous classification. Direct comparison of our two wheezing measures is difficult, because the LCGA derived probability of wheeze over 3 months was based on a different question than the ISAAC wheeze and uncertainty estimates from the original LCGA model were not propagated into the LCGA-based probability of wheeze. The consistency of these observations using two different measures of wheeze lends support to these findings. Although steroids affect the strength of the correlation between FeNO and wheeze (44), the positive interaction remained after adjusting for or excluding children with reported use of corticosteroids (data not shown).

Urinary concentrations of MBzP recently have been shown to be associated with a non-specific marker of systemic inflammation in a large population-based analysis. MBzP concentrations were associated with increased serum C-reactive protein (CRP) in a dose-dependent fashion among 8,346 NHANES participants (45). An interquartile range higher urinary MBzP concentration was associated with an average of 6.0% (95% CI 1.7% to 10.8%) higher CRP, and this association held in analyses restricted to younger NHANES participants 6-12 and 13-19 years old. However, the mechanism through which phthalates are associated with systemic or airway inflammation remains unclear. Some phthalate metabolites, especially MEHP and MBzP activate the ubiquitously expressed nuclear peroxisome proliferator-activated receptors (PPAR) α and γ (46), which are ligand-activated transcription factors important in a variety of physiological processes including airway inflammation and airway remodeling. For example, it has been shown that the expression of PPARγ is higher in the bronchial submucosa,
airway epithelium, and smooth muscle of asthmatics than controls and is associated with decrements in lung function (47).

Diurnal variations both in measures of urinary phthalates and FeNO have been reported, allowing for the potential for confounding of an association between them. Among adults and children enrolled in NHANES, MEP was higher during midday collections while MBzP was non-significantly higher during the evenings (7). However, a panel study of adults found that 76% of the variance in urinary MEP was explained by between-person differences (48). There are conflicting findings in the literature about whether FeNO has circadian variation (49), but at least one study in children reported higher FeNO measured in the morning as compared with evening (50). We recorded the time of day of FeNO, but not urine collection. Nonetheless, FeNO and urine were collected during a single office visit that typically lasted less than 2 hours. Therefore, the robustness of the findings after adjustment for time of day of FeNO collection reduces the likelihood that these observations were due to confounding by the time of collection.

One strength of the cross-sectional study design is that it allows for the observation of effects that may be short-lived in response to exposures that vary over time. Studies of exposure to air pollutants, like elemental carbon (22), have demonstrated increased FeNO hours after exposure, suggesting the importance of assessing the impact of pollutant exposures on FeNO in short time windows. Associations between FeNO and phthalate metabolites were evaluated cross-sectionally. However, it will be important in future studies to determine the relevance of chronic exposure to DEP and BBzP and elevated FeNO to airway disease.

This study had several limitations. In an observational study, urinary concentrations of particular phthalate metabolites also may indicate exposure to other chemicals that share sources or chemical properties with the parent phthalates. For example, the mothers of the children in
this analysis who reported the use of perfume or an index of uses of other personal care products during pregnancy had higher urinary concentrations of MEP, the main metabolite of DEP, than other pregnant women within the same cohort (51). These participants also can be expected to have higher exposure to other chemicals in those products that could confound observed associations, such as artificial fragrances as seen in sampling of indoor air (52). The substantially higher urinary concentrations of MEP in girls than boys warrants further investigation as the burden of asthma and respiratory disease differentially increases for girls in adolescence, when exposure to DEP might be expected to remain high (53). Further, with phthalates, exposures occur to mixtures of correlated compounds. For example, in this study MnBP and MBzP were highly correlated (rho = 0.68), which may be due to shared sources of exposure and the fact that MnBP is a minor product of BBzP (6%) (54). MBzP has been shown previously to be a more reproducible biomarker than MnBP in spot urine samples collected over six months from children in New York City (9), its concentration may serve as a better marker of the mixture of related compounds than does the concentration of MnBP.

Conclusion

We report cross-sectional associations between children’s urinary concentrations of three phthalate metabolites and FeNO, a marker of airway inflammation. Concentrations of both MEP and MBzP remained associated with FeNO in a four-pollutant model. The association of concentrations of MBzP and FeNO was significantly stronger among children in this study with as compared to those without reported wheeze. The children with wheeze are presumed to have hyper-reactive airways more susceptible to environmental exposures. These findings suggest a
role for a ubiquitous exposure on airway inflammation in a susceptible population. Future studies can prospectively follow these children to observe whether associations persist through childhood and the long-term consequences to respiratory health of increased airway inflammation in children.
Acknowledgements

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Disclaimer

The findings and conclusions in this paper are those of the authors and do not necessarily represent the views of the CDC.
References


Figure Legends

Figure 1. The association between urinary concentrations of three phthalate metabolites: MEP, MnBP, and MBzP and FeNO from separate models (n=313 observations from 244 children). Adjusted estimates for each quintile are shown as horizontal segments. The continuous linear association is shown as a wider grey line. Both quintile and continuous model estimates are adjusted and shown at mean levels of specific gravity, age, sex, race/ethnicity, time of day of FeNO collection, and ambient NO concentration.

Figure 2. The association between MEP or MBzP urinary concentrations and FeNO stratified by seroatopy of the child (n=188 and n=86 observations from 217 children). Boxplots show the distribution of FeNO concentrations is higher among atopes than non-atopes (p<0.001). The interaction terms testing for a difference in slopes were not statistically significant (p=0.51 and p=0.60), shown at the mean specific gravity, age, sex, race/ethnicity, time of day of FeNO collection, and ambient NO concentration. Main effects for both metabolites remained statistically significant after adjusting for seroatopy without interaction.

Figure 3. The association between MBzP urinary concentrations and FeNO varies by whether the mother reported on the ISAAC questionnaire, administered on the day of FeNO collection or at the next study visit (within one year), that the child had wheezing or whistling in the chest in the past 12 months (n=284 observations from 231 children). The interaction term between MBzP and the report of wheeze was positive and significant (p=0.016) in the adjusted model shown at the mean levels of specific gravity, age, sex, race/ethnicity, time of day of FeNO collection, and ambient NO concentration.
**Figure 4.** The association between MBzP urinary concentrations and FeNO is stronger among children predicted to wheeze \((n=313\) observations from 244 children). The area of points is proportional to the probability of wheezing predicted from a latent class growth analysis (continuous variable ranging from 0.01 to 0.66). The three lines display the MBzP and FeNO association for participants at the median, 75\(^{th}\) percentile, and the 95\(^{th}\) percentile of the probability of wheeze shown at the mean specific gravity, age, sex, race/ethnicity, time of day of FeNO collection, and ambient NO concentration from a multivariable GEE model. The interaction term between MBzP and the probability of wheezing was positive and highly significant in the model \((p = 0.006)\).
Table 1. Demographics of participants and distribution of urinary metabolites stratified by wheeze and seroatopy (n=244). †

<table>
<thead>
<tr>
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<th>Overall (n=244)</th>
<th>Seroatopy‡ (n=153)</th>
<th>Wheeze§ (n=64)</th>
<th>Overall (n=177)</th>
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<td>no (n=153)</td>
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<td>6.3 (1.2)</td>
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<td>70/153 (46)</td>
<td>36/64 (56)</td>
<td>84/177 (47)</td>
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<td>19/64 (30)</td>
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<td>104/153 (68)</td>
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<td>52/153 (34)</td>
<td>20/64 (31)</td>
<td>58/177 (33)</td>
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<td>First born, no older siblings, n (%)</td>
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<td>70/153 (46)</td>
<td>42/64 (66)*</td>
<td>92/177 (52)</td>
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<td>59/153 (39)</td>
<td>29/64 (45)</td>
<td>66/177 (37)</td>
<td>28/54 (52)</td>
</tr>
<tr>
<td>Urinary metabolites, unadjusted geometric means in ng/ml (95% CI)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEP††</td>
<td>111 (96-129)</td>
<td>109 (91-131)</td>
<td>122 (90-165)</td>
<td>116 (97-139)</td>
<td>109 (78-154)</td>
</tr>
<tr>
<td>MnBP</td>
<td>41 (36-46)</td>
<td>38 (32-45)</td>
<td>48 (38-60)</td>
<td>40 (35-47)</td>
<td>39 (29-53)</td>
</tr>
<tr>
<td>MBzP††</td>
<td>24 (20-28)</td>
<td>22 (18-27)</td>
<td>30 (22-41)</td>
<td>22 (18-27)</td>
<td>24 (16-34)</td>
</tr>
</tbody>
</table>
Footnotes for Table 1.

*P<0.05 difference in demographic variables between children with and without seroatopy or with and without wheeze.

†Potentially time varying variables (e.g., wheeze and seroatopy) are reported for the children at the youngest age measured among the children with metabolites and FeNO measured at multiple ages.

‡Specific IgE to dust mite, cockroach, or mouse allergens (≥0.35 IU/ml) in serum collected within one year of the FeNO measure.

§Children with a report of wheeze on the ISAAC questionnaire following FeNO collection were classified as having wheezed.

¶African-American race and Dominican Republic ethnicity were reported for the mother.

**Mother reported at closest questionnaire that she and her family could not afford needed food, rent, clothing or medical care or that gas/electricity was suspended because of bill non-payment in the previous 6 months.

††Corrected for updated CDC standards as of January 2012 as described in the online supplement.
**Table 2.** Adjusted* percent difference in fractional concentration of nitric oxide for a one log-unit higher phthalate metabolite urinary concentration from GEE regression models

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>% Difference (95% CI)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEP</td>
<td>6.5 (1.0, 12.4)</td>
<td>0.021</td>
</tr>
<tr>
<td>MnBP</td>
<td>8.5 (0.2, 17.6)</td>
<td>0.045</td>
</tr>
<tr>
<td>MBzP</td>
<td>6.8 (1.1, 12.9)</td>
<td>0.019</td>
</tr>
<tr>
<td>MEHHP</td>
<td>2.7 (-3.3, 9.1)</td>
<td>0.38</td>
</tr>
</tbody>
</table>

Single metabolites, separate models adjusted for seroatopy *(n=274)*

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>% Difference (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEP</td>
<td>6.8 (1.0, 12.9)</td>
<td>0.020</td>
</tr>
<tr>
<td>MnBP</td>
<td>8.4 (-0.1, 17.5)</td>
<td>0.053</td>
</tr>
<tr>
<td>MBzP</td>
<td>8.3 (2.3, 14.8)</td>
<td>0.007</td>
</tr>
<tr>
<td>MEHHP</td>
<td>2.1 (-4.3, 9.0)</td>
<td>0.53</td>
</tr>
</tbody>
</table>

All four metabolites in one model adjusted for seroatopy *(n=274)*

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>% Difference (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEP</td>
<td>6.6 (0.5, 13.1)</td>
<td>0.034</td>
</tr>
<tr>
<td>MnBP</td>
<td>-1.5 (-11.1, 9.1)</td>
<td>0.77</td>
</tr>
<tr>
<td>MBzP</td>
<td>8.7 (1.9, 16.0)</td>
<td>0.011</td>
</tr>
<tr>
<td>MEHHP</td>
<td>0.0 (-6.4, 6.9)</td>
<td>0.99</td>
</tr>
</tbody>
</table>

**Footnote for Table 2.**

*All models adjusted for specific gravity, sex, race/ethnicity, age, time of day of FeNO collection, and ambient NO concentration.
Figure One

Concentration of FeNO, ppb

Urinary concentration of MEP, log(ng/ml)

Concentration of FeNO, ppb

Urinary concentration of MnBP, log(ng/ml)

Concentration of FeNO, ppb

Urinary concentration of MBzP, log(ng/ml)
Fig 2A (top) Fig 2B (bottom)

### Non-seroatopic, n=188

- Urinary concentration of MEP, log(ng/ml)
- Concentration of FeNO, ppb

### Seroatopic, n=86

- Urinary concentration of MEP, log(ng/ml)
- Concentration of FeNO, ppb
Figure 3

Non-wheeze, n=218

Wheeze, n=66

Concentration of FeNO, ppb

Urine concentration of MBzP, log(ng/ml)
ONLINE DATA SUPPLEMENT

Children’s urinary phthalate metabolites and fractional exhaled nitric oxide in an urban cohort

Allan C. Just, Robin M. Whyatt, Rachel L. Miller, Andrew G. Rundle, Qixuan Chen, Antonia M. Calafat, Adnan Divjan, Maria J. Rosa, Hanjie Zhang, Frederica P. Perera, Inge F. Goldstein, Matthew S. Perzanowski
Methods

Urinary metabolite used to estimate DEHP exposure

Di(2-ethylhexyl) phthalate (DEHP) has several metabolites. We used the urinary concentration of mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) as the proxy for exposure to DEHP because it has a longer half-life and higher detection frequency than mono(2-ethylhexyl) phthalate (MEHP) (E1). In this dataset, those two metabolites had a rank correlation of 0.85 (p<0.001, n=313).

Correction of phthalate metabolites

Measured concentrations of monoethyl phthalate (MEP) and monobenzyl phthalate (MBzP) in urine were adjusted by a factor of 0.66 and 0.72, respectively, to correct for analytical standards of inadequate purity following a CDC communication in January 2012 (E2). This linear adjustment had no impact on the magnitude or significance of any of the reported associations. However, urinary metabolite concentrations of MEP or MBzP from already published studies using uncorrected metabolite concentrations reported by the CDC through December 2011 would need to be adjusted in the same manner prior to any comparison with these concentrations.

Sample collection of FeNO

The protocol for FeNO collection has been described previously following guidelines from the American Thoracic Society (E3-E5). FeNO was collected in mylar bags after diverting to discard the first portion of the breath, approximately 1-3 seconds of exhalation of dead-space air, using an offline device (#CBSK 01400, GE Instruments, Boulder, CO). Children were coached through attempts to gather three valid maneuvers in which they exhaled at 83 ml/sec and inhaled through the NO scrubber. Samples were not considered valid and were excluded if the
child 1) did not inhale through the NO scrubber and the ambient NO was >20 ppb, or 2) if ambient NO was >100 ppb (regardless of inhalation through scrubber). Data analysis used the mean of the valid attempts for each child during their visit.

Use of glucocorticoid steroids

At the time of FeNO collection, mothers were asked about their child’s use of medications. Specifically, mothers were asked, “Is the child currently taking any medications for asthma or allergies? What are they taking?” Children were classified as potentially taking corticosteroids if the response included any of the following 11 medication names: Flovent, Advair, Pulmicort, Beclovent, Vanceril, Acrobid, Azmacort, Budesonide, Prednisone, Prelone, Pediapred. Among the 313 observations, there were 18 (6%) classified as potentially taking corticosteroids and only one instance in which one of these medications was reported as in use on the day of the FeNO collection. As reported in the manuscript, adjustment for these children potentially taking corticosteroids did not alter the effect size estimates for the phthalate metabolites in the single metabolite models indicating that report of corticosteroids does not confound the observed associations. Finally, the interaction of MBzP and wheeze in association with FeNO remained essentially unchanged when restricted to exclude observations with reported use of corticosteroids (data not shown) which demonstrates that this group does not account for the observed interaction.

Postnatal urinary phthalates and incident seroatopy

While exposure to phthalates is likely to occur at all ages, a cross-sectional analysis with seroatopy may be inappropriate as the onset of allergic sensitization to aeroallergens is largely non-reversible and incidence is spread over the childhood period. However, repeated sampling of both phthalate metabolites urinary concentrations and seroatopy at various ages in this cohort
makes possible an analysis of the association of childhood exposure with later incident seroatopy. This analysis can be done on a larger subset of children in the CCCEH study without restricting to those with measures of FeNO.

Concentrations of phthalate metabolites were measured in urine samples collected at age 3 and 5 years at the CDC as previously described. Seroatopy at a particular age was defined as specific IgE (≥0.35 IU/ml) to dust mite, cockroach, or mouse in samples from age 2 or 3 years (combined), age 5, or age 7. Incident seroatopy at age 5 or 7 years was defined as new cases of seroatopy among those who had one or more samples collected at previous ages and had no previous positive specific IgE results. Thus, a child developing seroatopy between age 3 and 5 would not be included in the susceptible population at age 7. Logistic regression models using generalized estimating equations modeled incident seroatopy at age 5 and 7 and phthalate metabolite urinary concentrations two years prior adjusting for specific gravity, sex, race/ethnicity, age at atopy measure, and time between the phthalate and atopy measure.

**Results**

*Time of day and FeNO concentration*

We explored the association between the time of day that FeNO was collected and FeNO concentration. There was no association between time of day and FeNO after adjusting for covariates (particularly ambient NO concentration) and allowing for flexibility in the shape of the association. Using a generalized additive mixed model with penalized splines, the parameter estimating the time of day of FeNO collection simplifies to a non-significant linear term (p=0.41)
in a model adjusted for our set of other potential confounders (sex, race/ethnicity, age, and ambient NO concentration).

Postnatal urinary phthalates and incident seroatopy

There were 240 children with phthalate metabolites measured at age 3 years, no seroatopy at age 2 or 3, and a seroatopy measure at age 5, as well as 198 children with phthalate metabolites measured at age 5, no seroatopy in available samples from ages 2, 3, or 5, and a measure of seroatopy at age 7. Overall, there were 438 observations with these available data from 321 children. There were 41/240 (17%) and 34/198 (17%) new incident cases of seroatopy at age 5 and 7 years respectively. There was no association between any of the individual metabolites of interest and incident seroatopy in the following two years (Supplemental Materials Table E2).

We have reported previously that there was no association between a maternal urinary concentration of MBzP and the development of seroatopy by age 5 years in this population (E6). In this analysis we also did not see any association between phthalate metabolite concentrations measured at age 3 or age 5 and the development of seroatopy over the following two years.

Phthalate metabolites and wheeze

In both the subset with FeNO data and a larger group of children from the full CCCEH cohort with available data, there was no consistent association between individual urinary concentrations of the four metabolites of interest and concurrent report of wheeze at age 5 or at age 7 (Supplemental Materials Table E3).
References


**Table E1.** Demographics of mothers and child at enrollment among those included in the analyses \((n=244)\) and those recruited who were not included.

<table>
<thead>
<tr>
<th></th>
<th>Included ((n=244))</th>
<th>Enrolled but not included ((n=483))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mother</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, mean (SD)</td>
<td>25.1 (4.8)</td>
<td>25.2 (5.0)</td>
</tr>
<tr>
<td>African-American race, (n) (%)</td>
<td>80/244 (33)</td>
<td>174/483 (36)</td>
</tr>
<tr>
<td>Dominican ethnicity, (n) (%)</td>
<td>164/244 (67)</td>
<td>309/483 (64)</td>
</tr>
<tr>
<td>Maternal asthma, (n) (%) †</td>
<td>61/244 (25)</td>
<td>102/483 (21)</td>
</tr>
<tr>
<td>No high school degree, (n) (%)</td>
<td>81/244 (33)</td>
<td>176/470 (37)</td>
</tr>
<tr>
<td>Receiving Medicaid, (n) (%)</td>
<td>226/243 (93)</td>
<td>431/480 (90)</td>
</tr>
<tr>
<td>Material hardship, (n) (%) ‡</td>
<td>96/243 (40)</td>
<td>225/480 (47)</td>
</tr>
<tr>
<td>Smoker in home, (n) (%)</td>
<td>74/244 (30)</td>
<td>175/481 (36)</td>
</tr>
<tr>
<td><strong>Study child</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male, (n) (%)</td>
<td>119/244 (49)</td>
<td>232/483 (48)</td>
</tr>
<tr>
<td>First born, no older siblings, (n) (%)</td>
<td>127/244 (52)</td>
<td>201/483 (42)*</td>
</tr>
</tbody>
</table>

**Footnotes for Table E1.**

*\(P<0.05\) comparing children included with those enrolled but not included.

†Mother reported either during pregnancy or on a questionnaire 3 months after delivery that she had asthma.

‡Mother reported during pregnancy that in the past 6 months she and her family could not afford needed food, rent, clothing or medical care or that gas/electricity was suspended because of bill non-payment.
Table E2: Distributional summary for unadjusted urinary metabolite concentrations (ng/ml) measured in 313 spot samples from 244 New York City children 4.9 to 9.1 years old.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>n</th>
<th>&gt;LOD*</th>
<th>Min</th>
<th>25%</th>
<th>50%</th>
<th>75%</th>
<th>95%</th>
<th>Max</th>
<th>GM (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEP‡</td>
<td>313</td>
<td>100%</td>
<td>7</td>
<td>51</td>
<td>106</td>
<td>228</td>
<td>1267</td>
<td>7986</td>
<td>118 (103 to 136)</td>
</tr>
<tr>
<td>MnBP</td>
<td>313</td>
<td>100%</td>
<td>3</td>
<td>22</td>
<td>45</td>
<td>82</td>
<td>186</td>
<td>539</td>
<td>42 (37 to 46)</td>
</tr>
<tr>
<td>MBzP‡</td>
<td>313</td>
<td>100%</td>
<td>1</td>
<td>10</td>
<td>23</td>
<td>50</td>
<td>238</td>
<td>1498</td>
<td>23 (20 to 27)</td>
</tr>
<tr>
<td>MEHHP</td>
<td>313</td>
<td>100%</td>
<td>2</td>
<td>19</td>
<td>44</td>
<td>82</td>
<td>350</td>
<td>1690</td>
<td>43 (38 to 49)</td>
</tr>
</tbody>
</table>

Footnotes for Table E2.

‡Limits of detection (LOD) all less than 1 ng/ml

†Corrected for updated CDC standards as of January 2012 as described in the online supplement
Table E3: Spearman’s Rank Correlation between phthalate metabolite concentrations ($n=313$ urine samples).

<table>
<thead>
<tr>
<th></th>
<th>MEP</th>
<th>MnBP</th>
<th>MBzP</th>
</tr>
</thead>
<tbody>
<tr>
<td>MnBP</td>
<td>0.40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBzP</td>
<td>0.21</td>
<td>0.68</td>
<td></td>
</tr>
<tr>
<td>MEHHP</td>
<td>0.26</td>
<td>0.55</td>
<td>0.35</td>
</tr>
</tbody>
</table>
Table E4: Adjusted* percent difference in fractional concentration of nitric oxide for a one log-unit higher urinary phthalate metabolite concentration from sensitivity analyses of GEE regression models restricting to a subset of seroatopy samples collected on the same day as FeNO.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>% Difference</th>
<th>(95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEP</td>
<td>7.3</td>
<td>(0.1, 15.1)</td>
<td>0.046</td>
</tr>
<tr>
<td>MnBP</td>
<td>9.2</td>
<td>(-0.5, 19.8)</td>
<td>0.063</td>
</tr>
<tr>
<td>MBzP</td>
<td>6.1</td>
<td>(-1.1, 13.8)</td>
<td>0.099</td>
</tr>
<tr>
<td>MEHHP</td>
<td>4.3</td>
<td>(-4.4, 13.8)</td>
<td>0.35</td>
</tr>
</tbody>
</table>

Footnote for Table E4.

*All models adjusted for specific gravity, seroatopy, sex, race/ethnicity, age, time of day of FeNO collection and ambient NO concentration.
Table E5: Adjusted Odds Ratios for a log unit change in children’s phthalate metabolite concentrations at age 3 or 5 and incident seroatopy over the following two years (n=438 observations from 321 children).

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>OR</th>
<th>(95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEP</td>
<td>1.07</td>
<td>(0.84 to 1.37)</td>
<td>0.56</td>
</tr>
<tr>
<td>MnBP</td>
<td>0.89</td>
<td>(0.66 to 1.20)</td>
<td>0.43</td>
</tr>
<tr>
<td>MBzP</td>
<td>1.08</td>
<td>(0.89 to 1.32)</td>
<td>0.42</td>
</tr>
<tr>
<td>MEHHP</td>
<td>1.07</td>
<td>(0.80 to 1.45)</td>
<td>0.64</td>
</tr>
</tbody>
</table>

Footnote for Table E5.

*Models adjusted for specific gravity, age at atopy measure, time since phthalate measure, sex, and race/ethnicity
Table E6: Adjusted* odds ratio estimates for report of wheeze in the past year on ISAAC questionnaires at age 5 and 7 for a log unit increase of urinary phthalate metabolite concentration from separate single metabolite models restricted to children in the FeNO analysis and those with available data in the larger CCCEH dataset.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>OR</th>
<th>(95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEP</td>
<td>0.81</td>
<td>(0.60, 1.08)</td>
<td>0.15</td>
</tr>
<tr>
<td>MnBP</td>
<td>0.92</td>
<td>(0.64, 1.32)</td>
<td>0.63</td>
</tr>
<tr>
<td>MBzP</td>
<td>0.94</td>
<td>(0.72, 1.22)</td>
<td>0.63</td>
</tr>
<tr>
<td>MEHHP</td>
<td>1.05</td>
<td>(0.75, 1.45)</td>
<td>0.79</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>OR</th>
<th>(95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEP</td>
<td>0.91</td>
<td>(0.74, 1.11)</td>
<td>0.35</td>
</tr>
<tr>
<td>MnBP</td>
<td>0.93</td>
<td>(0.71, 1.21)</td>
<td>0.59</td>
</tr>
<tr>
<td>MBzP</td>
<td>1.00</td>
<td>(0.83, 1.22)</td>
<td>0.98</td>
</tr>
<tr>
<td>MEHHP</td>
<td>1.12</td>
<td>(0.87, 1.45)</td>
<td>0.37</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>OR</th>
<th>(95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEP</td>
<td>1.03</td>
<td>(0.75, 1.42)</td>
<td>0.84</td>
</tr>
<tr>
<td>MnBP</td>
<td>1.05</td>
<td>(0.66, 1.67)</td>
<td>0.84</td>
</tr>
<tr>
<td>MBzP</td>
<td>1.12</td>
<td>(0.81, 1.54)</td>
<td>0.50</td>
</tr>
<tr>
<td>MEHHP</td>
<td>1.29</td>
<td>(0.92, 1.80)</td>
<td>0.14</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>OR</th>
<th>(95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEP</td>
<td>1.09</td>
<td>(0.86, 1.38)</td>
<td>0.49</td>
</tr>
<tr>
<td>MnBP</td>
<td>0.91</td>
<td>(0.66, 1.26)</td>
<td>0.58</td>
</tr>
<tr>
<td>MBzP</td>
<td>1.07</td>
<td>(0.85, 1.34)</td>
<td>0.57</td>
</tr>
<tr>
<td>MEHHP</td>
<td>1.14</td>
<td>(0.90, 1.43)</td>
<td>0.27</td>
</tr>
</tbody>
</table>

Footnote for Table E6.

*Models adjusted for specific gravity, sex, and race/ethnicity