

# Characterizing the joint effects of pesticide exposure and criteria ambient air pollutants on pediatric asthma morbidity in an agricultural community

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**Background:** Environmental contributions to pediatric asthma morbidity have been studied extensively in urban settings; exposures characteristic of agricultural and rural communities have received less attention despite a comparable burden of morbidity.

**Methods:** We obtained repeated urine samples ( $n = 139$ ) from 16 school-age children with asthma in the Yakima Valley of Washington State between July and October 2012. Biomarkers of organophosphate (OP) pesticide exposure (dialkyl phosphates [DAPs]) and asthma exacerbation (leukotriene E4 [LTE4]) were analyzed in samples. Corresponding 24-hour average particulate matter  $<2.5$   $\mu\text{g}$  ( $\text{PM}_{2.5}$ ) and maximum 8-hour ozone concentration data for the study period were available from local monitoring stations. We evaluated the independent and multi-pollutant associations between LTE4 and exposure to ambient air pollutants and DAPs using generalized estimating equations. For multi-domain and multi-pollutant models, we created categorized pollution combination levels and estimated the relative health impact of exposure to pollutant mixtures.

**Results:** In single-pollutant models, an interquartile range increase in exposures to DAPs was associated with increase in LTE4 levels ( $\beta$ : 4.1 [0.6–7.6] pg/mg).  $\text{PM}_{2.5}$  and ozone were also associated with increase in LTE4, though confidence intervals contained the null value. Increase in LTE4 levels was consistently associated with increase in median-dichotomized multi-pollutant combination exposures; the highest effect estimates were observed with joint highest (vs. the lowest) category of the three-pollutant exposure ( $\text{PM}_{2.5}$ , ozone, and OP;  $\beta$ : 53.5, 95% confidence interval = 24.2, 82.8 pg/mg).

**Conclusion:** Concurrent short-term exposure to criteria air pollutants and OPs in an agricultural community was associated with an increase in a marker of asthma morbidity.

**Keywords:** Pollutant mixtures; Agricultural settings; Pediatric asthma; Biomarkers

## Introduction

Pediatric asthma continues to be a significant public health issue with approximately 8.4% of children in the United States living

with the disease.<sup>1,2</sup> Asthma also remains one of the most frequent causes of pediatric hospitalization,<sup>3,4</sup> and costs the United States over \$50 billion in health expenditures and lost productivity annually.<sup>3,5–7</sup>

Children with asthma are disproportionately impacted by environmental agents.<sup>8</sup> The effects of these exposures on pediatric asthma morbidity has been well-studied, particularly in urban settings.<sup>9,10</sup> However, the contribution of environmental agents in rural and agricultural settings remains largely unexplored. As the sources and composition of these environmental pollutants vary between urban and rural settings,<sup>6,11</sup> associations widely described in the literature (predominantly urban) are unlikely to be generalizable to rural areas. Agricultural communities may have fewer urban ambient air pollution sources (e.g., motor vehicle traffic and industrial emissions), but have substantially more unique local sources like pollen and similar aeroallergens, and emissions from industrial-scale agricultural operations including windblown dust, animal agricultural emissions and pesticide

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Data and computing code required to replicate results reported in this manuscript are available upon demand from the authors, in accordance with the University of Washington and Colorado State University Institutional Review Board regulations.

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## What this study adds

The effects of ambient air pollution on pediatric asthma morbidity have been well-documented in epidemiological studies in urban communities. There has been less focus on health effects of the unique ambient exposures in rural and agricultural settings, especially in the context of simultaneous exposure to multiple diverse environmental agents. This study examined the impact of multiple environmental exposures characteristic of agricultural communities (ambient air pollutants and organophosphate pesticides) on pediatric asthma morbidity. We found that simultaneous short-term exposure to air pollutants and organophosphate pesticides was consistently associated with an increase in a biomarker of asthma morbidity.

drift from large crop-growing operations,<sup>12,13</sup> all of which have been suggested to contribute to respiratory disease.<sup>14,15</sup>

Although environmental health research has traditionally focused on estimating the effects of single-pollutant exposures, children are invariably exposed to and affected by a mixture of exposures unique to their environment.<sup>16–18</sup> Moreover, assessing the health impacts of environmental exposures in the context of the “one atmosphere” approach requires a more robust characterization of exposures to local sources of environmental pollution beyond single-pollutant risk or total exposure within specific pollutant groups.<sup>19–21</sup>

Exposure to agricultural pesticides, particularly organophosphate (OP) insecticides, has been linked to adverse respiratory outcomes in agricultural settings,<sup>22</sup> and children are especially susceptible to the effects of pesticide exposure.<sup>23,24</sup> However, appropriate characterization of this association in terms of pediatric asthma morbidity is limited.<sup>25</sup> Further, the respiratory health effects of pesticides in the context of other local air pollutants are not well understood. A multi-domain approach that considers the joint effect of multiple classes of environmental agents (specifically, ambient air pollutants and agricultural pesticides) may provide a more concrete representation of association between multi-pollutant exposure to environmental pollutants and respiratory morbidity.

Respiratory health effects for children with asthma in rural agricultural communities may be affected by multiple sources, including biogenic (e.g., dust, pollen) and anthropogenic pollutants. We focus specifically on (1) ozone and particulate matter <2.5  $\mu\text{m}$  in diameter ( $\text{PM}_{2.5}$ ), pollutant concentrations regulated by federal law, and among ambient criteria pollutants, are responsible for a majority of human health damages,<sup>26</sup> and (2) OP pesticides, a group of widely used insecticides with potential health hazards, and also subject to federal standards.

We adopted a conceptually simple method to evaluate independent and joint effects of exposure to air pollution ( $\text{PM}_{2.5}$  and ozone) and OP pesticide (using well-established metabolite biomarkers) on a biomarker of pulmonary inflammation and asthma exacerbation.

## Methods

### Study population

The Aggravating Factors of Asthma in a Rural Environment (AFARE) project was conducted in the Yakima Valley of Washington State. This region is characterized by a high density of large-scale agricultural operations including production of fruit crops and vegetables. Details about recruitment and baseline health evaluations have been reported previously.<sup>38</sup> Briefly, the AFARE study collected longitudinal data to explore and identify ambient environmental factors associated with pediatric asthma exacerbations in an agricultural community. The children were between 6 and 16 years of age at baseline and had no serious illnesses other than asthma. For this analysis, we used the repeated measures made on a subset of children for which urine specimens were collected in the AFARE cohort ( $n = 16$ ), at 6-day intervals over a 4 months period (July 2012–October 2012). All study procedures were approved by the Institutional Review Boards of the University of Washington and the Colorado State University.

### Asthma morbidity assessment

#### Urinary leukotriene E4 monitoring

We used urinary leukotriene E4 (LTE4) to assess asthma morbidity (pulmonary inflammation) in this study. LTE4 is a validated marker of systemic cysteinyl leukotriene activity, and an indirect marker of lung cysteinyl leukotriene activity, a lipid mediator known to play a central pathophysiological role in asthma.<sup>27,28</sup> Cysteinyl leukotrienes are eicosanoids produced by a variety of cells associated with inflammation. Measurement of LTE4 represents a noninvasive method to assess acute pulmonary inflammation among children with asthma.

LTE4 was measured from spot urine samples, scheduled to be collected study participants every 6 days during the study period. Samples were subsequently stored at  $-20^{\circ}\text{C}$  before analysis. Quantitative analysis of urine samples for LTE4 was performed in the University of Washington–Department of Environmental and Occupational Health Studies Functional Genomics Laboratory using the Cayman Human LTE4 EIA Kit (Cayman, Ann Arbor, Michigan) according to the manufacturer’s instructions. Creatinine concentration was also measured for each sample to account for urine dilution; this analysis was conducted by the Department of Laboratory Medicine at the University of Washington. Creatinine-adjusted concentrations were used for all final model analyses.

## Environmental pollutants and meteorological data

### Organophosphate pesticide monitoring

Spot urine samples were analyzed for OP pesticide metabolites at the same time LTE4 was assessed. Six urinary dialkyl phosphate (DAP) metabolites that result from the degradation of different OPs were measured in participants’ urine; dimethyl phosphate, dimethyl thiophosphate, dimethyl dithiophosphate, diethyl phosphate, diethyl thiophosphate, and diethyl dithiophosphate. Metabolite reporting limits were based on the limit of detection (LOD) for each DAP compound, and masses below the LOD were approximated as  $\text{LOD}/\sqrt{2}$ . Creatinine concentration was measured similarly to account for urine dilution.

We used summative measures of DAPs rather than measures of individual analytes, to provide a better indicator of total OP exposure,<sup>29</sup> and to account for circumstances where individual OP pesticides devolve to more than one DAP metabolite.<sup>30</sup> Summed urine DAP concentrations (total dimethyl alkylphosphate, total diethyl alkylphosphate, and total DAP pesticides [EDAP]) were estimated by summing molar concentrations (DAP concentration divided by their molecular weights) of metabolites. Creatinine-adjusted EDAP was used for final single-pollutant and multi-pollutant model analyses.

### Ambient $\text{PM}_{2.5}$ and ozone measurements

We obtained daily measurements of ambient concentrations of  $\text{PM}_{2.5}$  from the local US Environmental Protection Agency (EPA) central site air monitor in Toppenish, Washington. Ozone data were obtained from the US EPA Air Quality System Data Mart as an 8-hour daily maximum in parts per billion. Because the ozone monitors ( $n = 8$ ) are sparsely distributed in Central Washington, we used data from all available monitoring sites that had complete pollution data during the study period and averaged measurements from the three closest monitors within 100 miles of participant homes. We explored the effect of  $\text{PM}_{2.5}$  and ozone measured on multiple lag days; to correspond with the limited exposure window for OP exposure, final analyses were performed with weekly (7-day) average  $\text{PM}_{2.5}$  levels (as we did not have consecutive daily pollutant measurements), and lag-1 ozone levels.

### Meteorology

To capture meteorological conditions for the week before LTE4 measurements, we used data downloaded from the county-weather package in R which provides data from NOAA’s Global Historical Climatology Network on 24-hour average, maximum and dew-point temperature, precipitation, and wind speed.

### Statistical analysis

We evaluated associations of a marker of asthma exacerbation (LTE4) with exposure to ambient pollution (weekly average for  $\text{PM}_{2.5}$  and day-prior 8-hour maximum for ozone), and OP

pesticides (DAP) using generalized estimating equations (GEEs) with an exchangeable correlation matrix. In all our models, the mean outcome was modeled to be linear in response to the primary exposure of interest.

We presented the results in single-pollutant models as effect sizes per interquartile range (IQR) increases in exposure to make the associations comparable between the pollutants. For multi-pollutant models, we created categorized pollution combination levels and estimated the relative health impact of exposure to pollutant mixtures. This method assumes that there are similar functional characteristics (categorical effects) for individual components of joint exposures. Levels of individual exposure metrics (OP, PM<sub>2.5</sub>, and ozone) were split into dichotomous indicator categories (high and low) based on the median values observed in the cohort. Then we aggregated the high and low pollutant levels to form two-pollutant and three-pollutant exposure mixture categories as shown in Table 1. For a two-pollutant mixture (“OP + ozone,” “OP + PM<sub>2.5</sub>,” “PM<sub>2.5</sub> + ozone”), there would be three categories of pollutant mixture reflecting high exposure (both pollutants at high exposure levels), moderate exposure (one pollutant at high exposure level), and low (both pollutants at low exposure levels); and for the three-pollutant mixture (“OP + ozone + PM<sub>2.5</sub>”), there would be four categories of pollutant mixture reflecting high exposure (all pollutants at high exposure levels), moderate exposure (two pollutants at high exposure level), mild exposure (only one pollutant at high exposure levels), and low exposure (no pollutants at high exposure levels). Mixture exposure categories were then included as independent variables in GEE models, using the homogeneous “low” categories as the reference category, and controlling for confounders.

Covariates included in all models as potential confounders were selected a priori based on existing evidence of relationships between the covariate and both respiratory health and exposure to air pollution: temperature, wind speed, precipitation and relative humidity (averaged over the week before LTE4 measurements), week and month of the year as two possible markers of temporal trends (known high-risk periods for increased exacerbation include the return to school in the fall and respiratory virus season<sup>31,32</sup>), and subject-specific characteristics potentially associated with asthma and asthma exacerbation: sex, age, use of inhaled corticosteroids at baseline, a measure of severity at baseline (exhaled nitric oxide levels), and the number of individuals in household.

**Table 1**

**Summary of mixture categories used for multi-pollutant analysis**

Pollutant levels			Exposure category	
OP	Ozone	PM <sub>2.5</sub>	3-category	4-category
High	High		2	High
Low	High		1	Moderate
High	Low			
Low	Low		0	Low
High		High	2	High
Low		High	1	Moderate
High		Low		
Low		Low	0	Low
	High	High	2	High
	Low	High	1	Moderate
	High	Low		
	Low	Low	0	Low
Low	Low	Low		0
Low	Low	High		1
Low	High	Low		
High	Low	Low		
Low	High	High		2
High	Low	High		Moderate
High	High	Low		
High	High	High		3
				High

In sensitivity analyses, we repeated the multi-pollutant analysis after restriction to exposure days below the US EPA National Ambient Air Quality Standards for ozone and PM<sub>2.5</sub>. Further, to investigate whether the health impact of joint multi-pollutant categories was sensitive to the choice of thresholds, we assessed multiple combinations for cutpoint choices at the 25th, 50th, and 75th percentile thresholds: we varied the cutpoints that distinguish between high and low exposure, and then reran models with adjusted joint multi-pollutant exposure categories created from these new cutpoints.

Model diagnostics were also performed to explore the possibility of influential subjects using the “leave one out” method. These analyses did not indicate the presence of significant impact of a single observation on model fit or estimates. We used the quasi-information criterion as an estimator of the relative quality (model fit) of statistical models.

Analyses were performed using SAS version 9.4 (SAS Institute, Inc., Cary, North Carolina) for GEE and mixed model analyses, and R version 3.3.0 (R Foundation for Statistical Computing, Vienna, Austria) for exploratory and descriptive analyses.

## Results

Overall, the mean age of the 16 children included in this analysis was 12 years, and 56.3% were male (Table 2). Nearly all (93.8%) of the children self-identified as Hispanic/Latinx, 56.3% were from low-income families, and 87.5% relied on public health insurance/aid. Approximately 69% of the children were taking corticosteroid medication at the time of enrollment, and 12 children (75.0%) were identified to be skin prick positive to at least one aeroallergen. Based on a clinical examination performed at baseline, more than half of the subjects (56.3%) were classified as overweight (body mass index-for-age above the 85th percentile).

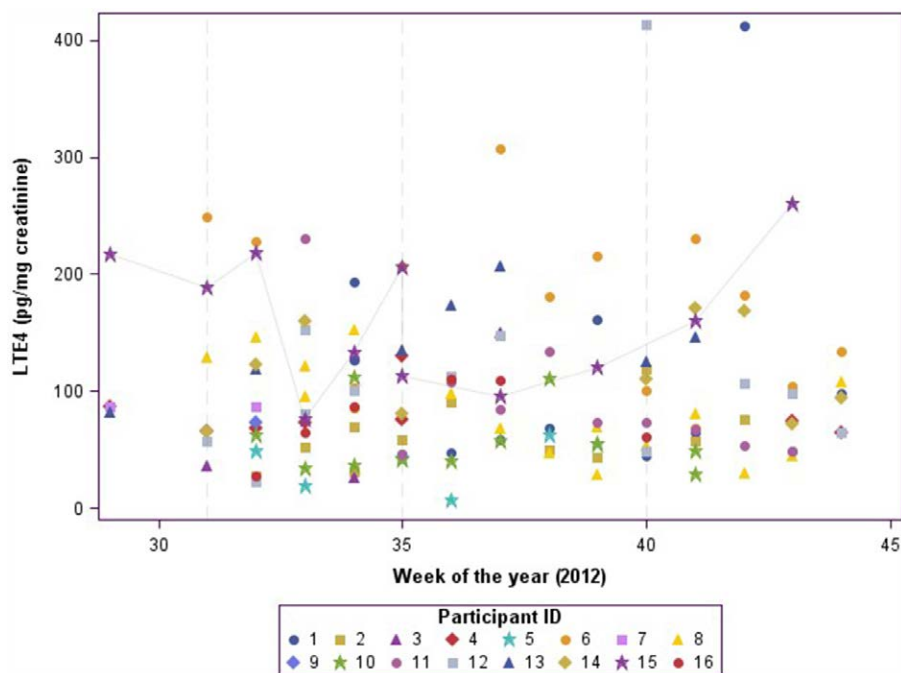
Compliance with the collection of urine samples varied: the total number of samples per subject ranged from 1 to 12 (median: 10.5). A total of 139 observations were obtained from

**Table 2**

**Characteristics of children in AFARE sub-cohort**

Variable	Level	%
Sex	Male	56.3
Birth country	United States	68.8
	Other	31.3
Ethnicity	Hispanic/Latino	93.8
	Non-Hispanic	6.3
Income	≤\$15k/year	53.3
	\$15k–<30k/year	26.7
	>30k/year	20.0
Residence	In town	81.3
	Rural/Farm	18.8
Total no. household members	<5	31.3
	≥5	68.8
Insurance	Public insurance or aid	87.5
	Private insurance/self	12.5
Skin prick test positive (atopy)	Yes	75.0
Inhaled corticosteroid use	Yes	68.8
	Mean	11.9
	Median	11.0
	Minimum	9.0
	Maximum	17.0
BMI for age (85th Percentile)	Above	56.3
	Below	43.7
Baseline fraction exhaled nitric oxide level (ppb)	Mean	22.1
	Median	12.0
	Minimum	6.0
	Maximum	120.0

BMI indicate body mass index.



**Figure 1.** Creatinine-adjusted urinary LTE4 levels for study participants over the study period. Vertical lines represent the beginning of a new month. Observed LTE4 series for one participant (15) is highlighted.

the 16 children over the study period. One observation was excluded because of incomplete data, resulting in 138 observations for analyses.

Individual-level creatinine-adjusted urinary LTE4 over the study period are illustrated in Figure 1. The median level of LTE4 among participants was 84.8 pg/mg creatinine (geometric mean: 84.8 pg/mg) over the study period.

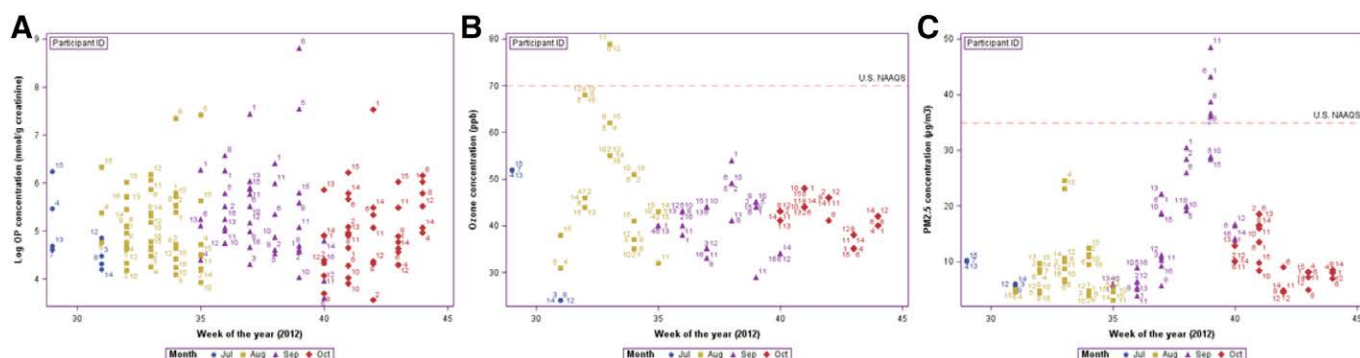
Twenty-four hour weekly average PM<sub>2.5</sub> concentrations had a median (IQR) of 8.7 (8.2) µg/m<sup>3</sup> over the study period with the highest values occurring in late September (Figure 2A). There were multiple weekly periods with average PM<sub>2.5</sub> exposure levels above the EPA 24-hour ambient air quality standards (35.0 µg/m<sup>3</sup>), likely coinciding with a wildfire in Washington state during this period.<sup>33</sup> The other predominant sources of PM<sub>2.5</sub> emissions in the region include fossil fuel combustion, waste disposal and agricultural crop and livestock-related dust.<sup>34</sup> The median (IQR) maximum 8-hour ozone value for individuals over the study period was 43.0 (10.0) ppb (Figure 2B). There were also multiple days with observations above EPA 8-hour daily maximum ambient air quality standard (70.0 ppb). The median (IQR) total OP metabolite (EDAP) level was 142.9 (197.3) nmol/g creatinine (Figure 2C). Spearman correlations of ambient air pollutants showed very weak positive correlations between EDAP and

both ozone and PM<sub>2.5</sub> (both  $\rho < 0.1$ ). The correlation between ozone and PM<sub>2.5</sub> was positive and slightly stronger ( $\rho = 0.2$ ).

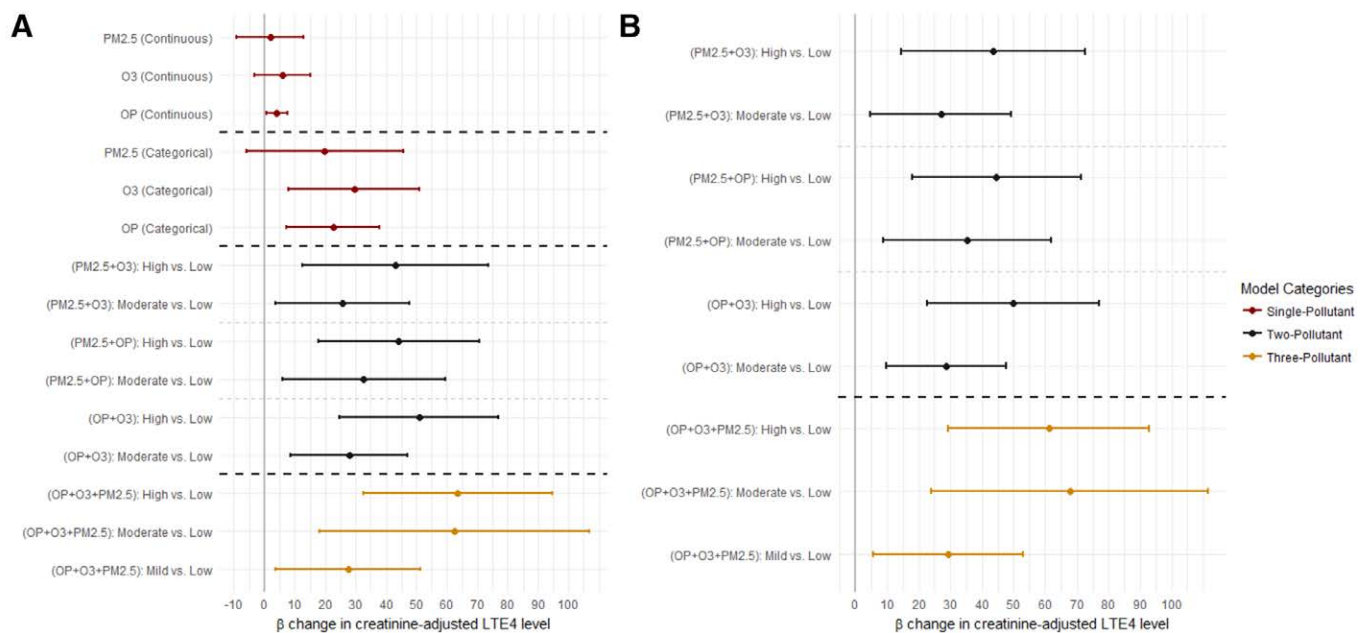
In addition, we observed no evidence of patterns in pollutant concentrations by residence (in-town vs. rural/farm), although <20% of participants resided in or next to farms (eFigure 1; <http://links.lww.com/EE/A38>). However, concentrations of the exposure (PM<sub>2.5</sub>, ozone and OP) and outcome (LTE4) measures exhibited a fair amount of temporal variability over the study period.

The associations of pollutants with LTE4 using single-pollutant (as continuous pollutant exposures), two-pollutant (two of OP, PM<sub>2.5</sub>, and ozone), and three-pollutant models (all three pollutant exposures) are presented in Figure 3. In single-pollutant models, an IQR increase in OP levels was associated with a LTE4 increase of 4.1 pg/mg creatinine (95% confidence interval [CI] = 0.6, 7.6). We also observed elevated associations between LTE4 levels and ozone ( $\beta = 5.8$ ; 95% CI = -3.3, 14.8) and PM<sub>2.5</sub> ( $\beta = 2.1$ ; 95% CI = -9.2, 13.4), although CIs included the null value.

All the models with median-dichotomized multi-pollutant combination exposures showed associations with increase in LTE4 levels (Figure 3). We observed the highest change in LTE4 effect estimate for the highest (vs. the lowest) category of the



**Figure 2.** Air pollutant and pesticide exposure levels for study participants over the study period. Numbers in plot area indicate participant IDs. Horizontal line represents US National Ambient Air Quality Standard. A, Creatinine-adjusted urinary log OP (DAP, OP) levels. B, Maximum 8-hour ambient ozone levels. C, 24-hour average ambient PM<sub>2.5</sub> concentrations.



**Figure 3.** Estimated effects ( $\beta$  change and 95% CIs) of pollutants on creatinine-adjusted LTE4 levels (in pg/mg creatinine). A, Single-pollutant and multi-pollutant models using the median cutoff for categorization. B, Multi-pollutant models using median cutoff for categorization, excluding observations below EPA cutoff values. All models were adjusted for sex, age, use of inhaled corticosteroids at baseline, number of individuals in household, temperature, wind speed, precipitation, and relative humidity. PM<sub>2.5</sub> indicates 24-hour-average exposure to particulate matter <2.5  $\mu$ m in diameter; ozone, 8-hour maximum concentration of ozone; OP, urinary measure of metabolite of OP exposure, total DAP.

three-pollutant exposure ( $\beta = 63.6$ ; 95% CI = 32.4, 94.7); mild and moderate exposure categories resulted in approximately 27.5 (95% CI = 3.6, 51.5) and 62.5 (95% CI = 18.1, 107.0) pg/mg creatinine increases in LTE4, respectively, compared with the lowest exposure category. Despite the significant overlap between estimates and CIs, we also observed a form of tiered dose-response pattern across the categories of exposure severity. In addition, effect estimates were similar for models that excluded observation points ( $n = 9$ ) above the EPA standards for ozone and PM<sub>2.5</sub> (Figure 3B).

In subanalyses of multi-pollutant models, we examined individual pollutant contribution to two-pollutant mixture categories. Within the limitations of overlapping CIs, we observed that for two-pollutant models, either moderate PM<sub>2.5</sub> or OP, and high PM<sub>2.5</sub> and OP were associated with increased LTE4 compared to the reference homogeneous low mixture category (Figure 4). In contrast, for two-pollutant mixtures with ozone, only the highest mixture categories containing ozone (compared with the reference homogeneous low mixture category) resulted in increased LTE4 effect estimate.

The results from the sensitivity analysis using combinations of the 25th, 50th and 75th percentiles as thresholds are shown in eTables 1 and 2; <http://links.lww.com/EE/A38>. We observed that the associations between high, moderate, mild, and low exposure groups generally persisted in these models. However, the magnitude of effect estimates (and 95% CI) varied with the differing cutpoints. For example, combination c4 which developed exposure categories based on the 25th percentile for PM<sub>2.5</sub> (5.3  $\mu$ g/m<sup>3</sup>), 50th percentile for ozone (40.0 ppb), and 25th percentile for OP (92.0 ng/mg creatinine), resulted in an estimated 17, 39, and 52  $\mu$ g/mg creatinine increase in LTE4 levels among the mild, moderate, and highest levels of joint exposures, respectively (compared with the lowest category).

Finally, we observed minimal differences between model fit/performance relative to selected cutpoints, based on the quasi-information criterion values (eTable 2; <http://links.lww.com/EE/A38>). The best fit models for the multi-pollutant association with LTE4 tended to involve lower cutpoints (at 25%) for ozone and OPs, and the higher cutpoints for PM<sub>2.5</sub> (at 75%).

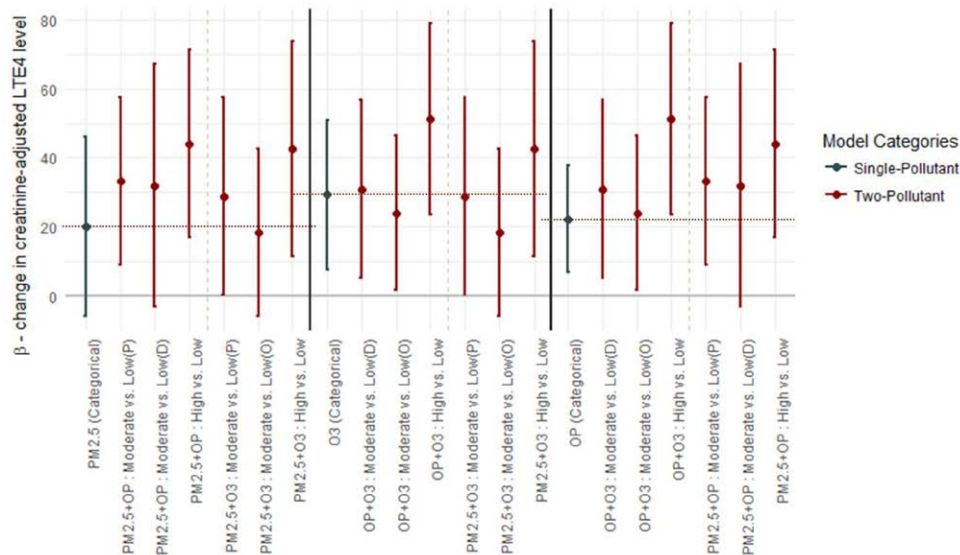
## Discussion

Geographical and population-based differences in the prevalence of asthma morbidity necessitate more refined assessment of the environmental exposures experienced by different populations. Our results provide insight into the effects of important criteria ambient pollutants on the respiratory health of children with asthma in a rural agricultural community, all in the context of contemporaneous exposure to OP pesticides.

To the best of our knowledge, no other study has considered environmental exposure to OP pesticides and criteria pollutant in joint health effects models; our study represents the first longitudinal, repeated measures study of joint assessment of community-level OP exposures, ambient air pollution and a marker of pulmonary inflammation among children in a largely agricultural setting.

Our findings highlight, within the limitations of this study, several important implications. First, single-pollutant models suggest independent positive associations between urinary LTE4 and short-term exposure to PM<sub>2.5</sub>, ozone, and OP pesticides, though only associations with exposure to OPs had a 95% CI that excluded null value. These findings, along with many previous studies using single-pollutant approaches, provide pertinent information about the potential role of the individual exposures, which is required to demonstrate relevance for the combination of these pollutants in multi-pollutant models.<sup>35,36</sup>

Next, the multi-pollutant models suggest increases in LTE4 was consistently associated with joint exposures combinations of PM<sub>2.5</sub>, ozone, and OPs. Our observation of ordered trends across categories of severity in two-pollutant and three-pollutant models may indicate increased risk of adverse health effects with increasing total mixture levels, though our small sample size resulted in significant overlap among the categories. We also observed a unique pattern of these trends with specific joint exposures; relative to the lowest categories, joint adverse associations with LTE4 levels observed for all higher levels of PM<sub>2.5</sub> and OPs, but only mixture categories with the highest levels of ozone showed increased/positive changes in LTE4, compared with the low mixture categories. This indicates that the relationship between ozone and LTE4 (and by extension inflammation)



**Figure 4.** Estimated effects ( $\beta$  change and 95% CIs) on creatinine-adjusted LTE4 levels (in pg/mg creatinine) from two-pollutant models indicating specific pollutant contribution to exposure mixture categories. Letter in parenthesis indicates pollutant with highest exposure level; PM<sub>2.5</sub>, particulate matter <2.5  $\mu$ m in diameter; O<sub>3</sub>, ozone; OP, total DAP (OP). Horizontal lines indicate level of effect estimates of single pollutant in comparison to multi-pollutants.

in mixtures is unlikely to be a simple linear one, with the worst effects seen at comparatively higher exposure levels, although underlying interaction mechanisms remain unclear.

Finally, the observed associations between exposure mixture categories were present at concentrations below NAAQS standards for PM<sub>2.5</sub> and ozone. Although these regulatory standards are predicated on single-pollutant research,<sup>16</sup> our results reiterate the shortcomings (especially among susceptible individuals) of the standards,<sup>37</sup> and signify a possible pathway to proffering standards based on multi-pollutant approaches.

The deleterious relationship between ozone, PM<sub>2.5</sub>, and pediatric asthma morbidity have been thoroughly studied and established by multiple observational and experimental studies.

Single-pollutant studies have found independent associations between short-term ozone and PM<sub>2.5</sub> exposure, and asthma-related symptoms, hospital visits and clinical measures of exacerbation. For example, Lewis et al<sup>37</sup> found that ambient PM<sub>2.5</sub>, PM<sub>10</sub>, 8-hour, and 1-hour peak ozone concentrations were associated with increased odds of respiratory symptoms in a set of children with asthma in Detroit. Similarly, Loftus et al<sup>38</sup> showed that PM<sub>2.5</sub> pollution in this AFARE agricultural setting resulted in increased asthma symptoms and decreased lung function.

Conversely, the impact of OPs on pediatric asthma morbidity has rarely been explored. In a previous study among children in the AFARE study, the authors showed that urinary pesticide metabolite levels (indicating short-term OP exposure) were significantly higher among the children in the AFARE cohort compared with children of similar age participating in the National Health and Nutrition Examination Survey, indicating additional exposure burden (most likely ambient and proximity-based) encountered in populations with significant agricultural activity.<sup>39</sup> They also pointed out that this OP exposure was associated with increased urinary LTE4 levels. Two other studies exploring urinary DAP metabolites among children from the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) birth cohort found a significant association between early-life exposure to OPs and respiratory symptoms, and lung function in childhood.<sup>40,41</sup> Although these symptoms and signs were consistent with pediatric asthma morbidity; their sample cohort was not focused on children with asthma. Moreso, postnatal short-term exposures to OPs were not explored.

The major research paradigm in environmental epidemiology research is to examine single-pollutant effects on health outcomes. The limited number of multi-pollutant studies often focus on a combination of criteria air pollutants, using either an additive main effects approach (including copollutants as coexposures in regression models), the interaction approach (as described by Dominici et al<sup>16</sup>), or other semiparametric and parametric approaches.<sup>42,43</sup> Although the effect of multi-pollutant exposures have been linked to pediatric asthma morbidity,<sup>44–49</sup> the difference in mixture components and approaches to quantify these pollutant mixtures limits direct comparability with our study results. Even less common are studies that explore noncriteria environmental pollutants, or multiple exposure domains. Research on multi-domain (in addition to multi-pollutant) exposures are important when considering the health effect of cumulative chemical exposure in communities with a mix of pollutant sources;<sup>36</sup> the individuals in such communities tend to be exposed to multiple diverse chemicals or environmental risk factors simultaneously.<sup>20</sup>

The exact mechanisms by which these three pollutants cause respiratory morbidity, individually or as part of a mixture, are poorly understood. However, across epidemiological and toxicological studies, airway inflammation and hyperresponsiveness are two mechanistic features consistently associated with all three pollutants.<sup>9,50</sup> Cysteinyl leukotrienes are a measure of endogenous release of inflammatory mediators and are recognized as a key mediator of airway inflammation.<sup>27,51,52</sup> Hence, LTE4, the stable end product of cysteine leukotriene metabolism can be considered as a logical marker of the endpoint of this inflammatory process.

Our approach to multi-pollutant analysis employed median dichotomization splits to generate exposure categories based on distributional properties of the single-pollutant exposure data in the cohort. This conceptually simple method builds on an unsupervised profile generation technique which transforms pollutant mixture concentrations into flexible variables that subsequently represent simple exposure profiles of the pollutant combination. We are able to generate interpretable effect estimates with reasonable inferential properties including better characterizations of the total environment, effect measure modification within the mixtures, and identify combinations of pollutants that may be the most harmful.

Other simplified methods for multi-pollutant analyses have been reported in the literature. For example, Hong

et al<sup>53</sup> presented a combined index for combinations of pollutant concentrations, calculated as the sum of mean scaled single-pollutant concentrations. Their index method is easy to interpret, but unsuitable for highly skewed data, and is unable to clearly delineate which mixture component or combination of pollutant levels is relatively more harmful. More recently, a study by Liu and Peng examined the cardiovascular health effects of three-pollutant mixtures (ozone, nitrogen dioxide, and fine particulate matter) in 85 United States counties, using a method called PollutANt Category KnittEd (PANCAKE) to categorize pollutant levels.<sup>54</sup> Their categories were based on thresholds of increasing magnitude, and PANCAKE created indicators for different mixture compositions. However, the PANCAKE method is more suited to ecological-level studies and requires large sample sizes to generate enough samples for exposure mixture categories.

There are several limitations in our study and analytic approach. First, using DAPs as a measure of ambient OP exposure is limited by the lack of specificity with respect to the OP from which they were derived, and reliability may be affected by human exposure to preformed DAPs in food or the environment.<sup>55,56</sup> We believe that any related measurement error will most likely be nondifferential, with possible attenuation of effect estimates. Moreover, a substantial body of literature has demonstrated significant temporal and spatial associations between ambient pesticide application/use/measurements and DAP levels.<sup>57–59</sup> Our measures of ozone and PM<sub>2.5</sub> were obtained from central monitors in proximity to children's homes. Such residential exposure assessment fails to account for time-activity patterns. Further, we were limited in this particular study in identifying spatial variation of exposures to environmental agents. Again, any errors resulting from this would most likely be nondifferential, and likely may have masked any true exposure-outcome associations by biasing results toward the null and increasing the standard errors association with effect estimates. Future studies should focus on better characterization of spatial exposure patterns in an agricultural community.

Further, to arrive at biologic plausibility for the joint effects of exposures on our outcome, we assume similar pathophysiological pathways for all three component pollutants. It is possible that the effects seen are due to simultaneously present differing mechanisms of action. For example, each pollutant may lead to respiratory morbidity through one or more of: direct insult on lung tissue receptors; indirectly through effects mediated by oxidative stress or inflammatory mechanisms; simultaneous direct and indirect mechanisms; or with the mechanism and effect of a specific pollutant acting as an adjuvant for another.<sup>9,60</sup> Toxicological data that appropriately quantifies the pathophysiological activity for individual pollutants may be required. However, it is unlikely that differences in individual pollutant pathophysiological mechanisms explains all of the observed effects.

Another possible source of exposure misclassification may be related to using dichotomized exposure cutpoints. To evaluate exposure cutpoint bias, we manually assessed the joint effects of pollutant exposures at multiple dichotomization splits. The observed results indicate a robustness of our chosen median cutpoints in this population.

Data constraints limited us to short-term lag exposure analyses, and limited characterization of the influence of seasons with respect to this particular agricultural community. Finally, we had no symptomatic/clinical marker of asthma exacerbation. However, multiple studies have highlighted correlations between acute exacerbation events and LTE4: Green et al<sup>52</sup> showed that urinary LTE4 levels among adults with asthma increased by over 30% during asthma exacerbations, compared with levels at follow-up; and a Rabinovitch et al<sup>61</sup> study indicated clinically significant decreases in pulmonary function (percent predicted

FEV<sub>1</sub> by 4.7%) per IQR increase in LTE4 among children with asthma. We do recognize that combining measures, such as biomarkers with clinical characteristics, most likely characterizes asthma exacerbation better than a single marker.<sup>62</sup>

Several issues need to be considered in interpreting our study results. Our choice of pollutants in single-pollutant or multi-pollutant models do not represent a full suite of possible pollutant exposures, even in this particular agricultural community. Moreover, those selected for our analyses are likely correlated with multiple other key pollutants and may only be acting as surrogates for unmeasured or poorly measured pollutants. We also acknowledge the possible contribution of indoor exposures that may act as allergens (such as mites and cockroaches, pets, gas stoves, and tobacco). However, the children in our study were enrolled in an asthma education program to address these common indoor factors before collection of urine samples.

Finally, exposures to the ambient air pollutants and pesticides were limited to a four-month block. Without patterns of variability across multiple time periods, we can only make cautious interpretations of the magnitude or significance of exposure-outcome associations. Again, more detailed studies that include time points from a larger number of seasons are required to provide a better characterization of temporal variations, and to validate our study methods.

The identification and mitigation of environmental triggers in a rural/agricultural setting is one effective component of community-based and clinic-based asthma management strategies, the success of which depends on the proper characterization of the relevant pollutant species beyond the commonly measured (and more urban) air pollutants. In this panel study, we explore the deleterious associations between a biomarker for pulmonary inflammation, and exposure to agricultural OP pesticides and two important criteria air pollutants among children with asthma. Additionally, we extend a multi-pollutant statistical framework to examine the joint effect of this distinct combination of ambient exposures that underscore the experience of simultaneous exposures to environmental triggers in an agricultural community.

More emphasis on region and population-specific analysis of pollutant mixtures and potential health effects is required, including development of tools and approaches for these epidemiologic analyses, with a focus on ultimately refining and enforcing more appropriate environmental standards.

## Conflicts of interest statement

The authors declares that they have no conflicts of interest with regard to the content of this report.

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