

Organophosphates in Urine and House Dust and Associations with Asthma Respiratory Health
Outcomes in an Agricultural Children's Cohort

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Abstract

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Organophosphate (OP) pesticides are extensively used to support agricultural practices in Washington State. OP exposure is associated with both asthmatic populations and agricultural communities. The objective of this thesis is to investigate the correlation between OP pesticide exposure in an agricultural community, with respiratory health outcomes, associated with asthma. As part of a Children's Cohort Study, Farmworkers (FW), Non-Farmworkers (NFW), each with a referent child, were recruited in the Yakima Valley of Eastern Washington. House dust and urine samples were collected in 3 seasons in 2 different study periods (2005 and 2011) according to pome fruit growth cycles. Health questionnaires were distributed to each household, and clinically recognized asthma-related respiratory data was collected by study staff. Concentrations of OP parent compounds in house dust samples, and dialkyl phosphate metabolites (DAPs) in urine samples were quantified and statistically evaluated against various

asthma-related respiratory outcomes collected on the administered health survey. Our results show a significant association between OP compounds and their metabolites with asthma-related respiratory health outcomes in our adult population. Among adults living in the agricultural community, an increase in dust OP compound and urinary metabolite concentration was significantly related to a decrease in asthmatic outcome. We found these associations between dust OP biomarker and asthmatic outcome and urine OP biomarkers with asthmatic outcome, were not significant in the children group. However, the exposure-health outcome association was significantly different between males and females among this group ($p= 0.043$) resulting in significantly different slopes for boys than girls. Previous studies from this research cohort have identified specific urinary microRNAs, including miR-223, that are associated with our FW and NFW occupational status and in a dose-response fashion to OP urinary DAPs from our study population. In this field, research studies have focused on miR-223 and its role as a regulatory molecule capable of influencing asthma pathogenesis. In our future directions we explore the urinary microRNA profiles associated with this pesticide-exposed population and their potential as asthma and respiratory disease biomarkers.

TABLE OF CONTENTS

	Page
1. BACKGROUND	1
1.1 Organophosphorus (OP) pesticides	1
1.2 Biomonitoring of OP pesticides.....	3
1.3 OP pesticides in agricultural settings	4
1.4 OPs and Asthma	6
2. HYPOTHESIS	7
3. SPECIFIC AIMS	8
4. METHODS	9
4.1 Cohort Introduction Description and Recruitment	9
4.2 Dust Sampling and Analysis.....	11
4.3 Urinary Metabolites Sampling and Analysis	11
4.4 Health Questionnaire and Respiratory Endpoint Assessment.....	12
4.5 Statistical Analysis of Dust with Respiratory Health Outcome.....	14
4.6 Statistical Analysis of DAPs with Respiratory Health Outcome.....	16
5. RESULTS	17
5.1 OPs in Dust and Asthma-Related Respiratory Health Outcome	17
5.2 DAP metabolites and Asthma-Related Respiratory Health Outcome.....	20
6. DISCUSSION	24
7. FUTURE DIRECTIONS	28
8. CONCLUSION	30
9. REFERENCES	32
10. SUPPLEMENTAL MATERIALS	38

1. BACKGROUND

1.1 Organophosphorus (OP) pesticides

Pesticides are used across the world to support both domestic and commercial agriculture practices. Organophosphate (OP) insecticides, which date back to the mid-1800s, grew popular after their use as nerve chemical warfare agents in WWII and are still considered one of most used pesticide classes worldwide (Mukherjee et al., 2020). In the United States, the EPA oversees and updates the regulatory status of different OP pesticides, and due to neurotoxic and developmental concerns, has already banned most of their residential use and began phasing out the commercial use for others, including the revoking all tolerance levels for chlorpyrifos in 2021, one of the most extensively used OP pesticide prior to regulatory updates (US EPA, “Chlorpyrifos.” 2022). However, the use of OP pesticides persists across the US agriculture market and flourishes globally (Mali et al., 2022). In developing countries, OP poisoning is a major public health issue, due to extensive, unmonitored, use and availability of OP compounds (Kaeley et al., 2022). The potential magnitude for global health impacts remains concerning, and differences in occupational safety practices, access to personal protective equipment (PPE), socio-economic status and availability of environmental health resources can make a population more at risk of developing toxic health effects (Buckley et al., 2004).

OPs are readily absorbed through the skin and mucous membranes (Cocker, 2002). OP exposure may occur through inhalation, ingestion, or dermal contact when handling and working with crops, and adverse effects are classified based on length of exposure, i.e. acute, subacute, and chronic

(Naughton et al., 2018). The ingested dose, route of absorption and rate of metabolic breakdown dictates symptomatology. A key impact of OP exposure is the inhibition of acetylcholinesterase (AChE), the enzyme responsible for breaking down acetylcholine (ACh) in the peripheral and central nervous systems at their nerve synapses (Robb, 2022). When absorbed, OP pesticides irreversibly bind to AChE and phosphorylate the serine hydroxyl group on the enzyme's active site, inhibiting its ability to transfer nerve signals at the synapses (Robb, 2022). This results in a buildup of ACh, a neurotransmitter responsible for signal transmission among nerves, causing overstimulation of the widespread nicotinic and muscarinic receptors (Wadia et al., 1974) which in turn leads to clinical manifestations and health complications across different body systems (Peter et al., 2014), including the cardiovascular system, gastrointestinal system, central nervous system, renal system, and respiratory system (Adeyinka et al., 2022). In addition to the recognized AChE inhibition mechanism, and the inhibition of other serine hydrolases, OP respiratory toxicity mechanistic studies have discovered additional pathways capable of inducing airway hyperreactivity at doses lower than ones capable of causing AChE inhibition (Lein et al., 2005), shedding light on the complexity of OP-induced airway inflammation and respiratory disease development. As described in a review by Shaffo et al. (2018), a significant body of literature suggests that OPs may induce respiratory toxicity via direct interactions, independent of AChE inhibition with muscarinic receptors, such as M3 receptor's activity in airway smooth muscle or the blockage of autoinhibitory M2 receptors on airway parasympathetic nerves. These pathways may be influenced by exposure characteristics. Chronically exposed subjects reveal that low level of OP pesticide exposure leads to significant lung dysfunction and exacerbates respiratory illness symptoms, as exposure duration increases (Fareed et al., 2013). Adequate monitoring and

assessment of exposure complexities is therefore necessary in studying health impacts of pesticides like OPs.

1.2 Biomonitoring of OP insecticides

Biological monitoring, or biomonitoring, consists of assessing exposure to chemicals by monitoring their presence (or their metabolites) in biofluids, such as blood or urine (Leng et al., 2017). Biomonitoring provides useful information regarding the amount and type of chemical individuals were exposed to from any source (soil, air, dust, water, and food). In the United States, biomonitoring in agricultural settings started in 1949 in Florida and is now mandatory in some, but not all, states (reviewed in Marsillach et al. 2013). After absorption, OPs are metabolized mostly in the liver. They break down into non-specific metabolites, known as dialkyl phosphates (DAPs) via bioactivation and/or detoxification pathways, and are eliminated in the urine (Bouvier et al., 2006). The breakdown of multiple OPs can release the same or different DAPs. They can be monitored in various biological samples, including urine, to serve as a quantitative measure of internal dose (Yusa et al., 2015). 6 non-specific (DAPs) are depicted in **Figure 1**. These include dimethyl phosphate (DMP), dimethyl thiophosphate (DMTP), dimethyl di-thiophosphate (DMDTP), diethyl phosphate (DEP), diethyl thiophosphate (DETP), and diethyl di-thiophosphate (DEDTP). In addition to the detection of these non-specific DAP metabolites in biological samples, detection of specific metabolites, such as 3,5,6-trichloro-2-pyridinol (TCP), which results from the metabolism of the OP pesticide chlorpyrifos, serve as pesticide specific biomarkers. Following most exposures, the parent compounds of these pesticides are typically found in small concentrations across biological samples (ATSDR 1997; FAO/WHO 1999); therefore, retrieving

intact OP from environmental samples, such as dust, has proved to be an effective tool to measure OP exposure in agricultural communities (Tamaro et al., 2018).

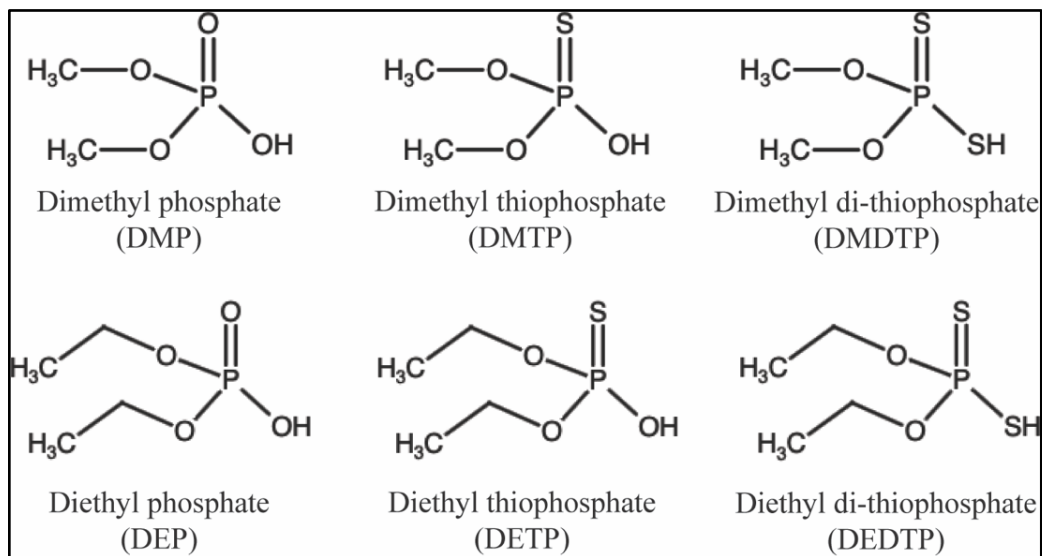


Figure 1. Chemical structures of common dialkyl phosphate metabolites (DAPS)

1.3 OP pesticides in agricultural settings

OP exposure is prevalent in agricultural areas, since these agents are still heavily used on crops, like in pome and fruit production in Washington State, where OPs are applied seasonally to control damaging insects (Tamaro et al., 2018). Farmworkers working in agriculture industries have an elevated risk for acute and chronic exposure to OP pesticides (Coronado, 2006). Multiple exposure routes to OPs have been established in this occupational group, and studies have identified several environmental vectors and pesticide take-home pathways (Thompson et al., 2014).

Farmworkers can track occupationally-used pesticides back to their vehicles used during their commutes to and from work and to their residences. These take-home pathways (**Figure 2**) can both prolong a farmworker's exposure and extend exposure to their families, including children,

in their home (Thompson et al., 2003). Lu et al. (2004) detected higher pesticide residues in the dust from homes of farmworkers compared to non-farmworkers and detected higher OP concentrations on farmworker children's toys compared to non-farmworker children (Lu et al., 2004). Differences in behaviors can make a subset of a population increasingly susceptible to individual exposure routes. In a report from the National Resources Defense Council, children of farmworkers were suggested to be the most highly pesticide-exposed group in the United States (Solomon et al.,1998), potentially attributed to their increased odds of pesticide ingestion due to hand-to-mouth tendencies and increased time spent on the floor. (Mills et al., 2001).

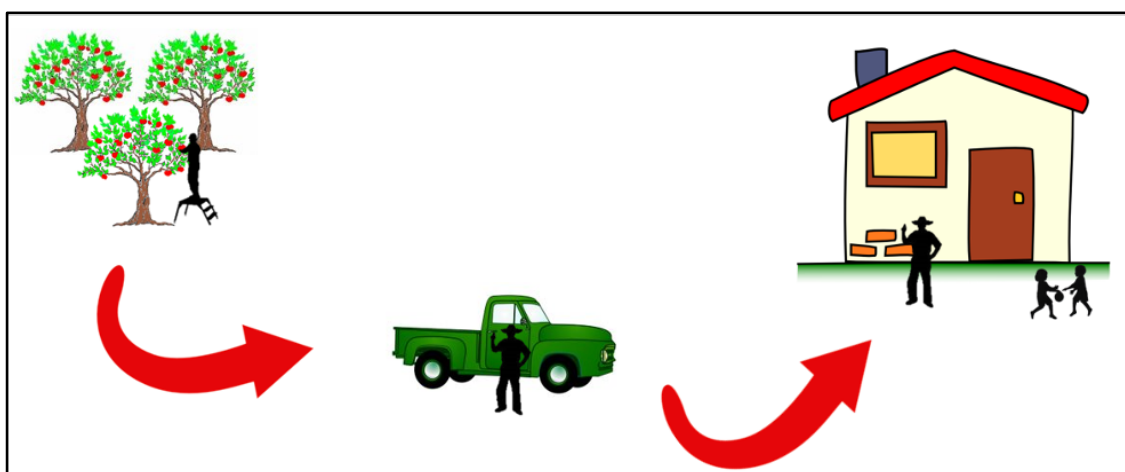


Figure 2: Take-home pathway: People working in the agriculture sector come into contact with pesticides on crops and these have been detected in vehicle dust and house dust, demonstrating evidence for take home pesticide pathway. Adapted from *Coronado et. al. 2006; Curl et al. 2002; Thompson et al. 2014*

In addition, children are particularly vulnerable to respiratory toxic effects of environmental toxicants (Benka-Coker et al., 2020). Metabolic rates and oxygen consumption tend to be higher than in adults due to higher inhalation rates per unit of body weight, meaning children may accumulate more pesticides and distribute them more readily than adults in the same environment (Moya et al., 2004). Respiratory organs are still undergoing development and maturation through

the first decades of life, affecting processes like absorption, metabolism, distribution, and excretion of toxic compounds (Saadeh et al., 2014). Differences in liver enzyme production, and lower activity of OP detoxifying enzymes, at early developmental stages may increase susceptibility to exposure (Mills et al., 2001). Studies exploring the relationship between OP biomarkers and adverse respiratory health outcomes specifically in farmworkers and their children are critical to characterizing exposure risk and complexity of exposure-induced respiratory mechanisms in susceptible populations.

1.4 Organophosphates and Asthma

Asthma is one of the most prevalent chronic pediatric diseases. The WHO estimates that by 2025, there will be over 350 million cases globally, most of them children (Pawankar et al., 2014; Masoli et al., 2004). Despite its widespread prevalence, asthma's pathophysiology, and the influence of gene-environment interactions on disease onset and progression, is not fully understood. Clinical features include variable airway obstruction, bronchial hyperresponsiveness and episodes of wheeze, cough, chest tightness, and shortness of breath (De Luca et al., 2010). The sudden onset and severity of these symptoms are often highly influenced by recognized asthma triggers. The Centers for Disease Control and Prevention (CDC) has established a variety of environmental exposures such as tobacco smoke, dust mites, outdoor air pollution, and mold as common asthma triggers (CDC, 2020). Asthma triggers remain extensive and variable, including humidity/changes in weather patterns, pollen, and chemicals known to cause respiratory tract aggravation (U.S. Department of Health and Human Services; NIH, 2022). As an environmental exposure, OP pesticides have been associated with asthma-like symptoms among agricultural workers, commercial applicators, and children of farmworkers, but in a review exploring the

epidemiological challenges in pesticides and asthma, Amaral et al. (2014) identified exposure definition as a major limitation across studies. These researchers state that in most cases, exposure assessment is based on self-reports, lacking a viable biomarker and information on the complex mixture of pesticides and surrounding exposure, their concentrations, and the time of exposure (Amaral et al., 2014).

2. HYPOTHESIS

The basis of this thesis' hypothesis is that pesticide exposure, specifically OP exposure has been linked with respiratory health endpoints. In our study population 20% of the children were diagnosed with asthma, which is higher than both the national and state average. Higher levels of OPs have been associated with agricultural populations and at higher levels in Farmworkers versus Non-Farmworkers in this cohort. The hypothesis of this master's thesis is that biomarkers of OP exposures are associated with asthma respiratory outcomes in an agricultural community. In this cohort, we have used multiple types of biomonitoring to characterize and understand the health effects of OPs. In this study we use OP pesticide parent compounds analyzed and quantified from dust samples and urinary DAPs to explore the OP exposure relationship to respiratory endpoints from a health-based questionnaire that are associated with asthma.

3. SPECIFIC AIMS

AIM #1: Evaluate the association between the concentration of OP parent compounds detected in house dust and asthma-related respiratory outcomes in an agricultural community.

Hypothesis: OP insecticide parent compounds in dust will be associated with asthma-related health outcomes in our population.

AIM #2: Using DAPs detected in urine samples, evaluate the association with asthma-related respiratory outcomes in an agricultural community.

Hypothesis: OP metabolites in urine will be associated with asthma-related health outcomes in our population.

EXPLORATORY AIM #3: Investigate the correlation of inflammatory microRNAs in a pesticide exposed group with asthmatic outcome.

Hypothesis: Inflammatory microRNAs, specifically ones associated with asthma, will be positively correlated with our exposed farmer-worker group.

4. METHODS

4.1 Cohort Introduction, Description and Recruitment

As part of a longitudinal cohort study, launched in 1999, the University of Washington's Center for Child Environmental Health Risk Research (CHC) has been conducting community-based participatory research in the Yakima Valley to examine the health impacts of pesticide exposure in local farm workers. The agricultural setting is further described in Thompson et al. (2014), but briefly the Yakima valley is an agriculturally prosperous region and is known for its production of apples and pears (pome fruit). Several longitudinal studies were launched, to investigate multiple exposure pathways, including take-home exposure pathways, lifestyle pathways, and environmental pathways collecting samples of house dust, vehicle soot, saliva, urine, and blood from both farmworker and non-farmworker families across different agricultural seasons (**Figure 3**).

In 2005, CHC's second study CHC2, was launched and implemented a community-based participatory research strategy to recruit about 200 households (as previously described in Thompson et al., 2014). The demographic breakdown of the cohort population and study participants is described in Smith et al (2015), but briefly, the participating adults were predominantly female (~80%) and living mostly in single family homes. The study cohort is mostly of Hispanic origin (<95%) and split into Farmworker (FW) and non-Farmworker (NFW) groups, each with a referent child 2-6 years old. FW status was defined as individuals that worked in pome fruit crops (apples and pears). 100 of the CHC2 households were selected at random to be recruited in CHC3, CHC's third follow-up study, conducted in 2011. 91 households from CHC2

joined the CHC3 study, and 9 additional new households were then recruited to participate. For this specific project, the 100 subset households from CHC2/CHC3 consisted of 60 FW and 40 NFW. Study materials and sampling protocols were approved and reviewed by the Institutional Review Board of the Fred Hutchinson Cancer Research Center (Seattle, WA), and the Human Subjects Division of the University of Washington. All participants participated in a formal informed Consent Process.

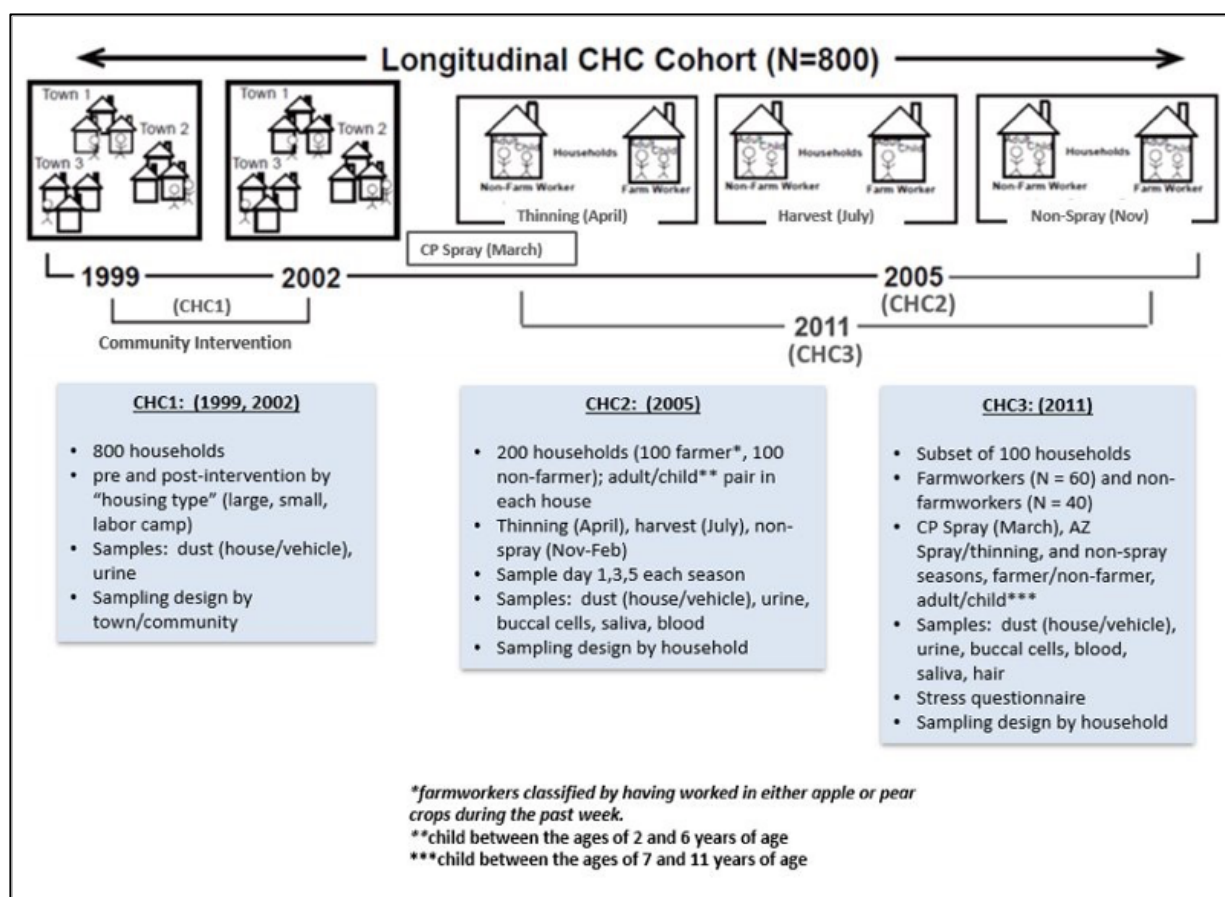


Figure 3: CHC Longitudinal Cohort Timeline and Studies: CHC1, CHC2, and CHC3. Community sampling design in CHC1, and household sampling design in CHC2 and CHC3. 200 households (100 FW and 100 NFW) in CHC2, and a subset of ~100 in CHC3. Samples collected include dust, urine, buccal cells, saliva, hair and blood. A health questionnaire (“Stress Questionnaire”) was distributed only to CHC3 households.

4.2 Dust Sampling and Analysis

House dust samples were collected according to pome fruit growth cycles from 200 households, across three seasons in 2005 (CHC2) and from 100 households, across three seasons (two samples per season) in 2011 (CHC3), each with at least one child. In CHC2, children were of ages 2-6, and in CHC3 7-12 years old. Dust samples were collected by vacuum by field researchers, first horizontally and then vertically, on 0.5 m x 0.5 m square area grid templates based on floor surface type as described in Thompson et. al (2014). In CHC2, a Nilfisk GS-80 vacuum cleaner was used, and in CHC3 a Metropolitan VM-500 High powered handheld vacuum was used to collect dust. 90% and 70% of samples collected had over 100 mg of dust in CHC2 and CHC3, respectively. 305 pesticides used in WA were initially reviewed for dust analysis. Out of 145 selected, 87 pesticides were successfully detected in the dust samples by the University of Washington's Environmental Health Laboratory and Trace Organics Analysis Center (EHL/TOAC). A total of 16 OP pesticides were evaluated for intact residues. (See **Table 1 in Supplemental data**).

4.3 DAP Urinary Metabolites Sampling and Analysis

FW and NFW adults and children participated in 5-day urinary collection periods, 3 times over the course of a year for CHC2 and 4 times for CHC3. In CHC2, the analysis of urinary DAPs was done by the CDC (Atlanta, GA), including creatine adjustment values, for all available urine samples, as described in Thompson et al. (2014). The CHC3 analysis of urinary DAPs was done by the EHL/TOAC, following the CDC protocol from CHC2, for all available samples in the spraying season, Day 1 and Day 5 samples for Thinning and Non-Spray season, and selected Day 7 (n=50) samples in the Thinning season, as described in Weldon et al. (2016). Briefly, according to our study protocol, developed by CHC and our Community Advisory Board (CAB), adult and

child urine was evaluated for 6 OP metabolites: dimethyl phosphate (DMP), dimethyl thiophosphate (DMTP), and diethyl di-thiophosphate (DMDTP), as well as Diethyl OPs metabolites: diethyl phosphate (DEP), diethyl thiophosphate (DETP) and diethyl di-thiophosphate (DEDTP). To perform the urinary DAPs analyses, lyophilization with gas chromatography-mass spectrometry and isotope dilution quantification were implemented and run according to CDC protocols, as described in Bravo et al. (2004). The total OPs analyzed in CHC2 and CHC3, in this study are metabolized into the following 5 DAP metabolites (**Table 1**).

<i>Organophosphate Pesticide</i>	<i>Class</i>	<i>Dialkylphosphate Urinary Metabolites</i>
<i>Azinphos-methyl</i>	<i>Dimethyl OP</i>	<i>DMP, DMTP, DMDTP</i>
<i>Malathion</i>	<i>Dimethyl OP</i>	<i>DMP, DMTP, DMDTP</i>
<i>Phosmet</i>	<i>Dimethyl OP</i>	<i>DMP, DMTP, DMDTP</i>
<i>Chlorpyrifos</i>	<i>Diethyl OP</i>	<i>DEP, DETP</i>
<i>Diazinon</i>	<i>Diethyl OP</i>	<i>DEP, DETP</i>
<i>Methamidophos</i>	<i>Dimethyl OP</i>	<i>DMTP</i>

Table 1. Urinary metabolites of organophosphate pesticides.

Abbreviations: OP, pesticide; DMP, dimethyl phosphate; DMTP, dimethyl thiophosphate; DMDTP), diethyl di-thiophosphate; DEP, diethyl phosphate; DETP, diethyl thiophosphate.

4.4 Health Questionnaire and Respiratory Endpoint Assessment

To establish respiratory endpoints, a questionnaire survey was administered to participants in 2011 during the non-spray season of the CHC3 study. The survey collected information on respiratory health history and allergic status of both adult and child participants. Ninety-eight households (58 FW and 40 NFW) answered the questionnaire on behalf of both the adult and the referent child. These 98 houses were used as the subset study population for this project's analysis. The respiratory-related questions included in the questionnaire were developed under the guidance of

Dr. Catherine Karr (UW DEOHS and Depts. of Pediatrics and Epidemiology), who specializes in pediatric asthma research, with a focus in agricultural communities, and adapted from the ISAAC (International Study of Asthma and Allergies to Childhood-- age 6-7 core module). Data was collected on respiratory health outcomes including the following: pneumonia, bronchitis, whooping cough, croup, asthma, hay fever, and other respiratory issues, and specific respiratory illness symptoms were surveyed: wheezing, chest pain and difficulty breathing, eczema and itchy rash. As similarly done with data from the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) birth cohort from California (Raanan et al., 2015), in this project, the asthma-related respiratory health outcome was based on a positive response to specific survey questions on respiratory symptoms. In our study, an “asthma-like respiratory health outcome”, represented by the y-axis label “Impaired Respiratory Health” in the Results section, is defined by a positive response to any of the following questions adapted from the distributed survey:

- *Have you or your child had pneumonia?*
- *Have you or your child had bronchitis?*
- *Have you or your child had croup?*
- *Have you or your child had pertussis?*
- *Have you or your child had other serious respiratory disease?*
- *Have you or your child ever had wheezing or whistling in the chest?*
- *Has a doctor diagnosed you or your child with asthma?*
- *Has your child ever had chest pain?*
- *Has your child ever had difficulty breathing?*

4.5 Statistical Analysis of Dust with Respiratory Health Outcome

To address the first specific aim and hypothesis of this project, we investigated how the self-reported data on respiratory outcomes obtained in the CHC3 study were related to the concentrations of OPs measured in house dust in the CHC2 and CHC3 studies. For the statistical analysis of this data, the respiratory outcomes are all binary outcomes depending on their presence or absence as reported by the study participants. The concentrations of the OPs in house dust are reported as nanomoles per gram of dust (nmol/g). Each was collected up to nine times across the two studies and summed to get total OP concentrations. To determine if there was a statistically detectable relationship between a respiratory outcome and the OPs, a logistic regression model was used. The model was evaluated by testing whether the relationship was statistically significant to answer the question; whether the exposure to the OP compounds was related to the respiratory outcome. The model used was:

$$\text{logit}(r) = a + \text{Sum}(c[i] b[i]) + \text{error}$$

Where:

Sum = summation across all times

r = respiratory outcome

c[i] = concentration in dust of OP at time *i*, units of nano moles

a = estimated intercept in logistic model

b[i] = estimated coefficient for time *i*

logit = logistic function which is $\log(p/(1-p))$ where *p* is the probability of the respiratory outcome

error = residual error.

For dust concentrations below the limit of detection an imputation technique was used. We used Markov Chain Monte Carlo (MCMC) method to estimate mean concentrations at each time point and the variability across all the time points. These estimates for each time point were then used to impute values for the measurements below the limit of detection and missing values, and 10,000 imputations were performed after a burn in of 10,000 simulations of the MCMC chains. This was implemented in the statistical computing software R (R Core Team, 2020) with the Bayesian Regression Models using 'Stan' (BRMS) package, which is an interface to Stan, an MCMC language. The MCMC model for these simulations was:

$$\log(c[i]) = \text{Sum}(I[i] d[i]) + \text{error}$$

Where:

Sum = summation across all times

c[i] = concentration in dust of OP at time i, units of nano moles

I[i] = indicator that measurement occurred at time i

d[i] = estimated mean concentration at time i

error = residual error.

The imputation for missing values using the MCMC model for a concentration at time *i* was computed by choosing a random value from the normal distribution with mean *d[i]* and standard deviation equal to the error (a separate random value was computed for each missing value in time period *i*). For values below the limit of detection in time period *i*, a random value below the limit of detection was chosen from the same distribution. The imputed values were then used in MCMC model equation above, along with the values that were not missing or below the limit of detection.

4.6 Statistical Analysis of DAPs with Respiratory Health Outcome

To investigate how the self-reported data on respiratory outcomes obtained in the CHC3 study is related to the concentrations of DAPs in the CHC2 and CHC3 studies, a similar logistic regression model was used. The model was evaluated by testing whether the relationship was statistically significant to the respiratory health binary outcome. The model used was:

$$\text{logit}(r) = a + \text{Sum}(c[i] b[i]) + \text{error}$$

Where:

Sum = summation across all times

r = respiratory outcome

c[i] = concentration in DAPs at time *i*, units of nano moles

a = estimated intercept in logistic model

b[i] = estimated coefficient for time *i*

logit = logistic function which is $\log(p/(1-p))$ where *p* is the probability of the respiratory outcome

error = residual error.

For values below the limit of detection the same MCMC imputation technique was used. The MCMC model for these simulations was:

$$\log(c[i]) = \text{Sum}(I[i] d[i]) + \text{error}$$

Where:

Sum = summation across all times

c[i] = concentration in DAPs at time *i*, units of nano moles

I[i] = indicator that measurement occurred at time *i*

d[i] = estimated mean concentration at time *i*

error = residual error.

The OP pesticides and their DAP metabolites are presented as a percentage of samples above detection limit, and these are depicted in **Supplemental Table 2**

5. RESULTS:

5.1 OPs in Dust and Asthma-Related Respiratory Health Outcome (Aim 1)

The association between the concentration of total OP parent compounds detected in house dust versus the asthma-related respiratory health outcome, referred to as “impaired respiratory health” across the logistic regression models, was statistically significant in our adult population ($p=0.039$). **Figure 4a**, shows this association in our adult study population. There were 23 cases in 96 persons. The decreasing slope shows that as total OP concentration in nanomole/gram of dust increased (over 2 orders of magnitude), probability of impaired respiratory health decreased, with most cases occurring at lower levels of OP concentrations. Similarly, in **Figure 4b**, the slope also is decreasing, however in this case the association between asthma-related health outcomes and OP concentration in dust is not significant with a p-value of 0.24. In this case, 30 children out of 96 were reported to have respiratory health outcomes associated with asthma as declared on our questionnaire. For both adults and children, there was a negative slope with increasing dose indicating that our asthma/respiratory outcomes were correlated with lower OP levels.

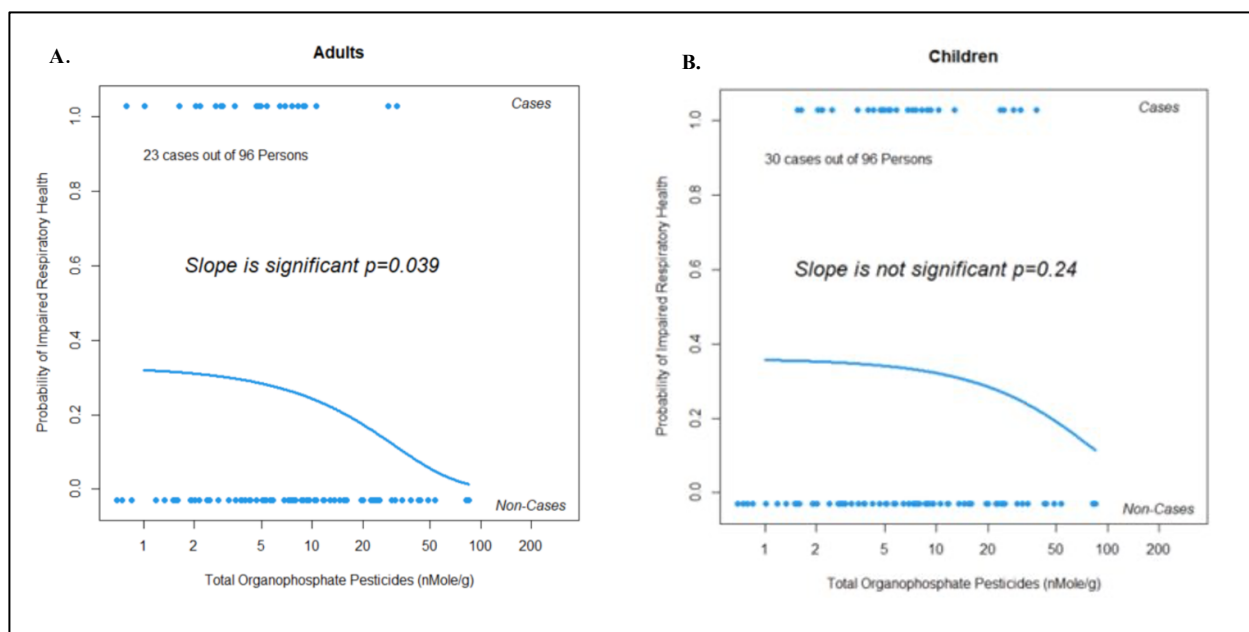


Figure 4: Association between OP Parent Compounds detected in house dust and Asthma-related Respiratory Outcomes in an Agricultural Community. 4a. Probability of Adult Impaired Respiratory Health vs. Total OPs in dust. **4b.** Probability of Child Impaired Respiratory Health vs. Total OPs in dust. These graphs represent the association between the OP parent compounds detected in house dust for 96 adults and 96 children (1 adult per household and 1 child per household). The y-axis reflects a positive response to the asthma-related respiratory health questions asked in our cohort's survey. The x axis shows the total OP concentrations in nanomole per gram of dust. The blue dots at the top represent cases, or individuals that were considered as having impaired respiratory health, i.e., asthma-related health outcomes, and the blue dots at the bottom represent non-cases, or individuals that did not declare any asthma-related health outcomes i.e. no impaired respiratory health.

To further explore the association in the model, additional analysis was conducted to see if sex and/or occupational status (FW/NFW) significantly affected the relationship. As shown in **Figure 5**, in adults, there were 3 cases out of 13 males, and 20 cases out of 83 females. In children, there were 12 cases out of 45 males, and 18 cases out of 51 females. When probability of impaired respiratory function was logistically regressed against total OP concentration, there were no significant differences in slopes between male and females in both groups. Finally, we looked at the difference in slopes between FW and NFW groups in both adults and children (**Figure 6a and 6b**). In adults, there were 12 cases out of 39 NFW and 11 cases out of 57 FW; in

children, there were 14 cases out of 40 NFW and 16 cases out of 57 FW and in both cases, the slopes between NFW and FW were not significantly different from one another.

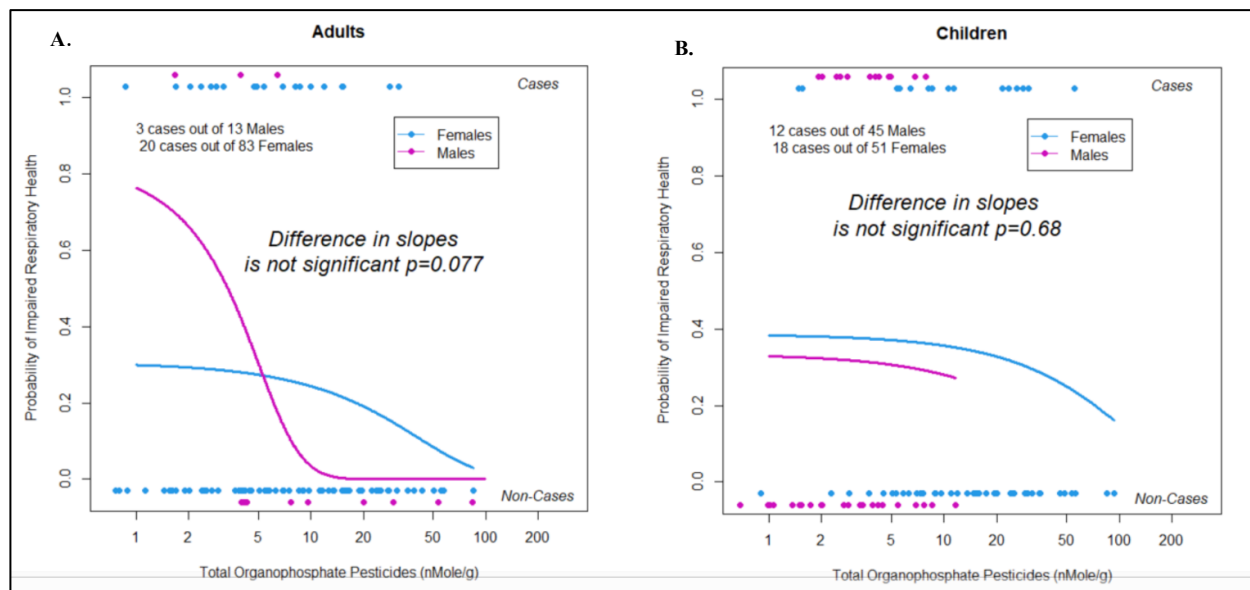


Figure 5: Association between OP Parent Compounds detected in house dust and Asthma-related Respiratory Outcomes in Males and Females of an Agricultural Community. 5a. Adult Impaired Respiratory Health vs. Total OPs in dust in Males and Females. **5b.** Probability of Child Impaired Respiratory Health vs. Total OPs in dust in Males and Females. The red dots represent cases and non-cases in males, and the blue dots represent cases and non-cases in females, for both the adult figure on the left and the child figure on the right. The red line represents the slope association in both figures for males and the blue line represents the slope association for females.

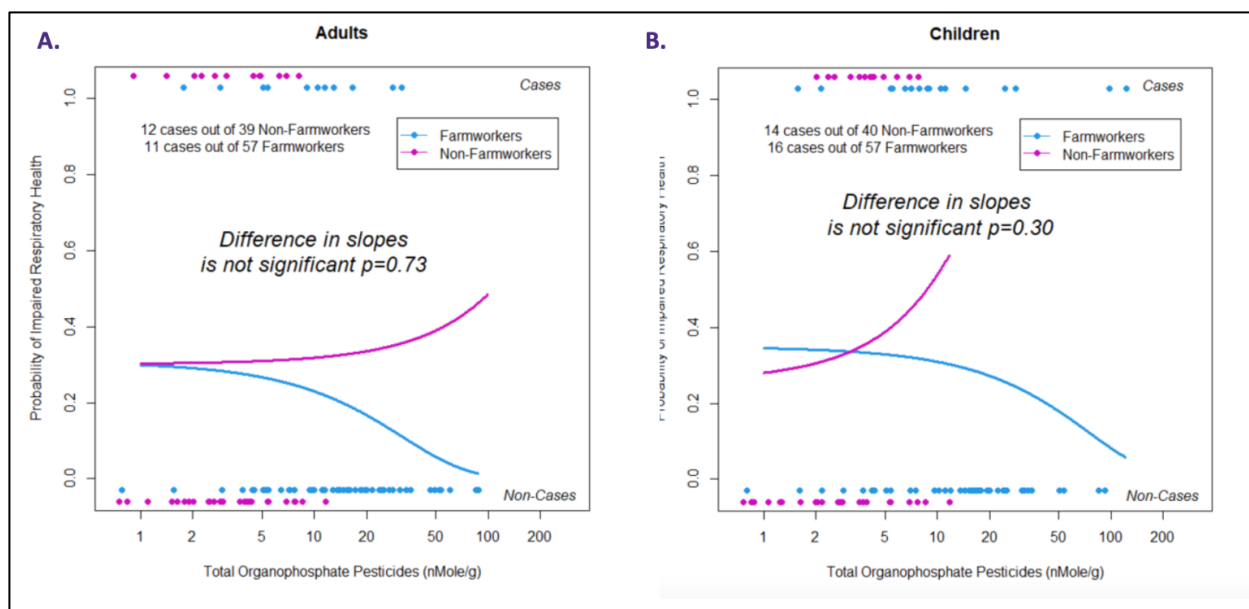


Figure 6: Association between OP Parent Compounds detected in house dust and Asthma-related Respiratory Outcomes based on Occupational Status in an Agricultural Community
6a. Probability of Impaired Respiratory Health vs. OPs in dust in Adult Farmworkers and Non-Farmworkers. **6b.** Probability of Impaired Respiratory Health vs. OPs in dust in Farmworker and Non-Farmworker Children. In adults, there were 12 cases out of 39 NFW (red) and 11 cases out of 57 FW (blue), and the slopes were not significantly different ($p=0.73$), In children, there were 14 cases out of 40 NFW and 16 cases out of 57 FW. Similarly, the slopes were not significantly different from one another ($p=0.30$)

5.2 DAP metabolites and Asthma-Related Respiratory Health Outcome (Aim2)

As shown in **Figure 7a**, we see that similarly to our dust analysis, for adults, the relationship of impaired respiratory health to the concentration of total urinary OP DAPS is significant at $p=0.009$. There were 23 cases in 97 persons and the relationship to probability of impaired respiratory health reflected a decreasing slope, showing that as urinary DAP concentrations in nanomole per ml of urine increased, probability of impaired respiratory health outcomes decreased. Like the total OP parent compounds collected in the dust samples, the total urinary DAPs were related to a decrease in probability of asthma-related respiratory health outcomes as described in our cohort's survey responses. In **Figure 7b**, the slope is increasing but, in this case the association is not significant, with a p -value of 0.20

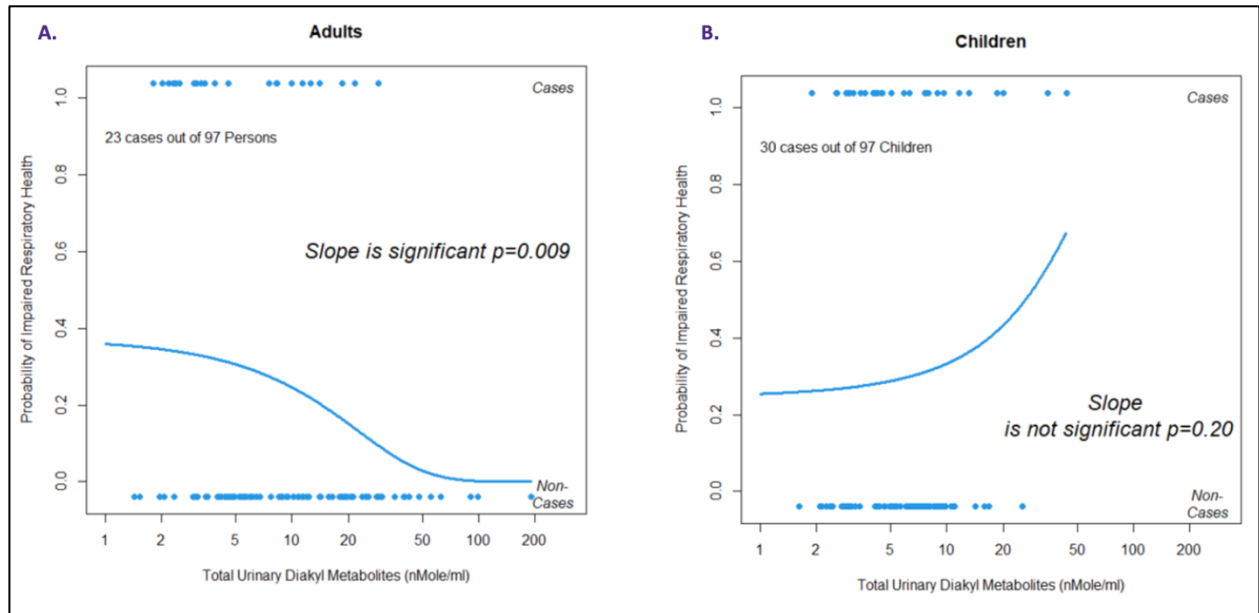


Figure 7: Association between urinary DAPs, and Asthma-related Respiratory Outcomes in an Agricultural Community. 7a. Probability of Adult Impaired Respiratory Health vs. Total DAPs **7b.** Probability of Child Impaired Respiratory Health vs. Total DAPs. These graphs represent the association between the urinary DAPs for 97 adults and 97 children (1 adult per household and 1 child per household). The y-axis reflects a positive response to the asthma-related respiratory health questions asked in CHC3. The x axis shows the total urinary DAP concentrations in nanomole per ml of urine. The blue dots at the top represent cases, or individuals that declared asthma-related health outcomes, and the blue dots at the bottom represent non-cases, or individuals that did not declare any asthma-related health outcomes (i.e., no impaired respiratory health).

To further explore the association of total DAPS and asthma-related health outcomes, we investigated whether sex played a significant role in the relationship (**Figure 8**). Similar to the adult results from Aim 1, when impaired respiratory health was logistically regressed against total DAPs in urine, there were no significant differences in the association between adult males and females ($p=0.067$) (**Figure 8a**). On the other hand, in the children, the difference in slopes between males and females was found to be statistically significant ($p=0.043$) (**Figure 8b**). Females in this group, with 18 cases out of 52, show a decreasing slope in probability of impaired respiratory health as total DAPs increase, whereas males in this group, with 12 cases out of 45 males, reflect

a positive trend in probability of impaired respiratory health, or asthma-related health outcomes, as total urinary DAPs increase. As reflected in **Figure 9a and 9b**, the difference in slopes for occupational status between FW and NFW groups, was not significant in both adults and children.

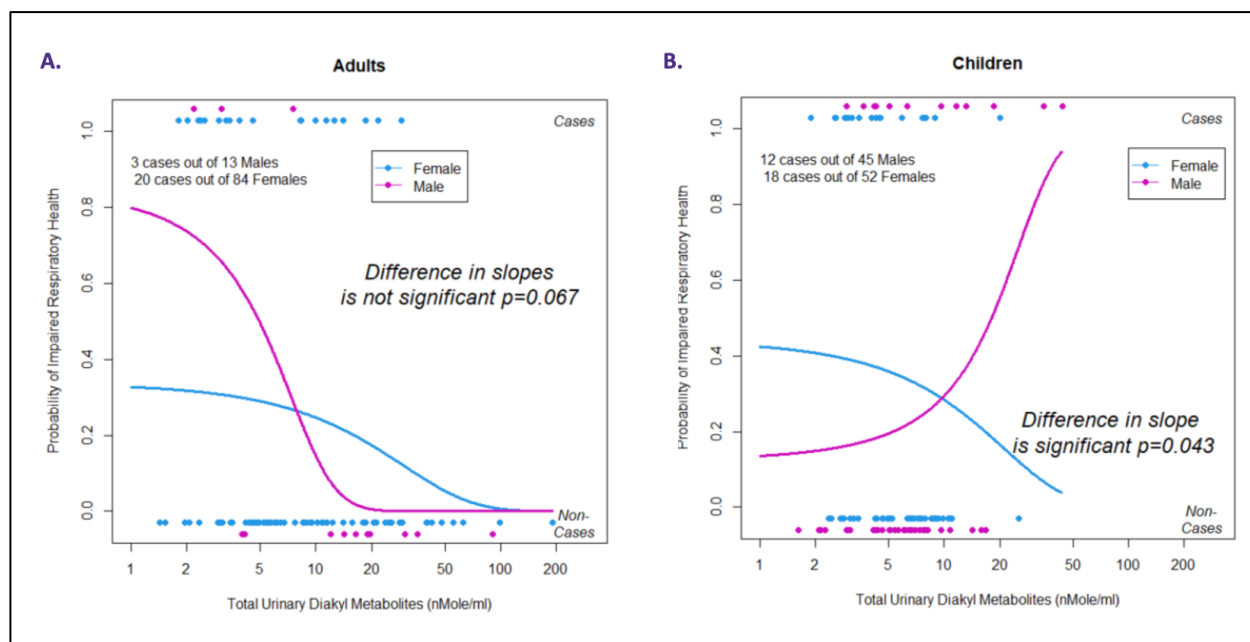


Figure 8: Association between Urinary DAPs and Asthma-related Respiratory Outcomes in Males and Females of an Agricultural Community. 8a. Adult Impaired Respiratory Health vs. Total DAPs in dust in Males and Females. **8b.** Probability of Child Impaired Respiratory Health vs. Total DAPs in dust in Males and Females. The red dots represent cases and non-cases in males, and the blue dots represent cases and non-cases in females, for both the adult figure on the left and the child figure on the right. The red line represents the slope association in both figures for males and the blue line represents the slope association for females.

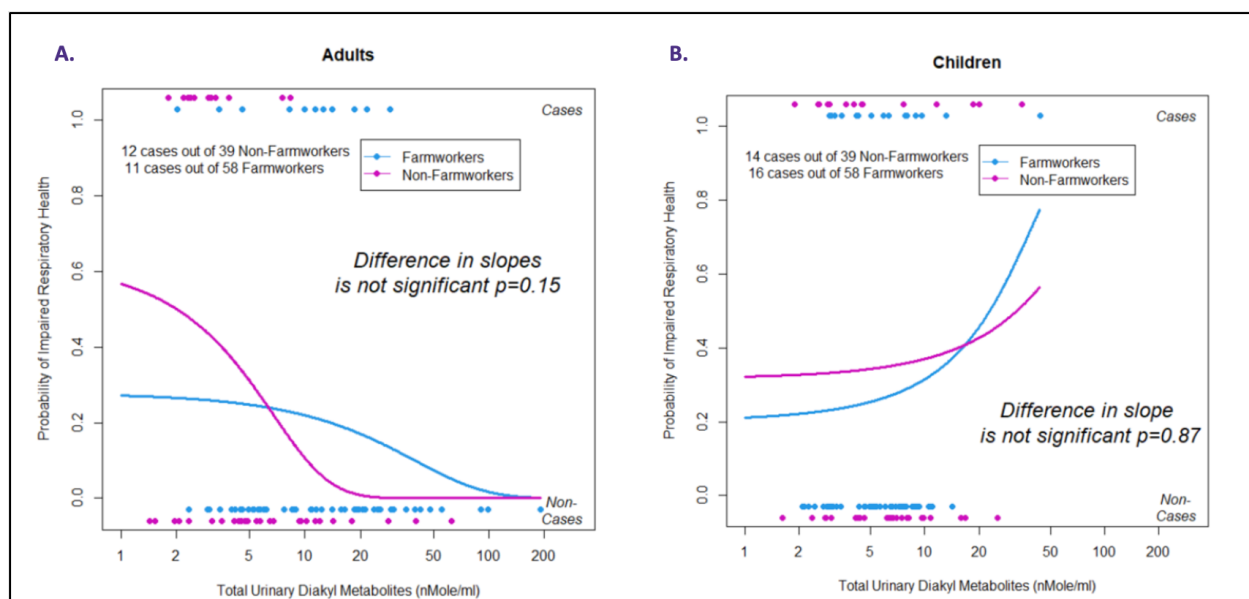


Figure 9: Association Between Urinary DAPs and Asthma-related Respiratory Outcomes in Farmworkers and Non-Farmworkers. 9a. Probability of Impaired Respiratory Health vs. Total DAPs in Adult Farmworkers and Non-Farmworkers. **9b.** Probability of Impaired Respiratory Health vs. Total DAPs in Farmworker and Non-Farworker Children. In adults, there were 12 cases out of 39 NFW (red) and 11 cases out of 58 FW (blue), and the slopes were not significantly different ($p=0.15$), In children, there were 14 cases out of 39 NFW and 16 cases out of 58 FW. Similarly, the slopes were not significantly different from one another ($p=0.87$),.

Additional analysis was completed with azinphos-methyl (AZM), individually, as it composed the highest percentage of OP pesticide compounds in dust. Impaired respiratory health was not significantly related for adults and children to the AZM pesticide concentrations in household dust, although it was near to being significant for adults at $p=0.061$ (**Supplemental Data Figure 1**).

6. DISCUSSION

In this project, we found that when probability of impaired respiratory health was logistically regressed on the total OP compounds from dust samples and DAPs from urine samples in the CHC2 and CHC3 studies, the association was significant in both cases for adults (**Figure 4a and Figure 7a**). The decreasing relationship shows that as OP concentrations increased, the asthma-related respiratory health outcome decreased—potentially implying that exposure to OPs, may be protective of asthma-related outcomes. This could be due to an effect of the pesticide on asthma triggers or the microbiome of our population. This preliminary analysis mandates further exploration to examine causation. Different studies show contrasting results when looking at pesticide relationships with asthma. They differ based on exposure metrics and types, occupational status, and outcome definition. One prospective cohort study by Hoppin et al., (2014) compared asthma and exposure data from the Agricultural Health Study (AHS) of farmworkers in Iowa and North Carolina to data from the public via the National Health and Nutrition Examination Survey (NHANES). The incidence of asthma rate in the AHS farmer population was 2.1 versus 4.0 (per 1000 person-years) in the NHANES general population. This study findings supported decreased asthma, both new-onset and ever asthma, in adult farmers. Similarly, there have also been many studies in children that support the concept that growing up around an agricultural environment leads to a strong protective effect against developing asthma (Riedler, et al. 2001). In our current results, the association was not significant in children, however when analyzing urinary DAPs versus asthma-related respiratory outcome, a significant difference in slope was seen between males and females in children, implying that sex could play a role in disease development. According to the Washington State Department of Health, the Washington Behavioral Risk Factor Surveillance System (BRFSS) supports the findings that among young children, boys are more

likely than girls to have current asthma and that this relationship changes by adolescence, when a higher prevalence in women than men arises (Washington State Department of Health, 2010). This relationship mandates additional exploration but is suspected to be attributed to hormonal changes (Postma, 2007). As shown in the results from **Figure 8b**, when separating the group of children into girls and boys the association to our asthma outcome was significantly different and the slopes seem to be in opposite directions, with the male group trending towards a positive slope and the girls trending to a negative slope. This could reflect that sex plays a role in the relationship of OP pesticides and asthma outcome in this cohort population. Considering the scientific fact that children are known to be more susceptible to many environmental toxicants, further investigation of how environmental exposure to pesticides may alter their response is merited. Child specific factors such as increased rate of lung development, variable enzyme levels, and significant changes in hormonal and metabolic activity across development (Saadeh et al., 2014) that have been proposed as mechanisms for explaining children's increased susceptibility and these should be further explored for environmentally-based asthma research using a life-course approach.

In a review article by Wunschel & Poole (2016), the study team summarizes the distribution of publications supporting occupational farming exposures as a risk factor/protective factor for asthma. The results are widespread and are provided in **Supplemental Table 3**. The variability that is seen in these studies identifies a critical need for additional investigation of the multiple possible factors that affect the incidence of respiratory health effects for children with asthma in rural agricultural communities.

Overall, exposures are complex and can be challenging to characterize. The Exposome Framework is defined as the totality of exposures that occur from conception onwards (Wild, 2005). It provides the ability to look across multiple pesticides that adults and children in agricultural settings may be exposed to at work or in their homes. Methods to apply an exposome approach include using multiple environmental and biomonitoring techniques, and to look at results across multiple studies, that consider developmental periods of susceptibility, cumulative exposures, over multiple time points (Guillien et al., 2021). A hallmark of our cohort is the characterization of these different pathways, applying an exposome approach—using a wide distribution of environmental data, multiple sampling methods, various biomarkers, and targeting susceptible periods of development in the children of our cohort population, over multiple years, and seasons—allows for further characterization of OP insecticide exposures, to begin exploring disease development, health outcomes, and potentially uncover new sensitive biomarkers of both exposure/responses. In this project the OP concentrations were measured over 2 orders of magnitude, across three seasons in two different years. A strength of this study is therefore its depth of exposure detail, its longitudinal nature—following a population over multiple years— its investigation of multiple time points across each season, and control for variability within our study groups. Another strength of this study is the use of multiple biomarkers for OP exposure in the same analysis model, allowing us to compare results across different studies with singular exposure metrics. Dust was collected at multiple points across different agricultural seasons, to adjust for temporal bias when comparing OP dust concentrations to respiratory health outcome. Urinary DAPs were collected at the same time as dust samples and run against the same end point potentially giving insight on biomonitoring differences that should be considered when making conclusions across studies.

The outcome of interest, asthma, is a critical endpoint with significant variability in susceptibility, symptomatology, and pathology (Lougheed et al., 2007). This limits our ability to find a reliable and unique biomarker for quantifying disease status. Limitations of our study include the use of self-reported disease symptoms, that could have been improved with the addition of an objectively measured outcome for respiratory function performance, such as pulmonary function, which has been used across other environmental respiratory research study teams, such as Salome et al. (2000). These researchers examined low level exposures of insecticides and asthma by monitoring changes in forced expiratory volume (FEV1), a respiratory functional biomarker. Other asthma researchers have looked at mechanisms using biomarkers such as T-cell response in agricultural populations. Duramad et al. (2006) looked at T-helper 1 (Th1) and T-helper 2 (Th2) cytokines as biomarkers of allergic asthma at 12 months and 24 months in children from the CHAMACOS agricultural cohort. Our future asthma studies could benefit from such additions.

Differences in results across studies on environmentally induced asthma could be due to a variety of both exposure and susceptibility factors, including different times and complexity of pesticide exposures. However, it was important to examine populations of concern and since approximately 20% of the children in this cohort have been diagnosed with asthma, a rate higher than the WA state average and the national average of 8% in the United States (WA Department of Health, 2010) it provided an opportunity to look at a variety of such factors. In this study, the respiratory health questions were selected to encompass asthma outcomes based on asthma clinical expert guidance to encompass a wide array of asthma and respiratory phenotypes and profiles. Despite these strengths, our analysis is subject to the limitation of having a smaller sample size.

Policy makers should address differences in exposure characteristics based on multiple pathways and focus on understanding differences between children and adult exposure and mechanisms of susceptibilities across life stages. Taking into consideration the different windows of vulnerability to disease and exposure across lifespan, could lead to efficient, protective regulations across populations.

Future studies that continue to consider the different windows of vulnerability to disease and exposure across lifespan, could enhance translation of our results. Studies on OP-associated respiratory morbidity in children with asthma should consider including clinically-defined measures of asthma exacerbation such as spirometry, medication use, and symptom days, incorporated with a similar exposome framework approach to the one in this study-- with multiple biomarkers of pesticide exposure, across different seasons, and targeting susceptible periods of development. In our cohort we plan to continue capturing the level of detail that makes up the exposome of our study population. Our extended data contains quantitative data on over 16 different pesticides, and we plan to look at interactions in between classes, beyond OPs. We also have microbial data from the dust samplings in our population, including data on dust mites, which is one example of an additional exposure that might influence the respiratory health outcomes concerned in this study. Future directions include investigating the effect of medically recognized asthma triggers, among other exposure agents, on the association of OPs and our respiratory health outcomes.

7. FUTURE DIRECTIONS

An Exploration of MicroRNAs as biomarkers of Exposure and Disease (Aim 3)

A unique aspect of our studies is the variety of environmental biomarkers to characterize exposure and response. Investigating exposure association with different biomarkers of diseases is important to identify potential mechanisms of toxicity, molecular and physiological effects of associated disease progression, or novel therapeutic targets.

MicroRNAs (miRNAs) are small, non-coding RNA molecules that are fundamental in regulating gene expression (Jorge et al., 2021). Throughout evolution, they remain highly conserved and are involved in a multitude of biological processes, acting as post-transcriptional regulators in cell proliferation, signal transduction, degradation, etc. (Weber et al., 2010). Their intensive role in the immune system has increasingly become apparent in the last decade, in both clinical research and environmental exposure research (Hirschberger et al., 2018).

Studies running genome wide analyses have revealed microRNAs can function as viable biomarkers of different environmental exposures. A study by Rani, et al. (2021) found that altered expression of selective microRNAs can be used as early warning biomarkers in a polycyclic aromatic hydrocarbon (PAH)-exposed population. Mall et al. (2013) demonstrated that urinary miRNAs remain stable throughout a variety of storage conditions, further emphasizing their potential as reliable biomarkers. In our cohort, Weldon et al. (2016), identified that urinary microRNAs could serve as viable biomarkers of occupational status and OP pesticide exposures. The expression of six microRNAs (miR-223, -518d-3p, -597, -517b, -133b, & -28-5p) was

positively associated with occupational and seasonal status. Five of these demonstrated a positive dose-response relationship with OP metabolites in our study population, and have been identified as key regulators in various hallmark pathways of OP toxicity, including AChE and butyrylcholinesterase activity (Eaton et al., 2008) (**Supplemental Data Figure 2**).

As prime examples of regulatory molecules, microRNAs have also been demonstrated to influence and respond to inflammation and are associated in literature with both disease status and environmentally-induced toxic pathways. Several of these microRNAs, specifically our highest detected microRNA miR-223, have been studied as potential biomarkers of asthma and various respiratory health endpoints (Heffler, 2017). Animal models have demonstrated a protective up-regulation of miR-223, and an attenuation of asthma inflammation. In a study by Xu et al (2020), knockout miR-223 mice instantly developed asthma outcome and overexpression of miR-223 via treatment with miR-223 agomirs (artificially constructed miRNA mimics) in the asthmatic group, neutrophilic inflammation was attenuated, supporting its potential protective role in asthma pathogenesis via its influence on inflammatory mechanisms (Xu et al., 2020). Mechanistic studies have established the potential pathways of microRNA-223's protective effects on asthma inflammation (**Supplemental Data Figure 3**), its upregulation and significant association within our OP-exposed population, could play a factor in the analyses conducted in this project.

Research considering the clinical regulation of miRNA expression as a potential novel therapy for improving efficacious asthma treatment has begun in recent years (reviewed in Feketea et. al., 2019). Further investigation into the pathways involved with the microRNAs altered in our cohort population may be used to interpret the biological processes, functions, and pathways involved in

pesticide exposures. Future directions include adjusting the analyses done in this project for miR-223 expression (upregulation/downregulation) as an OP biomarker, versus asthma-related health outcomes, to investigate the hypothesis that the upregulation of miR-223, could have a protective effect over the respiratory health outcome in this study for our agricultural population.

8. CONCLUSION

This study highlights the importance of research aiming to define the complexity of underlying environmental exposures that contribute to the risk of respiratory disease. Multidisciplinary approaches are critical for understanding the effects of exposure on health outcomes. Using detailed exposure sampling that considers multiple pathways of exposure and identifies multiple biomarkers of exposure and response allows for further mechanistic exploration of environmentally-induced respiratory toxicity.

In this thesis project, Aim 1 and Aim 2 concluded that OP biomarkers in dust and urine were found to be significantly associated with decreased probability of asthma-related respiratory outcomes in our cohort adult population; in our exploratory Aim 3, it was concluded that this relationship could potentially be influenced by an upregulation of miR-223 and other inflammatory cytokines, capable of inhibiting signaling pathways in the pathogenesis of asthma. Further exploration of these phenomena in clinical/genetic x environmental research is strongly encouraged. Finally, the potential of urinary microRNAs to serve as cause/effect biomarkers should be further investigated.

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10. SUPPLEMENTAL MATERIALS

Number of pesticides / compounds analyzed by type	Compound Name / Class of Pesticides
Organophosphate Pesticides (N= 16)	Azinphosmethyl, Phosmet, Malathion, Chorpyrifos, Diazinon, Coumaphos, Methyl-parathion, Methamidophos, Dimethoate, Dichlorvos, Phorate, Tetrachlorvinphos, Methidathion, Naled, Terbufos, Ethoprop
Other Pesticides by class (N= 71)	Pyrethroid (8), N-Methyl Carbamate (7), Neonicotinoid (7), Chlorophenoxy acid or ester (4), Imidazole (4), Urea (3), Azole (3), Anilide (2), Pyrimidine (2), Quinoline (2), Triazine (2), Aryloxyphenoxy propionic acid (1), Benzimidazole (1), Bipyridylum (1), Benzoic acid (1), Chlorophenol (1), Carbazate (1), Dicarboximide (1), Guanidine (1), Metal (1), Pyridine (1), Pyrazole (1), Phenol (1), Pyridazinone (1), Strobilurin (1), Tetrazine (1), Xylylalanine (1), Other (11)
Metals (N= 22)	Lithium, Titanium, Chromium, Manganese, Iron, Cobalt, Nickel, Copper, Zinc, Gallium, Arsenic, Selenium, Molybdenum, Silver, Cadmium, Antimony, Tungsten, Mercury, Thallium, Lead, Bismuth, Uranium
Phthalates (N= 18)	Butylbenzyl phthalate(BBP), Di(2-ethylhexyl)phthalate (DEHP), Di(n-butyl)phthalate (DBP), Diethylphthalate (DEP), Dimethylphthalate (DMP), Di(n-pentyl)phthalate (DPP), n-Butylcyclohexyl phthalate (BCHP), n-Butyl-n-decyl phthalate (BDP), Diallyl phthalate (DAP), Bis(2-n-butoxyethyl) phthalate (DBEP), Dicyclohexyl phthalate (DCP), Di-n-hexyl phthalate (DHP), Diisobutyl phthalate (DiBP), Diisodecyl phthalate (DiDP), Diisooctyl phthalate (DiOP), n-Octyl-n-decyl phthalate (DODP), Di-n-octyl phthalate (DOP), Di-n-propyl phthalate (DPrP)

Supplemental Table 1. Compounds and metals analyzed in dust samples

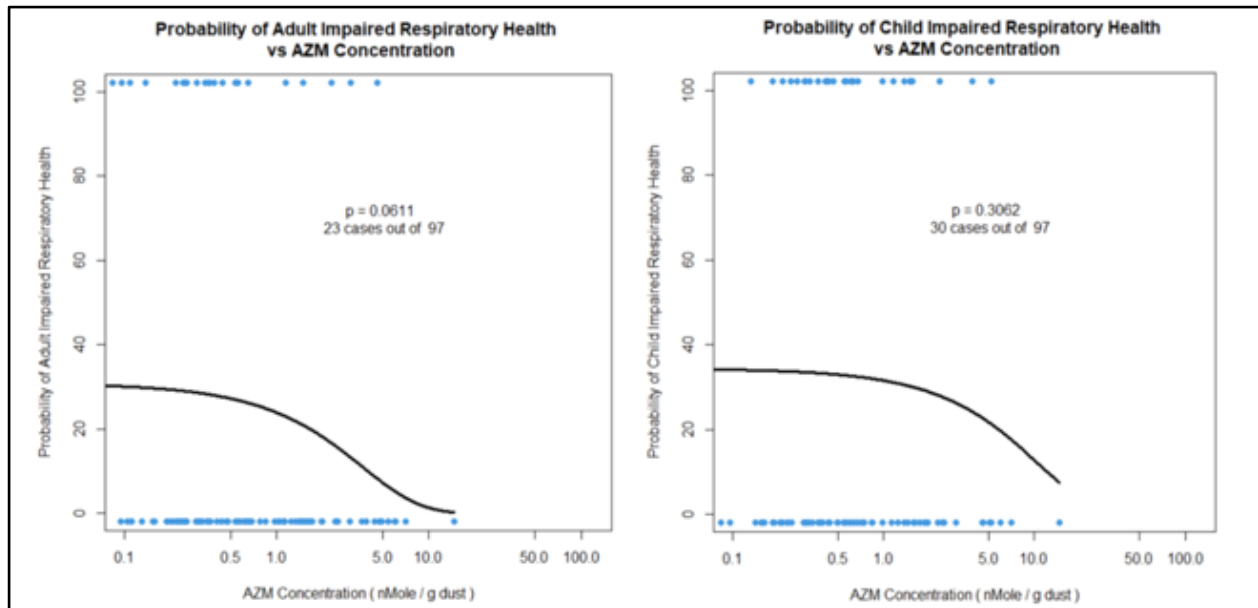
	Percent >LOD	Diakyl Metabolites					
		DMP	DMTP	DMDTP	DEP	DETP	DEDTP
Dialkyl Metabolites							
Percent>LOD	53%	56%	76%	58%	47%	57%	33%
Organophosphate Pesticide							
Azinphosmethyl	71 %	X	X	X			
Phosmet	82 %	X	X	X			
Malathion	63 %	X	X	X			
Methamidophos	7 %		X				
Chlorpyrifos	36 %				X	X	
Diazinon	59 %				X	X	

Supplemental Table 2. Organophosphate (OP) pesticides and their dialkyl phosphate (DAP) metabolites. Percent of samples of OP pesticides in dust and DAP metabolites of OPs in urine above detection limit.

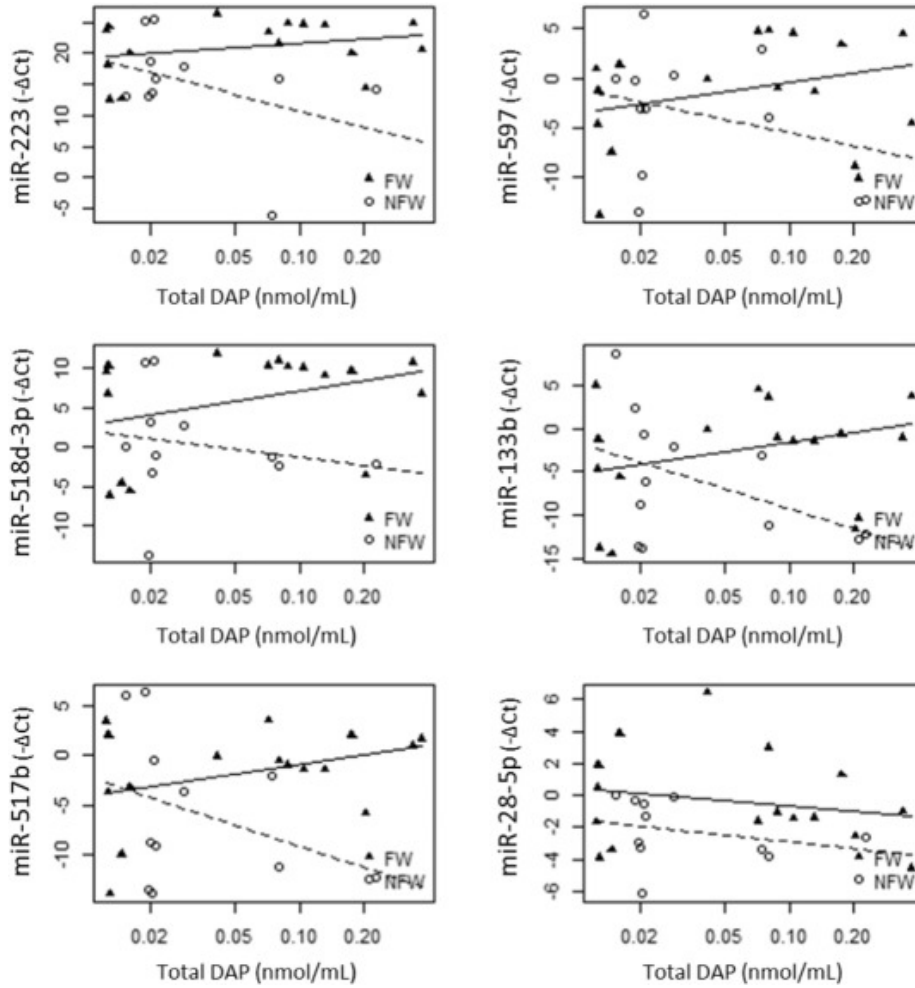
Study	Farming as Risk Factor for Asthma	Farming as Protective Against Asthma	No Effect
Douwes et al. (17)			
Current livestock and crop farmers		X	
Thaon et al.(4)			
Dairy	X		
Non-dairy livestock			X
Eduard et al. (19)			
Atopic asthma		X	
Nonatopic asthma		X	
Hoppin et al.(20, 26, 27)			
AHS study group		X	
Pesticide use	Atopic > Non-atopic		
HPEE	X		
Kogevinas et al. (23)			
All farmers	X		
Omland et al. (25)			
Swine	X		
Cattle	X		
Eduard et al. (3)			
Atopic with ≥ 2 livestock		X	
Nonatopic with ≥ 2 livestock	X		
Karjalainen et al. (24)			
Men and women	X		
Stoecklin-Marois et al. (28)			
Acculturated farm men			X
Acculturated farm women	X		

HPEE= High Pesticide Exposure Event.

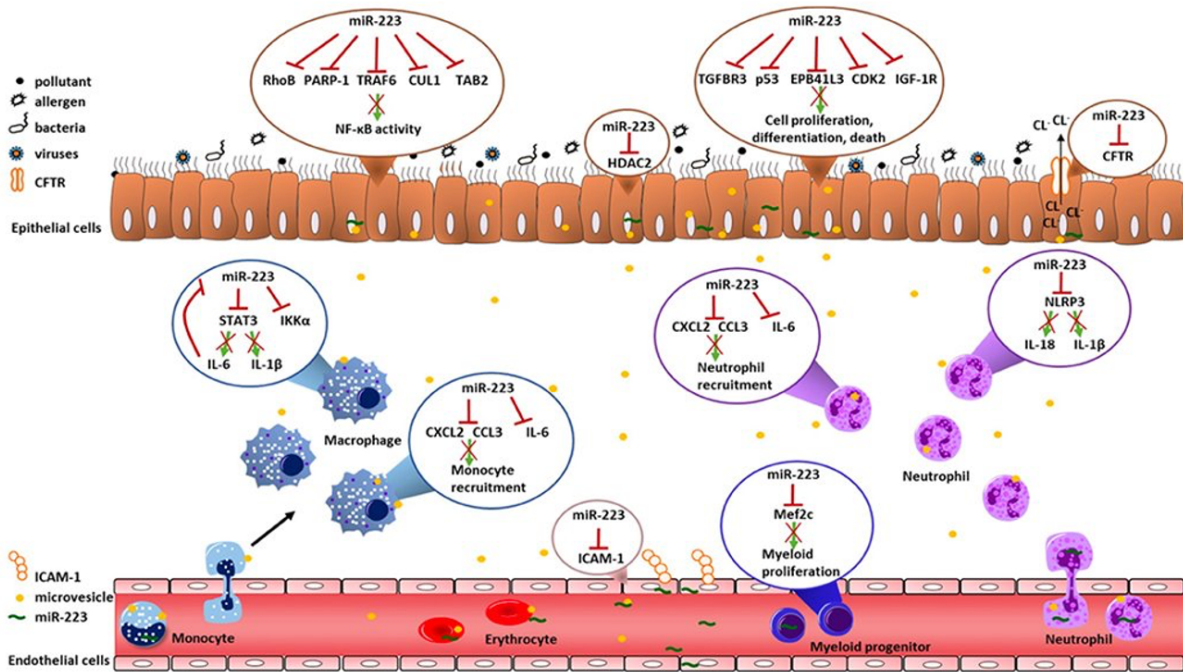
Supplemental Table 3: Summary of the studies in adult subjects supporting occupational farming exposures as either a risk factor for, or a protective factor against asthma (Wunschel et al. 2016).



Supplemental Figure 1. AZM Concentration in dust in CHC2 and CHC3 and Probability of Asthma-Related Health Outcomes on Questionnaire Survey in Adults and Children. The models show logistic regression of impaired respiratory health on the concentration of AZM pesticide. For adults we had 23 cases in 97 persons and the logistic regression was near statistically significant at $p=0.061$. For children we had 30 cases in 97 persons and was not statistically significant at $p=0.31$. The AZM concentrations were measured across 3 seasons in two different years.



Supplemental Figure 2: Regression of total DAPs and six FW-related miRNAs in FW and NFW adults in the post-harvest. FWs are indicated by triangles with solid trend lines and NFWs are indicated by open circles and hashed trend lines. Metabolites are reported as nmol total DAPs/mL urine and miRNA levels are reported as $-\Delta Ct$ where greater expression of miRNA corresponds with a positive trend. (Weldon et al., 2016)



Supplemental Figure 3: Overview of validated targets of miR-223 hypothesized to contribute to the pathogenesis of asthma and COPD (Roffel et al., 2020).