Integrating Biomarkers of Organophosphate Pesticides in an Agriculturally Exposed Population

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Abstract

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Exposure biomonitoring of organophosphate (OP) pesticides is a common practice to protect the health of agricultural workers. However, few studies have examined the association between markers of exposure and cholinesterase inhibition, the standard marker of effect, for these compounds or how genetic variation in the metabolic pathways responsible for the activation and detoxification of OP pesticides affects these associations. We evaluated the potential for pesticide exposure and cholinesterase inhibition levels of apple orchard farmworkers and their children in relation to non-farmworker households in the same region. We tested the hypothesis that 1.) OP pesticide exposure biomarkers were predictive of the inhibition of cholinesterase enzymes and 2.) genetic variants for metabolizing genes, including paraoxonase-1 (PON1) and cytochrome P-450s (CYP450s) altered this association. Higher levels of azinphos methyl in blood and dimethylphosphate urinary metabolites were observed in farmworkers adults and

children during periods of pesticide application (thinning season) compared to non-farmworkers. Levels of azinphos methyl in blood were significantly associated with higher levels of dimethyl phosphate metabolites in the urine. Significant associations between concentrations of these exposure biomarkers and levels of cholinesterase inhibition were observed during the thinning season. We observed no significant modification of these observations when incorporating PON1 phenotypic and genotypic information into our models in accordance with predictions from *in vivo* data. However, when considering quantification of exposure via urinary metabolites, we found significant modification of the exposure/effect relationship by genetic variants for a number of CYP450 enzymes. These observations suggest that significant differences in the level of activation or detoxification of OP pesticides occur between individuals differing in the genotype of certain CYP450s responsible for this metabolism. Despite not directly handling pesticides, individuals working in pomme orchards during periods of pesticide application and their children are exposed to OP pesticides and can experience cholinesterase inhibition. Furthermore, the risk of cholinesterase inhibition appears to be greater in individuals with certain genetic backgrounds. This information illustrates the significance of individual susceptibility and the metabolism of specific OP pesticides for cumulative risk assessments of these compounds.

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LIST OF ABBREVIATIONS

AChE – acetylcholinesterase

AREase - arylesterase

AZ – azinphos methyl

BuChE – butyrylcholinesterase

CES - carboxylesterase

CI – confidence interval

CP – chlorpyrifos

CYP450 – cytochrome P-450

DAP – dialkyl phosphate

DEP – diethyl phosphate

DETP - diethyl thiophosphate

DEDTP - diethyl dithiophosphate

DMP - dimethyl phosphate

DMTP - dimethyl thiophosphate

DMDTP - dimethyl dithiophosphate

GST – glutathione S-transferase

OP – organophosphate

PON1 – paraoxonase 1

PPE – personal protective equipment

SNP – single nucleotide polymorphism

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CHAPTER 1: Background and Significance

Organophosphate Pesticides

Of the insecticides used in the United States, organophosphate (OP) pesticides make up approximately 40% of all insecticides applied (USEPA 2011). These compounds exert their toxic effects by inhibiting the enzyme acetylcholinesterase (AChE) found in the nervous system and neuromuscular junctions after being metabolized to their toxicologically active oxon forms (Costa et al. 2008). While they degrade rapidly in the environment, humans can be exposed to OP pesticides and adverse health effects can result from these exposures. Federal regulatory agencies facilitated the voluntary elimination of certain OP pesticides over concern about adverse effects to children (USEPA 2002a) and such efforts likely contributed to a decrease in pesticide poisoning events observed between the 1990s and mid 2000s (Blondell 2007). Currently, the majority of OP pesticide applications are for agricultural purposes, thus characterizing the potential for exposures in agricultural workers is of public health importance.

Biomonitoring to Evaluate OP Exposures

OP pesticides can enter the body through dermal (van der Merwe et al. 2006) and oral routes of exposure (Garfitt et al. 2002; Bouchard et al. 2003), and some evidence points to respiratory absorption as well (Aprea et al. 1994). Once in the body, they can be metabolized to the toxicologically active oxon analog through a desulfuration reaction or inactivated through a dearylation reaction (Buratti et al. 2005). Biomonitoring of exposures can be performed by measuring the levels of the parent compound in the blood or urinary metabolites (Maroni et al. 2000; Barr and Angerer 2006). Measuring urinary metabolites has been the predominate method for monitoring of OP pesticides exposures since this biological matrix can be collected non-

invasively. Metabolite concentrations are 1.) reflective of recent exposures to both the parent pesticide and its preformed metabolites owing to their short environmental and biological half-lives (Carrier and Brunet 1999; Busby-Hjerpe et al. 2010), 2.) correlate well with residue levels on skin and clothes (McCurdy et al. 1994), and 3.) provides a more accurate measurement of exposure than self-reported contact rates (Perry et al. 2006).

Urinary metabolites for OP pesticides can be separated into two classes: specific metabolites and non-specific metabolites (Barr et al. 1999; Maroni et al. 2000; Barr and Needham 2002). The specific metabolites can be used to identify the OP pesticide to which the subject was exposed to as it is derived from the portion of the parent compound that provides its identity. The nonspecific metabolites, also called dialkyphosphates (DAPs), are present as dimethyl- or diethyl substituted phosphates. Figure 1-1 depicts the metabolism of azinphos methyl (AZ), an OP pesticide commonly sprayed on apple orchards in Washington State, and the formation of the dimethyl DAPs. As they are derived from many different OP pesticides, identification of the parent compound is not possible with DAPs but total exposures can be determined for multiple OP pesticides by measuring their total molar values (Wessels et al. 2003). This strategy has been used to evaluate total exposures from a number of OPs in monitored populations (Lu et al. 2001; Bradman et al. 2005; Young et al. 2005). It should be noted that preformed DAP metabolites can be found in foodstuffs (Lu et al. 2005; Zhang et al. 2008) and when ingested can enter the body and be excreted in the urine without further modification (Timchalk et al. 2007a; Busby-Hjerpe et al. 2010). As such, caution needs to be taken when interpreting levels of pesticide exposure from urinary biomarker data.

OP Pesticide Exposures in Farmworker Populations

Occupational exposures resulting from contact with OP pesticides regularly occur for workers who formulate, mix, or apply pesticides. Urinary metabolite levels in these individuals have been found to be higher than for controls not involved in pesticide-related work (Lee et al. 2007; Muniz et al. 2008; Kisby et al. 2009). Some states have monitoring programs in place to evaluate exposures in these agricultural workers in order to evaluate worker safety and prevent health impacts in these individuals (WSDLI 2010). Elevated DAP levels have also been found in farmworkers who generally do not handle pesticides directly (Muniz et al. 2008; Kisby et al. 2009). Among this group of workers, performing thinning tasks, which requires substantial physical contact with crop plants, has been linked to higher levels of urinary DAPs after spray events (Simcox et al. 1999; Coronado et al. 2004). Our previous research has shown that those who worked with pomme orchard fruits, that is apples and pears, which have the highest amount of active ingredient applied on fruit crops in Washington State (Table 1-1), had higher dimethyl phosphate urinary metabolites than farmworkers who did not work in those orchards (Coronado et al. 2006; Coronado et al. 2009). Moreover, the metabolite levels were higher among those who worked in both apple and pear orchards than for individuals who only worked with one of the crops. While intervention strategies like restricted reentry periods after spraying (Fenske et al. 2003) and use of personal protective equipment (PPE) can reduce exposures to workers (Keifer 2000; van der Jagt et al. 2004; Bradman et al. 2009; Protano et al. 2009; Vitali et al. 2009), there is currently no active program monitoring exposures and possible subsequent health effects in farmworkers as there are for those handling or applying pesticides directly.

Children's Exposure to OP Pesticides

Although occupational exposures to farmworkers and pesticide formulators, applicators, and manufacturers are easily identified and monitored (Jaga and Dharmani 2003), other populations are also at risk from exposures to OP compounds. In particular, children may experience higher exposures and be at greater risk of developing adverse health effects as a result of different diets, higher food consumption and metabolism (Selevan et al. 2000), hand-to-mouth activities, and immature capacities to detoxify OP compounds compared to adults (Faustman et al. 2000; Garry 2004). Data from the NHANES surveys show that detectable levels of OP metabolites are present in children within the general population; in fact, these levels are often higher in children and adolescents than in adults (Barr et al. 2004; Barr et al. 2005). When comparing children of different ages, several studies have found an inverse relationship between pesticide exposure and children's age (Loewenherz et al. 1997; Shalat et al. 2003; Curwin et al. 2007a). Although these findings have not been consistent across all studies (Koch et al. 2002; Curwin et al. 2007b), a correlation between exposure and age would correspond with the observation that time playing on household floors decreases with age (Black et al. 2005). Such activity likely allows pesticide residues to exist in the child's breathing space and to transfer to their skin where they can be absorbed or ingested during hand-to-mouth behaviors. Significant correlations have been found between levels of pesticides on floor surfaces, on toys, and on hand wipes (Lu et al. 2004; Quandt et al. 2004), illustrating transfer of pesticides between different surfaces and objects that children interact with.

Within the general population of children, dietary intake has been shown to be a significant contributor to OP exposure (Curl et al. 2003; C. Lu et al. 2006; Lu et al. 2008) and, taken with exposure through drinking water and residential use of pesticides, make up the major

exposure pathways for the typical American child (Fenske et al. 2000). A number of studies suggest that children of farmworkers have higher exposures to pesticides, as measured by residues in house dust and urinary metabolite levels, than children of non-farmworkers (Simcox et al. 1995; Loewenherz et al. 1997; Lambert et al. 2005). In some cases, urinary metabolite levels in farmworker children are more highly correlated to levels of pesticides in house dust than the same measures in non-farmworker children (Curwin et al. 2007b). These findings may be due in large part to the para-occupational route of exposure, also known as the take-home pathway, in which farmworkers bring residues into the home environment as a result of inefficient hygiene or safety practices.

In this model of exposure, adult farmworkers bring pesticide residues into the home through the use of a family vehicle for transport to and from their site of work, by not removing their work clothes prior to entering the home, and by not laundering their work clothes separately from the family's clothing (Fenske et al. 2000; Jaga and Dharmani 2003; Vida and Moretto 2007). Several lines of evidence are available to support exposures via the take home pathway. Levels of pesticides in vehicle and house dust have been correlated for farmworker households (Curl et al. 2002; Thompson et al. 2003; Coronado et al. 2006). Comparisons of house dust between farmworker and reference households equidistant to the nearest field or orchard found greater residues in the farmworker homes (Lu et al. 2000; Fenske et al. 2002). Elevated levels of total OPs and azinphos methyl were observed in house dust when workers did not remove their work clothes within two hours of entering the home compared to those who did so (McCauley et al. 2003). Concentrations of OP metabolites found in the urine of farmworker children have been found to be significantly correlated with the metabolites found in their parents urine (Curl et al. 2002; Thompson et al. 2003; Coronado et al. 2006) and with levels of the parent compound

in house dust (Curl et al. 2002; Coronado et al. 2006). These findings all support the take home pathway as a route of exposure for farmworker children.

Correlations between levels of pesticides in house dust and job or task also provide evidence for this pathway as some tasks likely leave higher levels of residues on a workers hands or clothes that they then subsequently can bring into the home. A comparison of workers with potentially high contact with pesticides to those with little to no likely contact found that the former group had higher levels of OP residues in house dust (McCauley et al. 2003). A study within our group examining the effect of agricultural task on pesticide exposure found that farmworkers performing "thinning" tasks was correlated with higher levels of azinphos-methyl residues in vehicle and house dust, as well as higher levels of DMTP in children's urine (Coronado et al. 2004). A subsequent study found that children living with a pomme-fruit worker had a higher frequency of detecting OP metabolites in their urine compared to children of non-pomme workers (Coronado et al. 2006). Furthermore, children of those who worked with both apples and pears had higher metabolite concentrations than children of farmworkers who handled only pomme fruits. These results illustrate that children living in farmworker households potentially have greater exposures to OP pesticides than do non-farmworker children.

Neurotoxicity of OP Pesticides

As a class of compounds, OP pesticides inhibit cholinesterase enzymes including AChE found in the nervous system and red blood cells and plasma butyrylcholinesterase (BuChE) of exposed humans and animals (Wessels et al. 2003). AChE plays an important role in the nervous system by regulating the effects of the neurotransmitter acetylcholine (ACh) in the synapses between neurons and at neuromuscular junctions (Costa 1997). When this enzyme is inhibited,

ACh levels build up in the synapse and provide continued stimulation to the post-synaptic cell, leading to tetanus, paralysis, and death after large acute exposures. Due to the similarity of the AChE present in the nervous system, the level of inhibition of blood AChE and BuChE enzymes can be used as surrogate markers to track the impact of OP pesticides on exposed individuals (Nigg and Knaak 2000; Cocker et al. 2002). Since there is large inter-person variability in enzyme activities, pesticide exposure monitoring programs utilize baseline measurements obtained during non-spray periods to evaluate the degree of effect (reduction in ChE activity from baseline) in exposed workers (Coye et al. 1986). The U.S. Environmental Protection Agency (USEPA) views cholinesterase inhibition greater than 20% as being biologically significant (USEPA 2000). In Washington State, the Department of Labor and Industries (WSDLI) requires agricultural pesticide handlers be tested for cholinesterase inhibition (WSDLI 2010). Should ChE inhibition exceed designated levels, this program dictates that a workplace evaluation be performed and/or the worker must be removed from pesticide related tasks. Programs like this help to reduce the acute effects of pesticide exposures on workers.

Exposures to OP pesticides are of concern beyond ChE inhibition as they may lead to neurologic effects. Such impacts have been observed in farmworker adults (Rothlein et al. 2006), but impacts in children are potentially of greater significance, since neurological alterations resulting from these exposures may endure for their entire lives. A number of studies with children and adolescents exposed to OPs have shown evidence for developmental toxicity and neurobehavioral defects as a result of these exposures (Jurewicz and Hanke 2008). In a study of adolescent applicators 9-18 years of age, a dose-response between years of pesticide exposure and behavioral deficits was observed (Abdel Rasoul et al. 2008). Using interviews to determine pesticide exposure, a significant negative correlation was found between several

measures in a behavioral battery of tests and exposure to pesticides in a group of Brazilian adolescent farmworkers (Eckerman et al. 2007). Furthermore, the greatest associations were in the youngest individuals, aged 10-11 years. In utero exposures as determined by measurement of DAPs or specific metabolites in maternal urine were been found to related to abnormal reflexes (Young et al. 2005; Engel et al. 2007) and mental development (Eskenazi et al. 2007; Eskenazi et al. 2008). In one study, prenatal exposure led to neurological deficiencies equal to 1.5-2 years of developmental delay (Harari et al. 2010). A trio of studies released in 2011 all found negative impacts of prenatal exposure to OP pesticides on IQ or cognitive development (Bouchard et al. 2011; Engel et al. 2011; Rauh et al. 2011). Associations have also been observed between in utero (Marks et al. 2010) or postnatally (Bouchard et al. 2010) OP pesticide exposures and having a diagnosis of ADHD in children. These findings suggest that current levels of exposure in children living in agricultural areas have neurological impacts that are potentially long-lasting and more monitoring of childhood exposures is necessary.

Genetic Variability in OP Pesticide Metabolism

For OP pesticides to exert their toxicity, they must be activated to their oxon intermediates. This reaction is catalyzed by the cytochrome P-450 (CYP) class of enzymes. A number of CYPs catalyze the desulfuration reaction that activates the parent OP pesticide compound to the oxon form (Buratti et al. 2002). This reaction is important toxicologically since the oxon is a more potent cholinesterase (ChE) inhibitor than the parent form (Dahm et al. 1962). The CYPs are also capable of facilitating a dearylation of the parent compound, producing the nontoxic dialkyl phosphates (DAPs) and specific metabolites that are excreted into the urine (Hodgson 2003). This reaction decreases the amount of parent compound that is converted to the

oxon and reduces subsequent ChE inhibition. The major CYP enzymes catalyzing these reactions include CYP1A2, CYP2B6, CYP2C19, CYP2D6, and CYP3A4 (Buratti et al. 2003; Sams et al. 2004; Mutch and Williams 2006) (see Appendix 1 for a review of the *in vitro* literature). Whether an individual CYP enzyme performs greater, lesser, or equivalent amounts of desulfuration compared to dearylation depends on the identity of the parent OP pesticide (Buratti et al. 2003; Sams et al. 2004). For example, when CYP2C19 metabolizes chlorpyrifos (CP), a greater amount of 3,5,6-trichloro-2-pyridinol (TCPy), the dearylation product for CP, is produced compared to CP oxon, while the same enzyme metabolizes parathion through the dearylation and desulfuration reactions at roughly equal rates (Mutch and Williams 2006). Given the central role of CYPs in the metabolism of OP pesticides, an understanding of these processes is essential for estimating risk resulting from exposures in human populations.

The genes encoding the CYPs are highly polymorphic and this genetic variation can translate to differences in phenotype for a given enzyme (Zhou et al. 2009). These differences in phenotype can impact how OP pesticides are metabolized by CYPs. Tang et al. (2001) observed higher amounts of dearylation products with E. Coli expressing the wild-type (CYP2C19*1B) allele after receiving 100 μM CP compared to those expressing the CYP2C19 *5, *6, or *8 alleles, all of which produce enzymes with lower activity compared to wild-type (Zhou et al. 2009). When comparing the effect of CYP3A4 genetic variants on the metabolism of 100 μM CP, Dai et al. (2001) observed greater rates of dearylation and desulfuration with the L293P (*18) variant, lower rates with the F189S (*17) variant, and no differences with the M445T (*3) or P467S (*19) variants compared to wild-type (*1). Genetic variability will likely have the greatest impact on the metabolism of an OP pesticide when a given CYP450 catalyzes a greater amount of desulfuration or dearylation. For example, Foxenberg et al. (2011) used a human

PBPK/PD model of OP pesticide metabolism by CYP2B6 and CYP2C19 to examine how varying the CYP content for these two isoforms would impact the level of oxon formed and subsequent ChE inhibition. As mentioned above, CYP2C19 predominately produces dearylation products with CP (Croom et al. 2010), so when the amount of this CYP was decreased in the PBPK/PD model, corresponding to a number of null genotypes present in human populations, a higher level of acetylcholinesterase (AChE) inhibition after exposure to CP was observed compared to the average value for this enzyme. Such an increase was not observed for parathion since CYP2C19 produces more equal amounts of dearylation and desulfuration products for parathion compared to CP (Foxenberg et al. 2007). As this genetic variation in CYPs can lead to different levels of oxon activation and ultimately the amount of ChE inhibition resulting from this metabolism, variant genotypes for CYPs can potentially confer sensitivity to individuals exposed to OP compounds and make them more susceptible to OP pesticide-induced health impacts.

Once activated to the oxon intermediate, OP pesticides can be detoxified by paraoxonase 1 (PON1) (Furlong 2007). Animal experiments utilizing supplementation with purified PON1 enzyme (Costa et al. 1990; Li et al. 1995) or knockout animals (Furlong et al. 1998; Shih et al. 1998; Lockridge et al. 2005) showed ChE activity after exposure to CP that was positively associated with the amount of PON1 present. PON1, therefore, plays an essential role in balance of activation and detoxification that determines the toxicity of certain OP pesticides.

Genetic variability in PON1 is associated with differences in metabolism of OP pesticide oxons. In particular, the position R192Q single nucleotide polymorphism located in the coding sequence affects the ability of PON1 to hydrolyze these oxon chemical species (Mackness et al. 1997). The impacts of this polymorphism on the hydrolysis of certain oxons has been related to

differences in catalytic efficiency, with the $PON1_{R192}$ isoform having roughly two and ten times greater catalytic efficiency towards the oxons of CP and parathion, respectively, than the $PON1_{Q192}$ isoform (Li et al. 2000). This lesser ability to metabolize oxons has translated to higher AChE inhibition in mice humanized with the $hPON1_{Q192}$ allele compared to animals with the $hPON1_{R192}$ genotype (Cole et al. 2005). In a group of pesticide applicators, Hofmann et al. (2009) observed significant alterations in the level of BuChE inhibition based on PON1 genotype and phenotype. These studies suggest that PON1 genotype and phenotype, may be a significant indicators of susceptibility to OP pesticide-induced health effects in exposed human populations (Costa et al. 2003; Furlong et al. 2005).

However, to date, only the oxons of CP and diazinon have been demonstrated to be toxicologically relevant substrates of PON1 (Li et al. 2000). For example, azinphos methyl (AZ) did not generate different levels of brain ChE inhibition in PON1 wild-type (PON +/+) and knockout (PON1 -/-) mice, strong evidence that AZ oxon is not a substrate for PON1 (Costa et al. 1999). PON1 did not provide protection when malaoxon, the oxon form of malathion, was administered to PON1 +/+, PON1 -/-, $hPON1_{R192}$, or $hPON1_{Q192}$ mice (Jansen et al. 2009). Moser and Padilla (2011), while showing CP oxon detoxification, observed no detoxification of malaoxon, the oxon of malathion, by PON1 when incubating human recombinant AChE with rat liver homogenate. These datasets suggest that PON1 status is not an appropriate marker of susceptibility for all OP pesticides to which humans may be exposed.

Buccal Epithelial Cells

In order to obtain the material needed to examine linkages between biological function and pesticide exposure, biological samples must be collected from study populations. Typically tissues or body fluids are collected based on their ability to act as surrogates for internal tissues (Rockett et al. 2004) or for their ability to be collected non-invasively (Esteban and Castano 2009). Buccal epithelial cells collected from the oral cavity with cytological brushes or mouthwashes are an efficient noninvasive manner to collect biological material (Salama et al. 1999), and have the potential to encourage higher response rates in molecular epidemiology studies than taking blood samples (Hansen et al. 2007). Buccal swab and mouthwashes have been used to isolate DNA for genotyping (Delpisheh et al. 2008; Kujawski et al. 2008) and for evaluation of genetic damage as a result of environmental exposures (Cavallo et al. 2005; Borthakur et al. 2008). Recent advances in multiplex genotyping technology allow for the simultaneous analysis of many thousands of markers at one time, and buccal derived DNA genotypes have high concordance with those generated with DNA isolated from blood (Feigelson et al. 2007; Woo et al. 2007a). With the increase in the number of markers included on these multiplex platforms, there is a concomitant increase in the total amount of DNA input for these tools and amplification may be required in order to meet the required amount. Buccal DNA amplified with the multiple displacement amplification (MDA) method (Dean et al. 2002) has shown high levels of concordance with blood-derived DNA on various multiplex genotyping platforms (Montgomery et al. 2005; Paynter et al. 2006; Moore et al. 2007). These results demonstrate the utility of buccal samples for determining genotypic information in human population studies.

In recent years, buccal epithelial cells have also served as a source of RNA for gene expression analyses. This has included both RT-PCR (Spivack et al. 2004; Kumar et al. 2005b) and microarray applications (Smith et al. 2006; Sridhar et al. 2008) to investigate alterations in gene expression as a result of tobacco smoke (Spivack et al. 2004; Smith et al. 2006; Sridhar et al. 2008), clinical radiation (Narayan et al. 2008), and environmental contaminants (Liu et al. 2007). The analysis of buccal gene expression has tremendous promise in identifying potential biomarkers of early response for exposures taking place through oral routes, for both animal and epidemiology studies. In a hamster model of oral squamous cell carcinoma, researchers observed up-regulation of CYP1B1 in buccal cells obtained by swabbing after administration of dibenzo[a,l]pyrene (Schwartz et al. 2008). In the case of human exposures to tobacco smoke, Sridhar and colleagues (2008) demonstrated a similar pattern of gene expression alterations in the buccal and bronchial epithelia of smokers when compared to nonsmokers. Liu and colleagues (2007) showed that levels of metallothionein transcripts in buccal cells were proportional to those found in blood tissues, and that the expression from both tissues was significantly lower in subjects diagnosed with arsenicosis compared to normal patients. All of these findings point to the potential use of buccal cells as a surrogate for internal tissues in diagnosing disease or health effects from environmental exposures.

In terms of using buccal RNA for gene expression analysis with pesticide exposed populations, studies have shown that pesticides can be detected in saliva samples. This has included animals models dosed with a specific compound (Lu et al. 1998; Lu et al. 2003; Timchalk et al. 2007b) and workers sampled in the field (Denovan et al. 2000; Hines et al. 2006; Chensheng Lu et al. 2006). *In vivo* animal studies have shown that pesticide concentrations in saliva are correlated with those measured in the plasma, indicating that this metric can be used to

estimate internal exposures (Lu et al. 1998; Lu et al. 2003). Other efforts have focused on using salivary cholinesterase inhibition as a marker of effect for OPs (Kousba et al. 2003; Henn et al. 2006). For example, salivary AChE inhibition and oxidative stress markers were measured in the saliva of animals treated with malathion (Abdollahi et al. 2004). Since buccal epithelial cells are in contact with saliva and blood vessels, they are likely to absorb pesticides from either of these two sources and any effects in these cells can be monitored through gene expression analysis. Therefore buccal cells are an attractive biological matrix for examining effects in populations exposed to OP pesticides.

In order for buccal sampling to be useful for these types of analyses, the RNA isolation procedure needs to be optimized to produce samples of sufficient quantity and quality. Spivack et al. (2004) utilized a standard column purification protocol (Qiagen RNEasy kit) incorporating the use of poly-C RNA as a carrier to isolate RNA from buccal cells collected with brushes. With the addition of 1 µg of poly-C RNA, the authors were able to obtain yielded 0.75 to 1.5 µg per brush, suggesting that carrier RNA makes up most of their sample. Despite this they and others using their protocol (Kumar et al. 2005a; Kupfer et al. 2010) have been able to isolate RNA and identify biological signals in their subjects. In the case of Liu et al. (2007), samples were collected after brushing with a toothbrush and rinsing with saline. RNA was then isolated using Trizol reagent and samples were cleaned up with RNEasy columns. Another protocol developed by Spira et al. (2004) involves the collection of buccal cells with a "plastic tool that is concave with serrated edges" that they designed followed by RNA isolation using Trizol. While the collection of the samples may cause some discomfort, they successfully demonstrated its use in profiling gene expression on microarrays after serially collecting the samples (Sridhar et al.

2008). With so many extraction protocols available, comparisons between RNA isolation methods should identify an optimal strategy to expand on the utility of these samples.

Study Population

As part of a Community-Based Participatory Research Project (CBPRP) collaboration with the Fed Hutchinson Cancer Research Center (FHCRC), we have been monitoring cohorts of Hispanic farmworker and non-farmworker families residing in the Lower Yakima Valley region of South Central Washington State to evaluate the potential for pesticide exposure. A number of reports have been published detailing the work performed with these communities (Curl et al. 2002; Thompson et al. 2003; Coronado et al. 2004; Coronado et al. 2006; Thompson et al. 2008; Coronado et al. 2009; Coronado et al. 2011). The study population consists of a cohort of one hundred farmworker and one hundred non-farmworker households in the Lower Yakima Valley region of South Central Washington State. Participants were recruited by trained bilingual, bicultural study staff at retail outlets, churches, and door-to-door solicitation. Each household represented an adult and one child between the ages of 2-6 years and were compensated $160 for participating during all three agricultural seasons, which included the thinning, harvest, and non-spray seasons. Farmworkers were limited to those who worked in the pomme (apple or pear) orchards during the two weeks prior to recruitment. Demographic statistics for the adults in the cohort have been published previously (Locke et al. 2009; Coronado et al. 2011) (Table 1-2).

A total of 204 households were contacted and interviewed during the thinning season (April-June) of 2005 and during the off/non-spray season (December-February). Over the course of the thinning and off seasons, 99 households maintained farmworker status, another 95 were consistently classified as non-farmworkers, and the remaining 10 households changed their

occupational status or were lost to follow-up. Those in the last category were dropped from the analyses presented in this document. The participating adults in both cohorts were largely female (80%) and the average age in both groups of participants was 31 years. Farmworkers were more likely to be married (91% vs. 76%), to have been born in Mexico (98% vs. 64%), and to speak primarily Spanish at home than non-farmworkers (94% vs. 51%). The data collection methods and study protocols were reviewed and approved of by the Human Subjects Review Board at the University of Washington and the Institutional Review Board (IRB) at the Fred Hutchinson Cancer Research Center. The sample collection design is presented in Figure 1-2.

Hypotheses and Specific Aims

The work presented in this document addresses our overall hypothesis that 1.) markers of exposure for OP pesticides are associated with cholinesterase markers of effect, and 2.) these relationships are modified by genetic variation in metabolic pathways responsible for the activation and detoxification of these compounds.

H_{O1}: Exposure metrics for OP pesticides are not associated with cholinesterase inhibition

H_{A1}: OP Pesticide Exposure metrics are associated with inhibition of cholinesterase enzymes

H_{O2}: Cholinesterase inhibition resulting from OP pesticide exposure is not modified by genetic variation in pathways responsible for the metabolism of OP pesticides.

H_{A2}: Genetic variation in OP pesticide metabolic pathways alter cholinesterase inhibition after exposure to OP pesticides

Specific Aim 1: Evaluate the association between levels of OP pesticide parent compounds in blood and urinary metabolites and inhibition of the cholinesterase enzymes in farmworkers and non-farmworkers.

Specific Aim 2: Determine the impact of PON1 status on the relationships between markers of exposure and effect observed in Specific Aim 1.

Specific Aim 3: Determine the impact of genetic variability in CYP450s and GSTs on the relationships between markers of exposure and effect observed in Specific Aim 1.

Figure 1-1 Proposed Metabolism Scheme for Azinphos Methyl

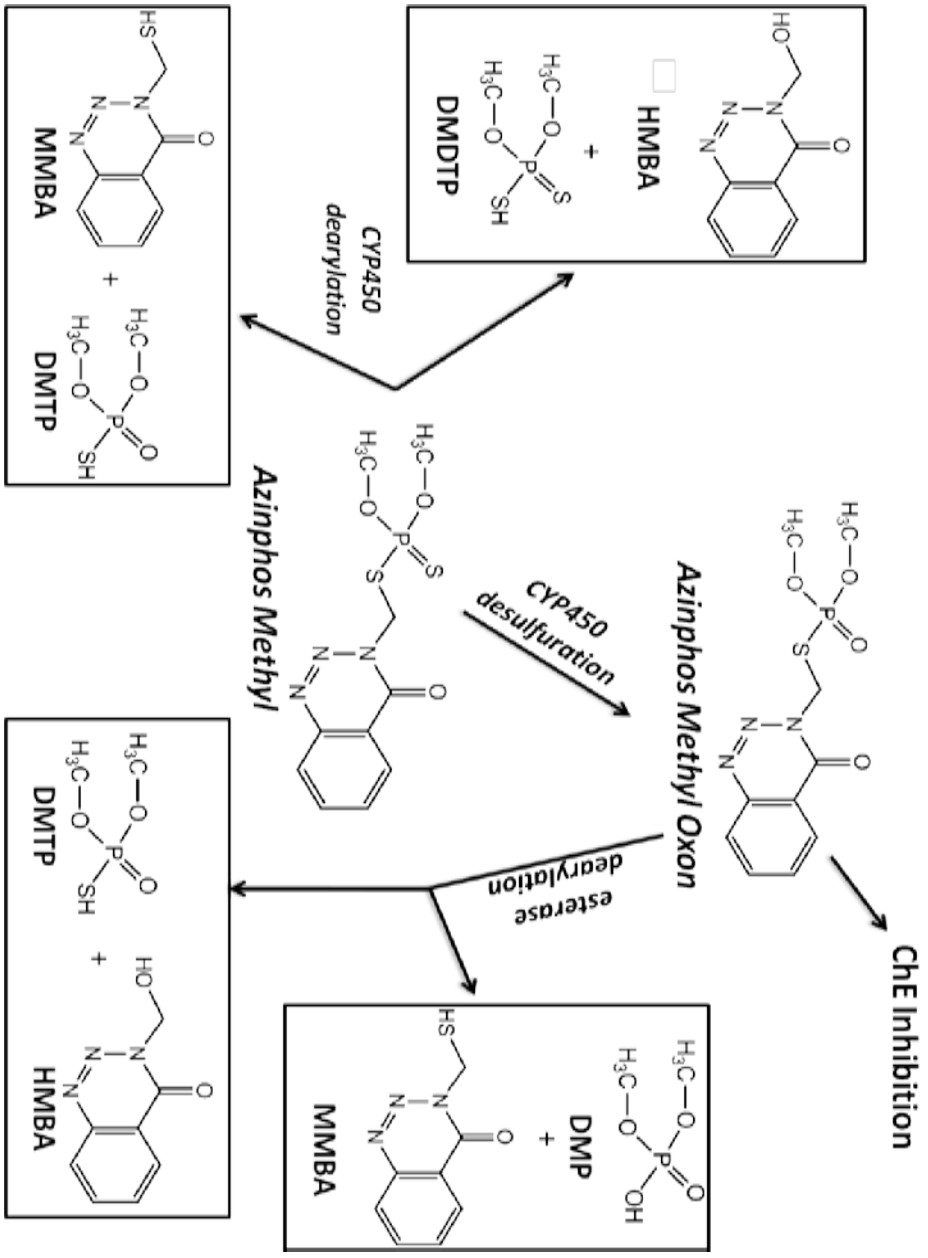


Fig 1-1 Proposed metabolic scheme for azinphos methyl (AZM). AZM can be detoxified to mercaptomethyl benzazimide (MMBA) and DMTP or DMDTP and hydroxymethyl benzazimide (HMBA) via a CYP450-mediated dearylation reaction. Alternatively, it can be activated to the oxon form through a desulfuration reaction also catalyzed by a number of CYP450s. The oxon moiety can be dearylated by esterases to form MMBA and DMTP or DMP and HMBA. Modified from Lin et al. (1980) and ATSDR (2008)..

Figure 1-2 Community-Based Particapatory Research Project (CBPRP) Sample Collection Design

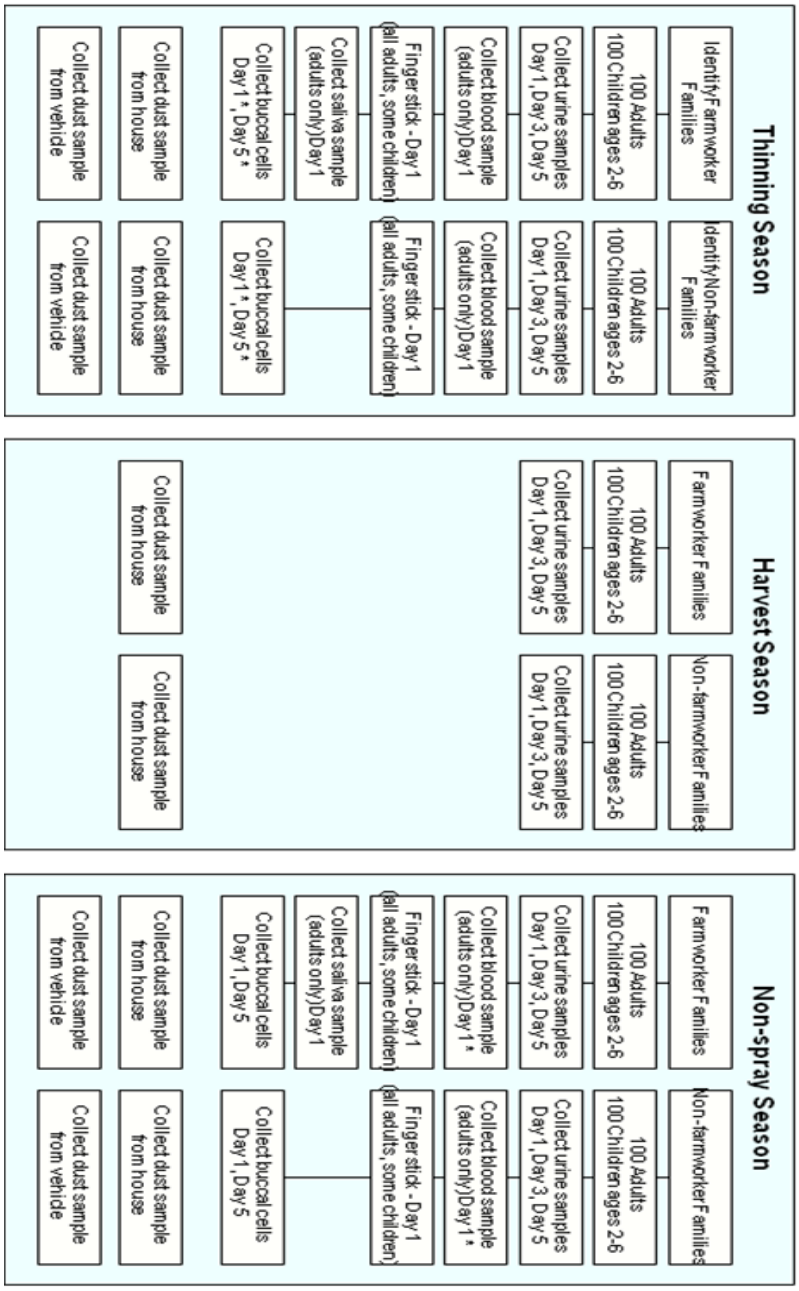


Fig 1-2. Sample collection design for the Community-Based Particapatory Research Project (CBPRP) Sample Collection Design. Samples were collected during the thinning (April-June) and off/non-spray (December-February) seasons in the 2005-2006 agricultural year. Harvest season samples were not analyzed in the research presented here.

Table 1-1 Amount of Organophosphate Active Ingredient Applied on Select Washington State Fruit Crops

		Total Active Ingredient Applied in Washington State (1,000s lbs)				
Fruit Crop	Pesticide	2001	2003	2005	2007	2009
Apples	Azinphos Methyl	241.4	289.0	196.4	236.3	94.0
	Chlorpyrifos	234.0	217.0	186.7	186.8	125.9
	Diazinon	5.7	5.0	13.3	33.4	31.5
	Malathion	1.6	NA	NA	NA	NA
	Phosmet	138.3	70.0	87.1	41.2	24.3
Sweet Cherries	Azinphos Methyl	21.6	24.0	16.8	NA	5.4
	Chlorpyrifos	21.6	31.7	26.5	NA	20.1
	Diazinon	3.4	7.7	5.6	NA	3.1
	Malathion	18.3	24.0	8.8	NA	2.1
Pears	Azinphos Methyl	22.8	28.7	18.7	NA	8.5
	Chlorpyrifos	17.1	21.6	13.2	NA	8.5
	Diazinon	2.9	NA	0.1	NA	0.7
	Phosmet	29.9	18.8	14.9	NA	1.9
Raspberries	Diazinon	6.2	7.9	7.3	NA	NA
	Malathion	5.6	5.5	5.6	NA	NA

Sources: Agricultural Chemical Use Database, National Agricultural Statistics Service (NASS), USDA

2001-2007 data - http://www.nass.usda.gov/Statistics_by_State/Washington/Publications/Ag_Chemical_Use/index.asp

2009 Data - http://www.nass.usda.gov/Data_and_Statistics/Pre-Defined_Queries/Ag_Chemical_Pesticide_Usage/index.asp

NA – Not available

Table 1-2 Descriptive statistics for Farmworker and Non-farmworker Adults

Characteristics	Farmworkers (n=101) n	Farmworkers (n=101) %[a]	Non-farmworkers (n=100) n	Non-farmworkers (n=100) %[a]	*P* value[b]
Sex					
Male	21	20.8	19	19.0	0.750
Female	80	79.2	81	81.0	
Age (y)					
17-24	12	11.9	15	15.1	0.093
25-29	20	19.8	27	27.3	
30-34	46	45.5	28	28.3	
35 and older	23	22.8	29	29.3	
Mean±standard error	31.5±5.7		31.3±7.1		
Household income (US$)					
≤10,000	22	21.8	18	18.2	<0.001
10,001-15,000	18	17.8	13	13.1	
15,001-25,000	40	39.6	32	32.3	
25,001-35,000	21	20.8	19	19.2	
>35,000	0	0.0	17	17.2	
Marital status					
Married/partnered	92	91.1	76	76.0	0.004
Other	9	8.9	24	24.0	
Language spoken at home					
More Spanish	94	94.0	51	51.0	<0.001
More or equal with English	6	6.0	49	49.0	
Birthplace					
Mexico	98	98.0	64	64.0	<0.001
United States	2	2.0	36	36.0	

[a]Sum of percentages may not total to 100% due to rounding.
[b]*P* values correspond to χ^2 tests of homogeneity between farmworker and non-farmworker cohorts.

Table 1-2 Descriptive statistics for farmworker and non-farmworkers. This data illustrates that the majority of the cohorts are female and that there are statistically significant differences in household income, marital status, language spoken at home, and birthplace between the two groups. Reproduced from Locke et al. (2009).

CHAPTER 2: Organophosphate Pesticide Biomarkers of Exposure Are Associated with Cholinesterase Inhibition in an Agriculturally-Exposed Population

Introduction:

Organophosphate (OP) pesticides make up approximately 40% of all insecticides applied in the United States (USEPA 2011). When absorbed into the body, these compounds can become metabolized to their toxicologically active oxon forms and inhibit the enzyme acetylcholinesterase (AChE) found in the nervous system and neuromuscular junctions (Costa et al. 2008). While elimination or restriction of certain pesticides has occurred over concern for health effects in children, the majority of remaining applications occur in agricultural settings and there is still a potential for impacts in these communities.

Biomonitoring of exposures to OP pesticides is performed by measuring the levels of the parent compound in the blood or urinary metabolites (Maroni et al. 2000; Barr and Angerer 2006). Measuring urinary metabolites has been the predominate method for monitoring of OP pesticides exposures since this biological matrix can be collected non-invasively. Metabolite concentrations are reflective of recent exposures to both the parent pesticide and its preformed metabolites owing to their short environmental and biological half-lives (Carrier and Brunet 1999; Busby-Hjerpe et al. 2010), correlate well with residue levels on skin and clothes (McCurdy et al. 1994) and provides a more accurate measurement of exposure than self-reported contact rates (Perry et al. 2006).

Elevated concentrations of urinary metabolites have been found in farmworkers who generally do not handle pesticides directly (Muniz et al. 2008; Kisby et al. 2009). Among this group of workers, performing thinning tasks, which require substantial physical contact with

crop plants, has been linked to higher levels of urinary DAPs after spray events (Simcox et al. 1999; Coronado et al. 2004). Children of farmworkers are also at risk of exposures to OP pesticides as studies suggest that children of farmworkers have higher exposures to pesticides, as measured by residues in house dust and urinary metabolite levels, than children of non-farmworkers (Simcox et al. 1995; Loewenherz et al. 1997; Lambert et al. 2005). These exposures may be due in large part to the para-occupational route of exposure, also known as the take-home pathway (Fenske et al. 2000; Jaga and Dharmani 2003; Vida and Moretto 2007), and has been supported by several lines of evidence from our center (Curl et al. 2002; Thompson et al. 2003; Coronado et al. 2006). Clearly, farmworkers and their families are at higher risk of exposures to OP pesticides than non-farmworkers.

OP pesticides inhibit cholinesterase enzymes including AChE found in the nervous system and red blood cells and plasma butyrylcholinesterase (BuChE) of exposed humans and animals (Wessels et al. 2003). AChE plays an essential role in the nervous system by regulating the synaptic transmission between neurons and at neuromuscular junctions (Costa 1997). Due to the similarity of the AChE present in the nervous system, inhibition levels of the blood AChE and BuChE enzymes can be used as surrogate markers to track the impact of OP pesticides on exposed individuals (Nigg and Knaak 2000; Cocker et al. 2002). Programs and standards have been created to monitor and protect worker health when handling or applying OP pesticides directly (USEPA 2000; WSDLI 2010).

In order for exposure biomonitoring data to be useful, associations have to be made with markers of effect. To date, few studies have examined any correlation between levels of urinary metabolites and ChE inhibition (Sudakin and Stone 2011). Using samples and data collected from our CBPRP, we sought to determine whether farmworkers and their children had higher

exposures to OPs than non-farmworkers, whether farmworker adults exhibited ChE inhibition during the thinning season, and whether specific OP pesticide markers of exposure were associated with ChE inhibition.

Methods:

Sample Collection

Interviews and biological sampling were performed with the CBPRP cohorts of farmworker and non-farmworker cohorts during the thinning season (April-June) of 2005 when OP pesticides like AZ are applied to the orchards (Lu et al. 2000) and during the off season (December-February) of 2005-2006 when few to no pesticide applications took place. During a five day period in each season, spot urine samples, approximately 15 ml per adult and child, were collected on days 1, 3, and 5, aliquoted, and frozen. On day 1, fingerstick samples were collected in two capillary tubes for cholinesterase measurements using a Genie lancet from adults. Three tubes (25 ml) of venous blood samples were also collected from adult participants on day 1 in order to measure parent OP pesticide compound. The venous blood samples were refrigerated overnight and then centrifuged to separate plasma, buffy coat, and serum.

Exposure Measurements

Blood sera and urine were sent to the Pesticide Laboratory in the National Center for Environmental Health at CDC for analysis. Levels of OP pesticide parent compounds were measured in blood sera using isotope dilution gas chromatography-linked high resolution mass spectrometry (GC-HR-MS) (Barr et al. 2002). The OP pesticides measured, and their limit of detections in ng/g plasma, were: AZ (0.04), bensulide (0.001), chlorpyrifos (CP) (0.06), coumaphos (0.02), dimethoate (0.07), methidathion (0.005), pirimiphos methyl (0.02),

propetamphos (0.04), temephos (0.02), tetrachlorvinphos (0.1), tribufos (0.03), and trichlorfon (0.1). The method was optimized for azinphos-methyl by measuring the monoisotopic mass at m/z 317.006 for quantification and a fragment ion at m/z 158.021 for confirmation using mass spectrometry. The extraction efficiency was 87+/-7% with a relative recovery of 102 +/- 3%. The relative standard deviation over the period of the analyses was 11%. Dimethyl- and diethyl-substituted metabolites in urine samples were measured by gas chromatography-linked tandem mass spectrometry (GC-MS-MS) (Bravo et al. 2004). Limits of detection for dimethyl phosphate (DMP), dimethyl thiophosphate (DMTP), and dimethyl thiophosphate (DMDTP) were 0.6, 0.2, and 0.1 µg/L urine, respectively. Those for diethyl phosphate (DEP), diethyl thiophosphate (DETP), and diethyl dithiophosphate (DEDTP) were 0.2 µg/L, 0.1 µg/L, and 0.1 µg/L, respectively.

Cholinesterase Activity and Inhibition

AChE and BuChE activity values were determined from the fingerstick samples using the Test-mate kit (EQM Research, Inc., Cincinnati, OH, www.eqmresearch.com). ChE values were adjusted for hemoglobin content and are expressed as units/g hemoglobin. Within our lab, we have observed high concordance in the ChE inhibition measurements and the Test-mate Kit and laboratory-based analysis (correlation = 0.75, data not shown). Cholinesterase inhibition during the thinning season was represented as the percentage of enzyme activity during the thinning season compared to the off season value.

Statistics

Normal distributions were observed for concentrations of urinary DAPs following log-transformation. Therefore the DAP data were log-transformed for all of the following analyses. A ‘components of variance’ model was used to estimate the geometric mean DAP concentrations

and 95% confidence intervals (CI) in each agricultural season and the within- and between-person components of variance. This model includes a standard deviation component describing how individuals varied between each other and a component describing the day-to-day variability within a given season for a single person. Each subject has one mean value and the between-person standard deviation describes how that mean value varies across the population. A second standard deviation describes how each person varies day-to-day about their mean value within an agricultural season.

We utilized a multivariate normal (MVN) model to describe the three dimethyl metabolites, DMP, DMTP, and DMDTP, analyzed on days 1, 3, and 4 of the thinning and off seasons. A MVN distribution allows for correlations between the metabolites to inform us about the distribution of censored values present at concentrations below the limit of detection (LOD) (Griffith et al. 2011). In a single urine sample, concentrations of the DAP metabolites present above the LOD are correlated with each other. This correlation informs us about the probability distribution for individual DAPs when they are below the LOD. In this data set, up to 45% of the values for a metabolite on a given day were below the LOD. By utilizing the correlations between the three dimethyl or diethyl metabolites and the values in the same sample above the LOD, we were able to perform more reliable statistical estimation and inference than using a constant value, such as half of the LOD, for the censored observation as is commonly done.

The components of variance model can be expressed as

(1) $\ln(X_{jk}) \sim MVN_9(\theta_k + \mu_j , \Sigma_w)$ for j= 1,2,…,n; k = 1,2,3

$\mu_j \sim MVN_3(0, \Sigma_b)$

where:

MVN_9 is a 9 dimensional multivariate normal distribution of the three dimethyl or diethyl metabolites for three collection days

MVN_3 is a 3 dimensional multivariate normal distribution describing between-person variability of the three dimethyl or diethyl metabolites

X_{jk} = vector of the measured metabolite concentrations on the kth collection days of the jth person

θ_k = vector of the means of the 3 metabolites for three collection days

μ_j = vector of the means of the 3 metabolites for the jth person

Σ_w = the within person variance-covariance matrix (9x9) for metabolites

Σ_b = the between person variance-covariance matrix (3x3) for metabolites

n = the number of people measured

For observations below the LOD for a particular metabolite, the value of the LOD is used to describe the upper limit of the censored lower left hand tail of the probability distribution described above. The components of variance model sets up the conceptual framework for the analysis of the data. Because of the large number of censored values, distributions of the model parameters were estimated using a Bayesian Markov chain Monte Carlo (MC-MC) method known as Gibbs sampling. Bayesian methods treat parameters as random variables and thus provide posterior estimates of the distribution of parameters based upon the data and prior distributions of the parameters. In our analysis to minimize the effects of the prior distributions on the final estimates, we utilized uninformative priors that provide little preference for any particular value of the parameter. In our calculations, a flat prior for the mean values and a Wishart distribution for the precision (inverse variance) of the MVN distributions were used.

Calculations were performed with the WinBUGS 1.4.3 software program (www.mrc-bsu.cam.ac.uk/bugs) (Lunn et al. 2000). We generated 50,000 MC-MC simulations and discarded the first 10,000 as burn in simulations to allow the MC-MC procedure to converge.

Wilcoxon ranked sign tests were used to examine differences in level of OP pesticide parent compounds in blood. Differences in urinary metabolite concentrations based on occupational status and agricultural season were assessed via analysis of variance (ANOVA). Linear regression models were used to evaluate relationships between ChE inhibition and markers of exposures. In the case of parent compound, the value for the serum concentration on day 1 was used to model exposure. For the urinary DAP metabolites, the geometric mean concentration for days 1, 3, and 5 generated with the MVN model were used to estimate exposure on the respective days. To estimate the slopes of the regression lines between markers of exposure and ChE inhibition, the intercept was assumed to be 100% of the baseline measurement taken during the off season. To determine differences in the fraction of farmworker and non-farmworkers having ChE inhibition exceeding 20% inhibition, a chi-square test was performed. All statistical tests were performed with the R statistical software package (http://www.r-project.org/)

Results:

In this study we characterized the potential for pesticide exposures in farmworkers and non-farmworkers working in the Yakima Valley, Washington state, and their children during the 2005-2006 thinning and off seasons. OP pesticide parent compounds were measured in a total of 348 blood sera samples collected from adult study participants during the thinning and off spray seasons. Two or fewer detects were found for bensulide, coumaphos, dimethoate, methidathion,

pirimiphos methyl, propetamphos, temephos, tetrachlorvinphos, and trichlorfon. In contrast, AZ, CP, and tribufos were detected in 45 (13%), 27 (8%), and 9 (3%) sera samples, respectively. Tribufos was present in samples collected from four farmworkers and 2 non-farmworkers during the thinning season and three non-farmworkers during the off season. Due to sample size limitations, only AZ and CP were considered for further analysis.

Levels of AZ exceeded the limit of detection (LOD, 0.04 ng/g serum) in 44 of 98 (45%) farmworkers and 0 of 94 (0%) non-farmworkers during the thinning season (Fig 2-1) (Wilcoxon test, $p < 0.001$). For farmworkers, the range of values for the thinning season were < LOD-6.19 ng/g. During the off season, no detectable levels of AZ were found in farmworkers while 1 of 82 (1%) non-farmworkers had detectable AZ with a concentration of 2.36 ng/g serum. The levels of AZ in farmworkers during the thinning season were significantly greater than concentrations measured in the same individuals during the off season (Wilcoxon test, $p < 0.001$).

For CP, 4 of 98 (4%) farmworker and 12 of 94 (13%) non-farmworker samples were present above the LOD of 0.06 ng/g serum during the thinning season (Fig 2-2). The range of values was < LOD-0.15 ng CP/g serum for farmworkers and < LOD-1.16 ng CP/g serum. In the off season, 8 of 74 (11%) farmworker and 3 of 82 (4%) non-farmworker samples had measurements exceeding the LOD, with ranges of < LOD-0.58 ng CP/g serum and < LOD-0.10 ng CP/g serum for farmworkers and non-farmworkers, respectively. No comparisons between occupations during the same season or between seasons for the same occupation were statistically significant (Wilcoxon test, $p > 0.05$).

Urine samples collected from adults and children were analyzed for the dimethyl (Tables 2-1 and 2-3) and diethyl (Tables 2-2 and 2-4) metabolites. Among the dimethyl metabolites, DMTP had the lowest percentage of non-detects for farmworker and non-farmworker households

during both agricultural seasons, followed by DMDTP and DMP. DMP measured in urine from farmworker households during the off season had the highest percentage of non-detects, ranging from 42-46% for adults and 38-43% for children. DETP had the smallest proportion of samples below the limit of detection for the diethyl DAPs, followed by DEP and DEDTP. Among these, the highest proportion of non-detects was found for DEDTP in the off season farmworker samples with a range of 58-62% of samples measured.

Significant within-person correlations were observed in farmworkers and non-farmworkers for all dimethyl metabolites and most diethyl metabolites, the exception being the correlations between DEP and DEDTP in both the thinning and off seasons (Fig 2-3). When we evaluated within season comparisons, the within-person correlations were greater in the farmworkers than non-farmworkers during the thinning season, while there were no significant differences between the groups during the off season. In the majority of cases, the variation in the 95% CI for the between-person correlation was greater than that for the within-person correlation for any two metabolites.

The results of the urinary analyses and modeling for our adult participants are represented in Figure 2-4 as geometric mean concentrations and 95% confidence intervals (95% CI) of the three sampling days for the three dimethyl (Fig 2-4A) and diethyl (Fig 2-4B) metabolites in the farmworkers and non-farmworkers during the thinning and off season. For the dimethyl metabolites, concentrations were highest in the farmworkers during the thinning season when AZ and phosmet, the two dimethyl OP pesticides most commonly applied in the region, were sprayed. DMTP was the metabolite with the highest concentrations in farmworker urine, with a geometric mean concentration across all three days of 62.87 μg/L (95% CI: 47.82, 82.41 μg/L) compared to 5.05 μg/L (95% CI: 4.23, 6.03 μg/L) in non-farmworkers ($p < 0.001$). DMP and

DMDTP were also higher in farmworkers during the thinning season: Farmworker DMP and DMDTP levels were 13.80 µg/L (95% CI: 10.68, 17.80 µg/L) and 5.93 µg/L (95% CI: 4.56, 7.69 µg/L), respectively, compared to 2.31 µg/L (95% CI: 1.88, 2.82) and 0.77 µg/L (95%CI: 0.63, 0.92 µg/L) in the non-farmworkers ($p < 0.001$ for both metabolites). During the off season, levels of these metabolites decreased significantly in farmworkers compared to the thinning season values ($p < 0.001$ for all three metabolites): concentrations of DMTP, DMP, and DMDTP were 6.71 µg/L (95% CI: 5.60, 8.05 µg/L), 1.06 µg/L (95% CI: 0.70, 1.56 µg/L), and 0.51 µg/L (95% CI: 0.39, 0.67 µg/L), respectively, across the three sampling days in the off season. Values for the non-farmworkers during the off season did not differ from their thinning season values ($p > 0.05$) and concentrations of DMTP and DMDTP, 6.07 µg DMTP/L (95% CI: 5.00, 7.37 µg/L), and 0.66 µg DMDTP/L (95% CI: 0.54, 0.80 µg/L), respectively, did not differ from farmworker values ($p > 0.05$). The off season non-farmworker DMP levels, 2.91 µg/L (95% CI: 2.29, 3.70 µg/L), were roughly three times higher than those for farmworkers ($p < 0.001$). A comparison was made between the DMTP values obtained in this study and data for the general population garnered from the National Health and Nutrition Examination Survey (NHANES) 2003-2004 (CDC 2011) and is presented in Fig 2-6.

Levels of the diethyl metabolites measured in adult urine samples collected during either season were lower than the comparable dimethyl metabolites (Fig 2-4B.). For farmworkers during the thinning season, geometric means of diethyl metabolites across the three sampling days were 1.69 µg DEP/L (95% CI: 1.24, 2.29 µg/L), 0.84 µg DETP/L (95% CI: 0.68, 1.05 µg/L), and 0.14 µg DEDTP/L (95% CI: 0.11, 0.18 µg/L). Non-farmworker geometric means of DEP, DETP, and DEDTP were 1.55 µg/L (95% CI: 1.15, 2.09 µg/L), 0.61 µg/L (95% CI: 0.51, 0.73 µg/L), and 0.17 µg/L (95% CI: 0.14, 0.21 µg/L), respectively, during the thinning season.

No differences were observed between farmworkers and non-farmworkers for DEP and DEDTP, whereas farmworker concentrations of DETP were significantly higher than non-farmworker values during the thinning season ($p = 0.025$). In the off season, concentrations of diethyl metabolites in farmworker urine decreased significantly compared to the thinning season values ($p < 0.001$ for DEP and DEDTP and $p = 0.033$ for DETP), with geometric means of 0.16 µg/L (95% CI: 0.08, 0.31 µg/L) for DEP, 0.60 µg/L (95% CI: 0.45, 0.78 µg/L), and 0.04 µg/L (95% CI: 0.02, 0.06 µg/L) for DETP. Off season values for non-farmworkers were not significantly different from the concentrations measured during the thinning season: 1.45 µg DEP/L (95% CI: 1.00, 2.08 µg/L), 0.76 µg DETP/L (95% CI: 0.62, 0.92 µg/L), 0.17 µg DEDTP/L (95% CI: 0.13, 0.22 µg/L). The non-farmworker concentrations of DEP and DEDTP were significantly greater than those for the farmworkers during the off season ($p < 0.001$).

We also analyzed the geometric means and 95% confidence intervals for dimethyl (Fig 2-5A) and diethyl metabolites (Fig 2-5B) measured in children's urine collected during the thinning and off seasons. During the thinning season, farmworker children had geometric mean concentrations across the three sampling days of 5.76 µg DMP/L urine (95% CI: 4.65, 7.11 µg/L), 16.79 µg DMTP/L (95% CI: 13.78, 20.39), and 2.43 µg DMDTP/L (95% CI: 1.95, 3.04). Non-farmworker children had values of 3.74 µg/L (95% CI: 3.06, 4.57 µg/L), 7.16 µg/L (95% CI: 5.74, 8.90 µg/L), and 0.89 µg/L (95% CI: 0.71, 1.09 µg/L), for DMP, DMTP, and DMDTP, respectively, during the thinning season. All three dimethyl metabolites were present in farmworker children's urine at higher concentrations than for non-farmworker children's samples ($p = 0.005$ for DMP and $p < 0.001$ for DMTP and DMDTP). During the off season, the urinary concentrations for farmworker children decreased significantly compared to the thinning season values ($p < 0.001$ for all three metabolites) to 1.50 µg/L (95% CI: 1.05, 2.11 µg/L) for

DMP, 6.21 µg/L (95%CI: 5.02, 7.67 µg/L) for DMTP, and 0.58 µg/L (95%CI: 0.44, 0.77 µg/L) for DMDTP. In comparison, the non-farmworker child values for DMP, DMTP, and DMDTP during the off season were not significantly different from the thinning season ($p > 0.05$): 3.91 µg/L (95%CI: 3.00, 5.07 µg/L), 7.58 µg/L (95% CI: 6.06, 9.48 µg/L), and 0.91 µg/L (95% CI: 0.74, 1.13 µg/L), respectively. The off season, non-farmworker child DMP and DMDTP concentrations were significantly greater than those for farmworker children ($p < 0.001$ for DMP and $p = 0.021$ for DMDTP).

Concentrations of diethyl DAP metabolites were also calculated for child participants (Fig 2-5B). Farmworker children and geometric mean concentrations across the three thinning season sampling days of 1.83 µg DEP/L (95%CI: 1.37, 2.46 µg/L), 0.62 µg DETP/L (95% CI: 0.50, 0.76 µg/L), and 0.12 µg DEDTP/L (95% CI: 0.09, 0.15 µg/L). The non-farmworker children had concentrations of 1.96 µg DEP/L (95% CI: 1.45, 2.63 µg/L), 0.64 µg DETP/L (95% CI: 0.53, 0.77 µg/L), and 0.15 µg DEDTP/L (95% CI: 0.12, 0.18 µg/L) during the same season and were not significantly different from the farmworker child concentrations ($p > 0.05$). During the off season, farmworker children had concentrations of DEP, DEDTP, and DEDTP of 0.25 µg/L (95% CI: 0.13, 0.46 µg/L), 0.68 µg/L (95% CI: 0.51, 0.91 µg/L), and 0.02 µg/L (95% CI: 0.01, 0.05 µg/L), respectively. Children in farmworker households had significantly lower DEP ($p < 0.001$) and DEDTP ($p < 0.001$) during the off season compared to the thinning season. The off season geometric means for non-farmworker children were 1.85 µg DEP/L (95% CI: 1.31, 2.55 µg/L), 0.78 µg DETP/L (95% CI: 0.63, 0.95 µg/L), 0.16 µg DMDTP/L (95% CI: 0.12, 0.21 µg/L). During the off season, farmworker children had significantly lower urinary concentrations of DEP ($p < 0.001$) and DEDTP ($p < 0.001$) than non-farmworker children.

Because this study evaluated both blood as well as urinary OP pesticide biomarker levels, significant associations between AZ and urinary metabolites could be observed in adult participants. Levels of DMP and DMTP were significantly correlated with serum concentrations of AZ, with correlations (r) of 0.50 (95% CI: 0.42, 0.56) and 0.46 (95% CI: 0.36, 0.52), respectively (Fig 2-7). DMDTP and total dimethyl metabolites were also correlated with serum AZ levels but to a lesser degree: 0.14 (95% CI: 0.09, 0.21) for DMDTP and 0.26 (95% CI: 0.20, 0.31) for total dimethyls. In the case of CP and the diethyl DAPs, the urinary metabolites were not significantly correlated with levels of CP measured in sera: correlation values between CP and the urinary metabolites were -0.05 (95% CI: -0.19, 0.10) for DEP, -0.08 (95% CI: -0.21, 0.07) for DETP, -0.03 (95% CI: -0.17, 0.11) for DEDTP, and -0.06 (95% CI: -0.20, 0.08) for total diethyl metabolites.

Levels of cholinesterase inhibition were calculated for RBC AChE and plasma BuChE. Finger-stick samples were obtained during the off and thinning agricultural seasons. Off season activity levels were considered baseline values and the amount of inhibition was determined by taking the ratio of the thinning season values to the baseline measurement for the same individual. Frequency distributions for inhibition of these enzymes in farmworkers and non-farmworkers are presented in Figure 2-8. Most subjects had levels of ChE inhibition below 20% during the thinning season compared to the off season baseline. A greater number of farmworkers had AChE and BuChE activity less than 80% of baseline, equivalent to 20% inhibition, than non-farmworkers but this difference was not statistically significant (Chi-square test; $p > 0.05$). The 20% inhibition level is significant as it marks the first action level for the WSDLI Cholinesterase Monitoring Program. For BuChE, 21 of 86 (24%) farmworkers had inhibition levels exceeding 20% and 5 (6%) farmworkers had levels above 40%. One

farmworker had BuChE inhibition exceeding 80%. Among the 29 non-farmworkers with cholinesterase activity data, 6 (21%) and 1 (3%) non-farmworkers had BuChE inhibition levels exceeding 20% and 40%, respectively. For AChE, 5 of 88 (6%) farmworkers had inhibition levels above 20% and one (1%) of these individuals had inhibition above 30%. In contrast, only one of 29 (3%) non-farmworkers had AChE inhibition exceeding the 20% threshold, with a value of 42% specifically. Three individuals, all farmworkers, had inhibition levels for both enzymes exceeding 20%. Under WSDLI, levels of AChE and BuChE inhibition above 40% and 30%, respectively, are thresholds at which a pesticide handler should be removed from the field and allowed to recuperate until the enzyme activity returned to within 20% of the baseline. No significant correlations were found between inhibition levels of AChE and BuChE (data not shown).

To determine if ChE inhibition was correlated with exposure biomarkers measured in this population, we generated linear models relating enzyme inhibition levels to quantities of parent compound measured in the blood (Fig 2-9) and concentrations of urinary metabolites during the thinning season (Table 2-5). We found significant linear relationships between levels of AZ in the blood serum and AChE inhibition (Fig 2-9A) and BuChE inhibition (Fig 2-9C): activity of AChE as percent of baseline decreased 2.9 % per ng AZ/g serum (95% CI: -4.3, -1.5%) ($p < 0.001$) and the activity of BuChE decreased 4.5% per ng AZ/g serum (95% CI: -7.7, -1.3%) ($p = 0.006$). The same analysis showed no significant association between levels of CP in the blood and inhibition of either AChE (Fig 2-9B; -3.2% per ng CP/g serum [95% CI: -18.8, 12.4%]) or BuChE (Fig 2-9D; -20.5% per ng CP/g serum; 95% CI: -55.0, 14.1%).

Testing the relationship between urinary metabolites and cholinesterase inhibition produced significant results for the regressions between inhibition of both AChE and BuChE and

levels of dimethyl urinary metabolites (Table 2-5). Decreases in AChE activity relative to baseline were observed with increases in DMP and DMTP, but not DMDTP: -15.5%/µM DMP (95% CI: -27.2, -3.7%) (p = 0.01), -2.9%/µM DMTP (95% CI: -4.9, -0.9%) (p = 0.005), and -12.4%/µM DMDTP (95%: -30.2, 5.4%) (p = 0.17). In a similar fashion, BuChE activity relative to the baseline decreased significantly by -27.0 %/µM DMP (95% CI: -53.6, -0.4%) (p = 0.047), -6.3 %/µM DMTP (95% CI: -10.8, -1.9%) (p = 0.006), and -49.5 %/µM DMDTP (95% CI: -88.4, -10.6%) (p = 0.013). No significant relationships were observed between the diethyl DAPs and inhibition of either AChE or BuChE: enzyme activity changed by -54.9% AChE/µM DEP (95% CI: -164.4, 140.8) (p = 0.30), -140.8% AChE/µM DETP (95% CI: -344.7, 63.1) (p = 0.18), -211.8% AChE/µM DEDTP (95% CI: -800.3, 376.8) (0.48), -140.4% BuChE/µM DEP (95% CI: -472.2, 191.4) (p = 0.41), -438.9% BuChE/µM DETP (95% CI: -990.4, 112.6) (p = 0.12), and -1104.4 % BuChE/µM DEDTP (95% CI: -2,399.5, 190.6) (p = 0.095).

Discussion:

With this work we have further characterized OP pesticide exposures in a group of farmworkers who do not directly handle or apply OP pesticides and have shown that multiple exposure biomarkers are associated with early biomarkers of response. We collected blood and urine samples from farmworker and non-farmworker cohorts in the thinning (April-June) and off/non-spray (December-February) seasons of 2005-2006. We measured the levels of the OP pesticides AZ (Fig 2-1) and CP (Fig. 2-2) in the blood plasma and dimethyl and diethyl metabolites in the urine samples (Figs 2-4 & 2-5). The parent AZ compound was detected in approximately half of adult farmworkers enrolled in our study during the thinning season whereas no detectable amounts were observed during the off season. The pattern for CP

exposure was much more varied and was not consistent with the periods of pesticide application, suggesting another source of exposure such as diet. Although AZ has been phased out of most applications, its use in apple orchards has been extended until 2012 (USEPA 2009). Based on the data presented here, farmworkers were exposed to AZ during these years.

We also examined the concentrations of DAP metabolites found in urine samples collected on days 1, 3, and 5 of a five-day sampling period during the thinning and off seasons. By collecting multiple samples in a given season we were able to analyze the intra-person variability in DAP metabolite levels. We utilized a MVN model in conjunction with Bayesian MC-MC methods to estimate the geometric mean concentrations for the three dimethyl DAPs across seasons. We observed significantly higher concentrations of all three dimethyl urinary DAPs in the thinning season than the off season in samples collected from farmworker households (Figs 2-4 & 2-5). Furthermore, these concentrations were higher than those for non-farmworker households during the thinning season. There were significant differences between the groups in the off season but to a lesser degree than during the thinning season. The thinning season DMTP values for adult farmworkers and both farmworker and non-farmworker children exceeded those found in the NHANES surveys of the general public (Fig. 2-6). The dimethyl metabolites were also significantly correlated with the levels of AZ measured in the adult blood sera (Fig 2-7). These results suggest an occupational component, including the take-home pathway, for farmworker household exposures to dimethyl OP pesticide over those for non-farmworkers living in the same geographic region during the thinning season.

For the diethyl metabolites, levels were similar between farmworker and non-farmworker households during the thinning season but were lower in farmworkers during the off season (Fig 2-4). The difference between the two groups was significant in the off season and the reasons for

this observation are not yet completely understood. Since the decrease was observed from the period of pesticide application to the off season, individuals residing in farmworker households may have been exposed to a diethyl OP pesticide other than CP during the thinning season. Changes in diet may also play a factor since the levels of diethyl metabolites measured were at least an order of magnitude below that observed for the dimethyls (Fig 2-4). Exposure to a different diethyl-substituted OP pesticide or preformed diethyl metabolites could explain the lack of correlation between the urinary diethyl metabolites and CP measured in the blood sera of adults. These exposure results reflect the seasonal nature of OP pesticide application and their low environmental persistence owing to short biological half-lives.

Findings of higher OP pesticide exposures in farmworkers and their children compared to control populations have been previously reported by our group as well as others (Simcox et al. 1999; Curl et al. 2002; Thompson et al. 2003). Bradman et al. (2005) measured detectable levels of DAPs and metabolites specific to the OP pesticides malathion and CP in farmworker mothers. A population of farmworkers in Monterey County, California, was found to have elevated levels of DMP and the malathion-specific metabolite malathion dicarboxylic acid when compared to NHANES data (Salvatore et al. 2008). Individual and total dimethyl DAPs were measured by Muniz et al. (2008) and Kisby et al. (2009) who found that DMP, DEP, DMTP, DMDTP, and total dimethyl DAPs were present at higher concentrations in farmworker and pesticide applicator urinary samples than those of controls. Similar to our findings, McCurdy et al. (1994) observed significant correlations between levels of the three dimethyl urinary metabolites in exposed workers. Additionally they found a significant correlation between the levels of AZ on skin and clothing and the levels of dimethyl urinary metabolites. Aprea and colleagues (1994) also observed high correlations (> 0.7) between levels of AZ and CP-methyl residues on the skin

of workers and amounts of urinary metabolites measured in the urine. Taken together, these results demonstrate that urinary biomarkers are sensitive indicators of occupational pesticide exposures in farmworkers during times of application, despite the fact that these individuals do not handle, formulate, or spray the pesticides.

We also observed higher urinary concentrations of dimethyl metabolites in farmworker children during the thinning season (Fig 2-5). Because the only difference between the farmworker and non-farmworker households was that farmworker adults worked in the pomme orchards, this is strong evidence of an occupational component to farmworker children's exposure to OP pesticides during periods of application. This observation is in agreement with data previously gathered characterizing the take-home pathway for households in which farmworker adults performed thinning tasks in pomme orchards (Curl et al. 2002; Thompson et al. 2003; Coronado et al. 2006). In addition to proximity to sprayed fields, this pathway is a significant contributor to the exposure burden experienced by these children over those not living in agricultural regions (Fenske et al. 2000) and likely results from the transport of pesticide residues into the home or family vehicle where children can interact with them (Fenske et al. 2000; Jaga and Dharmani 2003; Vida and Moretto 2007). Growing evidence from this and other study populations (McCauley et al. 2001; Curwin et al. 2007b) strongly point to the need for intervention strategies to be implemented to reduce children's exposures to agricultural chemicals. This process has begun in the Yakima Valley populations presented here (Thompson et al. 2008).

We investigated whether OP pesticide biomarkers of exposure were related to blood AChE and plasma BuChE inhibition, the classic markers of effect for these chemicals. A number of individuals had ChE inhibition exceeding the action levels set by WSDLI (2010) (Fig

2-8). When relating the exposure variables with AChE inhibition in a linear regression model, a significant relationship between AZ in the blood sera or dimethyl urinary metabolites and inhibition of AChE and BuChE were observed, with inhibition levels increasing with increasing amounts of the exposure biomarkers (Fig. 2-9 and Table 2-5). Neither the amount of CP nor the diethyl metabolites were significantly associated with cholinesterase inhibition. These findings are significant as they indicate that cholinesterase activity can be related to both the active compound measured in an invasively obtained matrix or metabolic products present in a non-invasively collected sample. Given that subjects may be more likely to provide biological samples obtained noninvasively (Hansen et al. 2007), these findings may open up further exploration of the utility of urinary metabolites in pesticide biomonitoring.

Few studies have examined associations between DAPs and ChE inhibition (Sudakin and Stone 2011). Atherton et al. (2009) observed a significant negative relationship between total DAPs, expressed as the sum of DMP, DMTP, DEP, and DETP, in the urine and overall AChE activity, which was not adjusted for baseline measurements in any manner. While this is an important observation, conclusions that can be drawn from such data may be confounded by the large amount of inter-person variability in AChE activity in unexposed individuals (Coye et al. 1986; Cocker et al. 2002). A better estimate would be to determine the level of ChE activity relative to a baseline value or ChE inhibition as we have done here, since it more closely reflects effects in the central nervous system than AChE activity alone. McCurdy et al. (1994) found a significant negative correlation between RBC AChE activity, but not plasma BuChE activity, expressed as a percentage of the baseline value and total dimethyl metabolites in a population of peach orchard workers. This observation for AChE matches the present finding of higher levels of inhibition with increasing concentrations of dimethyl metabolites in the urine. More studies

need to be performed with multiple AChE and BuChE activity measurements obtained in a given season to estimate the levels of intra- and inter-person variability and to evaluate their relationships with urinary DAPs and pesticide-specific metabolites in physiologically-based, pharmacokinetic (PBPK) studies.

Biomonitoring of OP pesticides with urinary metabolites can also be used to evaluate non-cholinesterase effects in exposed populations. For example, correlations have been found between OP pesticide exposure and markers of oxidative stress. Muniz et al. (2008) and Kisby et al. (2009) found significant correlations between urinary DAPs and comet tail length in lymphocytes taken from farmworkers and pesticide applicators. Studying indoor pesticide sprayers, Lee et al. (2007) observed significant correlations between DMP and total DAPs and either leukocyte or urinary 8-OHdG, an oxidized DNA nucleoside indicative of DNA damage. Atherton et al. (2009) also found an association between urinary 8-OHdG and total DAPs in addition to a significant correlation between leukocyte DNA damage ascertained by the comet assay and total DAPs. These observations add to the utility of using urinary markers of OP pesticide exposure to estimate biological effects from these pesticides.

Study design always needs to be considered in epidemiologic studies. As is mentioned elsewhere (Locke et al. 2009), the participants recruited for this study represent a convenience sample. Therefore, the results presented here may be a reflection of self-selection bias: those individuals who believe they are more likely to be exposed to OP pesticides occupationally may have been more willing to participate in the study than others who did not share that belief or concern. However, we were able to recruit farmworkers and non-farmworker cohorts that had similar distributions of age and sex, allowing us to have internal validity to make proper comparisons between the groups. Surveys conducted by the U.S. Department of Labor (USDOL

2005) show that the average age of farmworkers is 31 years of age, which matches the average age in the cohorts described here. However, these surveys also found that 79% of farmworkers were male. The higher percentage of females in our cohorts is likely due to the recruitment criteria of having at least one child in the household and would therefore have excluded single males and men traveling for work away from their families. Since the members of these cohorts represent households of concern for the take-home pathway of exposure, we believe the results presented here are meaningful for the larger population of households in the Yakima Valley and other agricultural regions that may also experience that route of exposure.

Considerations also need to be made regarding exposures through non-occupational sources. OP parent compounds and preformed metabolites may be present in foodstuffs consumed by those enrolled in the study (Lu et al. 2005; Zhang et al. 2008). If they are not metabolized further they could influence the levels of metabolites measured in urine samples (Timchalk et al. 2007a; Busby-Hjerpe et al. 2010). In this study, we recruited farmworkers and non-farmworkers from the same communities in the Yakima Valley of Central Washington and evaluated the potential for exposures in the context of when the major pesticide applications occur. We have shown that the higher levels of exposure in farmworkers occur during the thinning season when the dimethyl OP pesticides are most heavily applied. Furthermore, the levels of dimethyl urinary metabolites were significantly correlated with amounts of AZ measured in the blood, suggesting a significant contribution of DAPs through the metabolism of the parent compound. Therefore, we suggest that differences in exposures to dimethyl OP pesticides between the two cohorts will more accurately reflect occupational and dietary sources during the thinning season and dietary sources alone during the off season.

As a whole, our findings indicate that farmworker populations experience occupational exposures to OP pesticides that are associated with cholinesterase inhibition and the take-home pathway is a significant contributor to children's exposures during times of pesticide application. Additional resources need to be invested in identifying intervention strategies that are able to reduce occupational exposures in farmworkers. A number of studies have shown that the use of PPE in the field can reduce contact with OP pesticide residues and reduce the concentrations of DAPs measured in urine samples (Keifer 2000; van der Jagt et al. 2004; Bradman et al. 2009; Protano et al. 2009; Vitali et al. 2009). Specific tasks like thinning (Simcox et al. 1999; Coronado et al. 2004) or working with pomme fruits (Coronado et al. 2006; Coronado et al. 2009), or behaviors like consuming crop fruits in the field (Bradman et al. 2009) can increase exposures. Intervention strategies targeting these activities along with educational programs regarding the importance of occupational hygiene and proper PPE use are ongoing (Thompson et al. 2008). Continued biomonitoring will be a valuable tool in evaluating the effectiveness of these interventions.

Figure 2-1 Frequency Distributions of Azinphos-Methyl in Blood Sera of Farmworkers and Non-Farmworkers

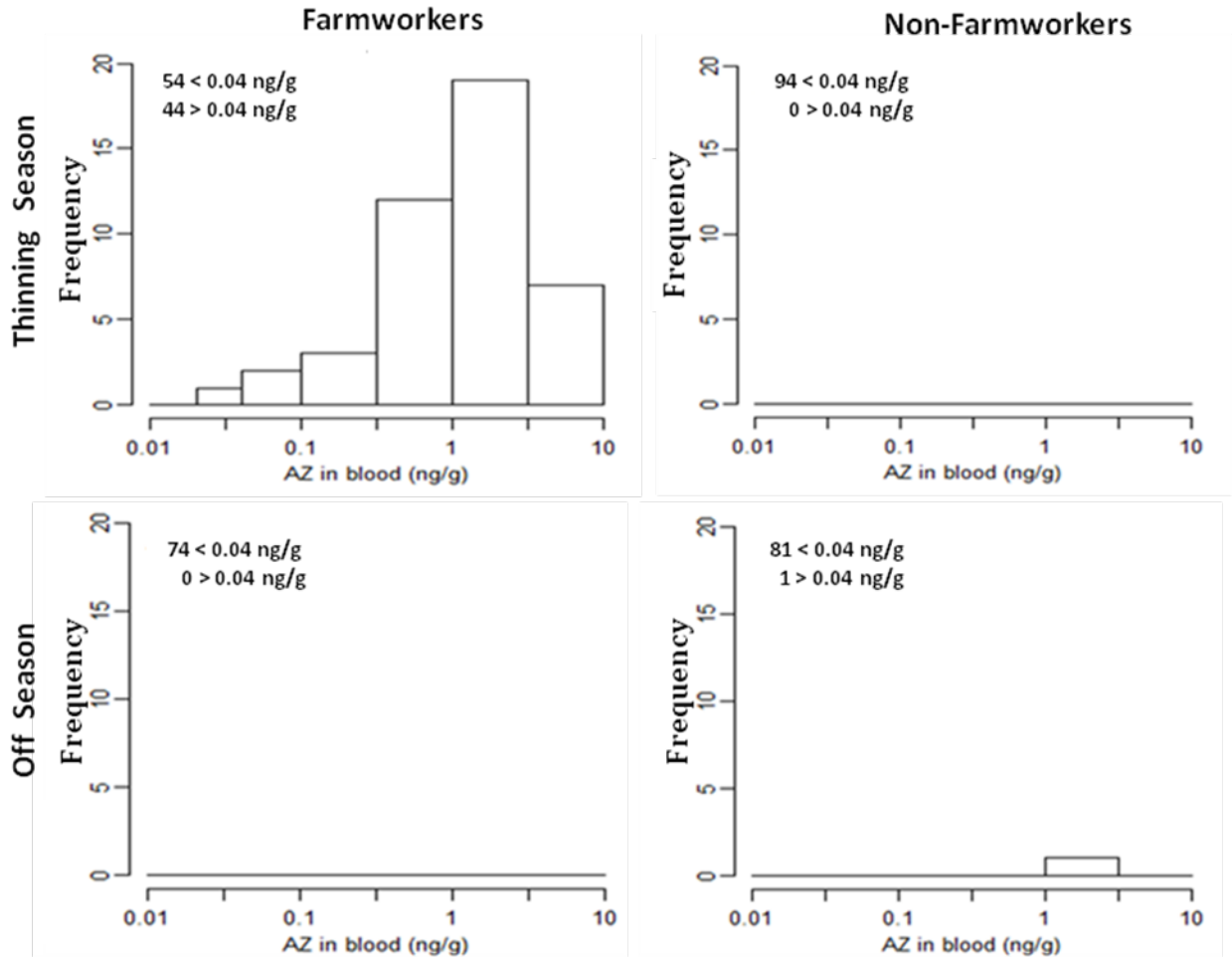


Fig 2-1. Frequency distributions of azinphos-methyl (AZ) in blood sera of farmworkers and non-farmworkers. Blood samples collected from farmworkers and non-farmworkers were analyzed via gas chromatography/mass spectrometry by the Pesticide Laboratory in the National Center for Environmental Health at CDC. Only farmworkers had AZ detectable in the blood during the thinning season; 44 farmworkers had levels above 0.04 ng/g serum limit of detection during the thinning season compared to none in the off season. One non-farmworker had observable levels (2.36 ng/g serum) in the off season. Thinning season farmworker AZ levels were significantly higher than non-farmworker concentrations in the same season and farmworker concentrations in the off season (Wilcoxon test, $p < 0.001$).

Figure 2-2 Frequency Distributions of Chlorpyrifos in Blood Sera of Farmworkers and Non-Farmworkers

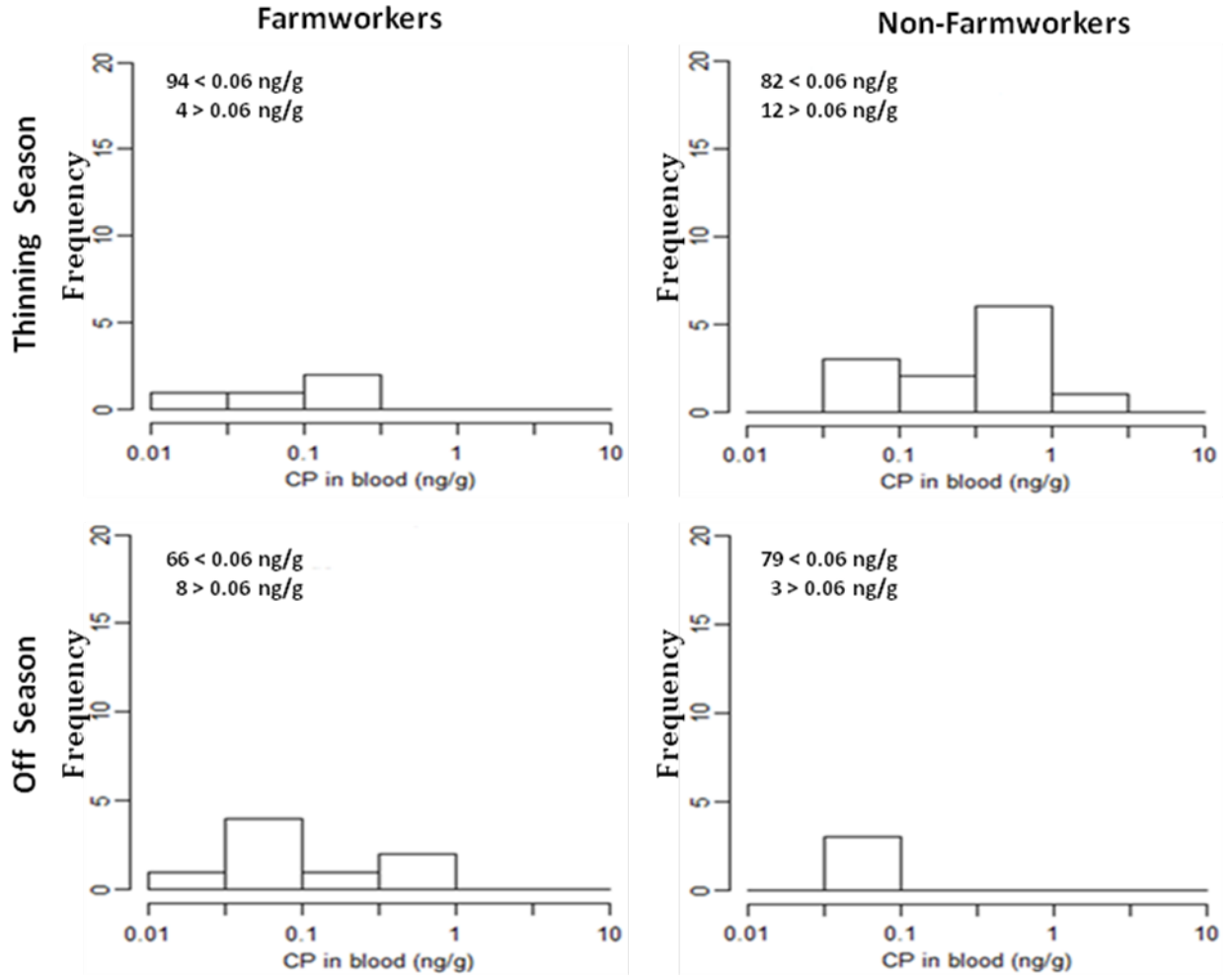


Figure 2-2
Frequency distributions of chlorpyrifos (CP) in blood sera of farmworkers and non-farmworkers. Blood samples collected from farmworkers and non-farmworkers were analyzed by gas chromatography/mass spectrometry at the Pesticide Laboratory in the National Center for Environmental Health at CDC. CP was more frequently detected above the LOD of 0.06 ng/g serum in farmworkers in the off season than in the thinning season while it was detected in the non-farmworker samples more often in the thinning than in the off season. No farmworkers had detectable levels in both the thinning and off season, while only one non-farmworker had detectable levels in both seasons. No significant differences in blood levels of CP were observed (Wilcoxon test, $p > 0.05$).

Figure 2-3 Between- and Within-Person Correlations for Dimethyl and Diethyl Urinary Metabolites.

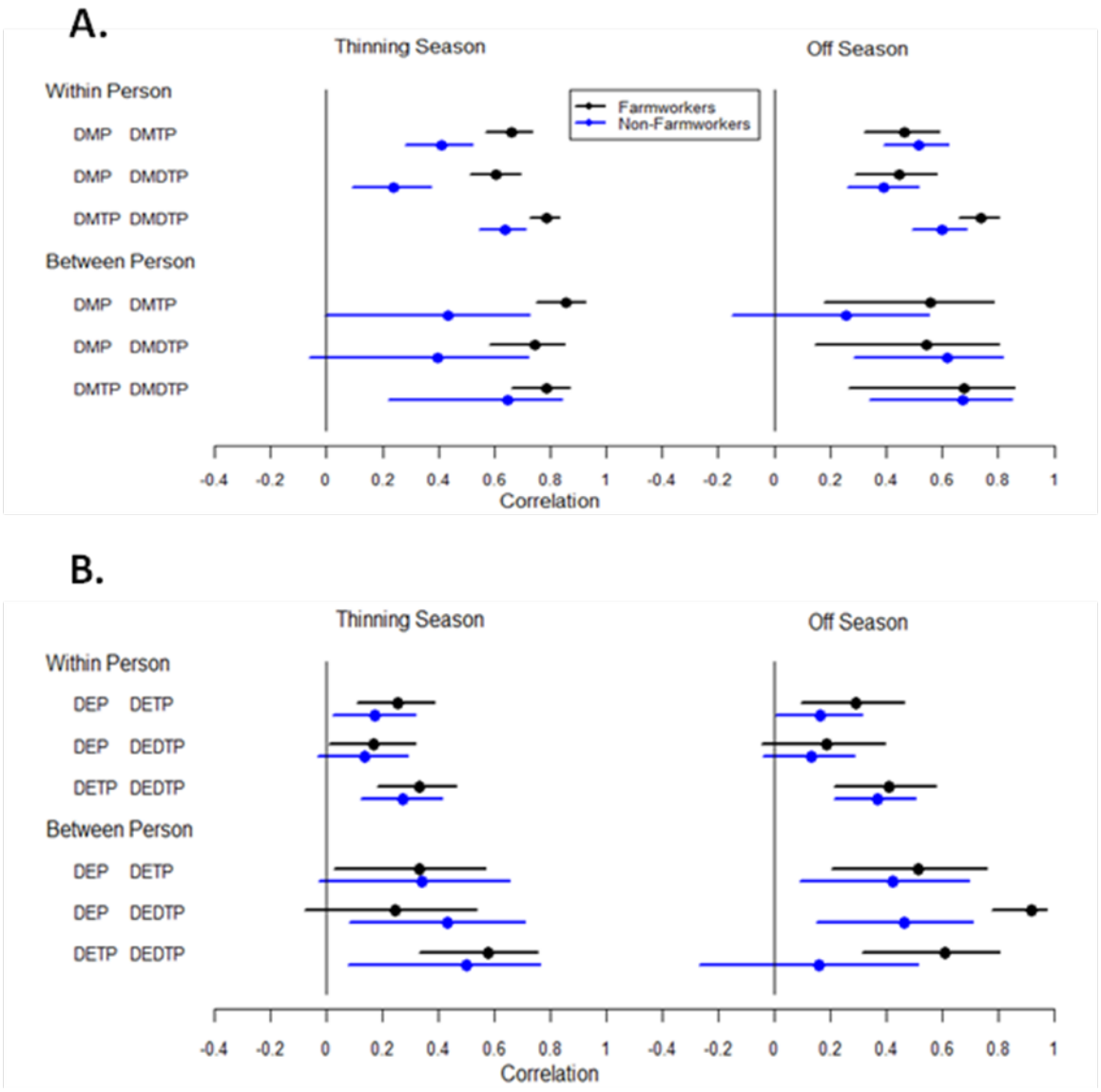


Figure 2-3. Between- and within-person correlations for A) dimethyl and B) diethyl urinary metabolites. The correlations and 95% confidence intervals for each pair of dimethyl or diethyl metabolites measured during the thinning and off seasons are represented. These correlations were incorporated into a multivariate normal (MVN) distribution in conjunction with Markov-Chain Monte Carlo simulations to estimate values for the DAP urinary metabolites as described in the Methods section.

Figure 2-4 Profiles of Dimethyl and Diethyl DAP Urinary Metabolites in Adult Participants

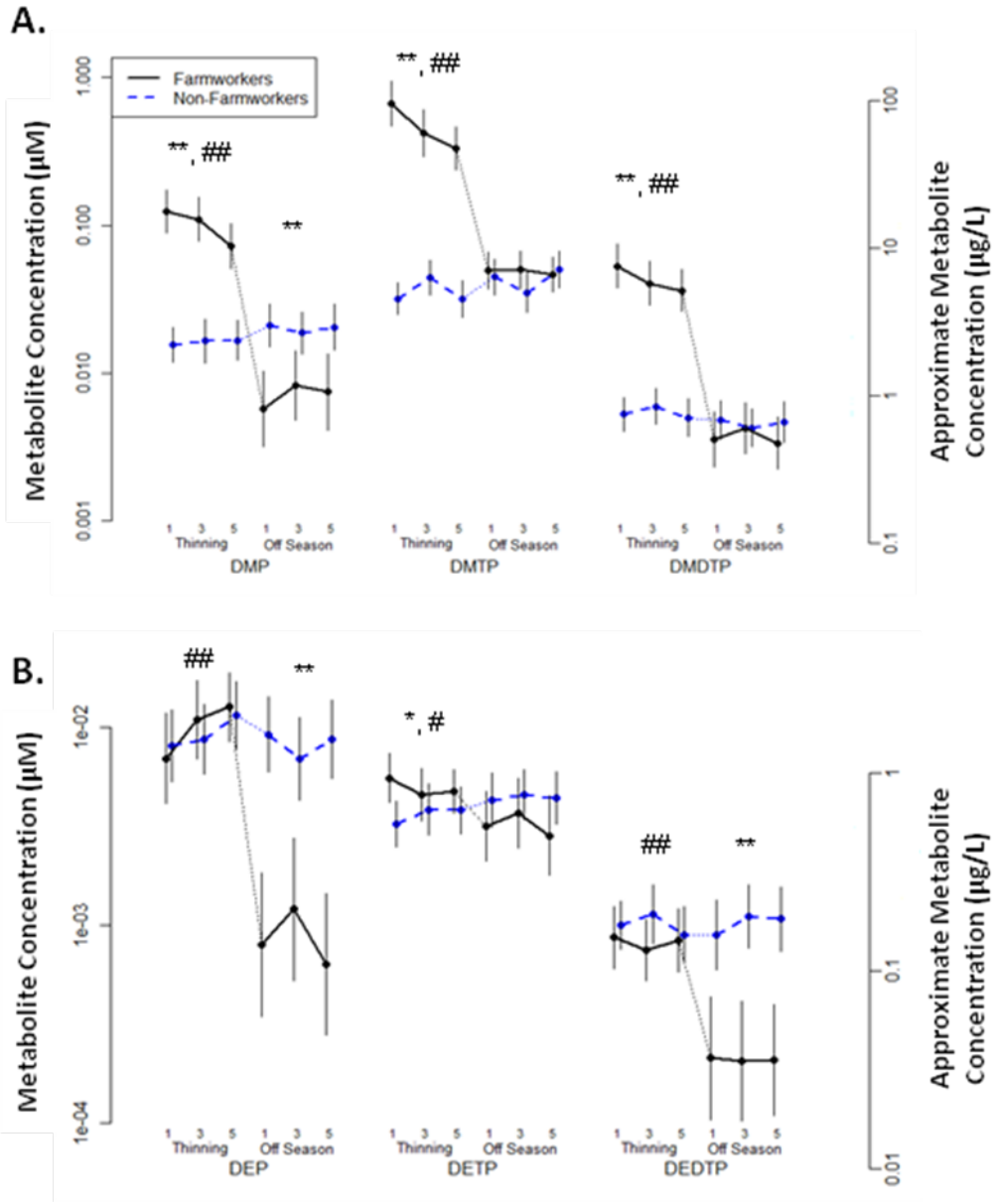


Figure 2-4 Profiles of A.) dimethyl and B.) diethyl DAP Urinary Metabolites in Adult Participants. Urine was collected from farmworkers and non-farmworkers during days 1, 3, and 5 of sampling periods during the agricultural thinning (April-July) and off (Nov.-Feb.) seasons and metabolites were measured using HPLC-MS/MS. A multivariate normal (MVN) components of variance model and Monte Carlo-Markov Chain simulations were performed to estimate values below the limit of detection (see Methods section). Data are represented as geometric means and 95% confidence intervals and the vertical axes are approximate concentrations (µg/L) and molar (µM) values in log-scale. * symbols indicate significant differences between farmworkers and non-farmworkers in the same season, while # indicate significant differences between the thinning and off season values for farmworkers. *, #: $p < 0.05$; **, ##: $p < 0.001$.

Figure 2-5 Profiles of Dimethyl and Diethyl DAP Urinary Metabolites in Child Participants

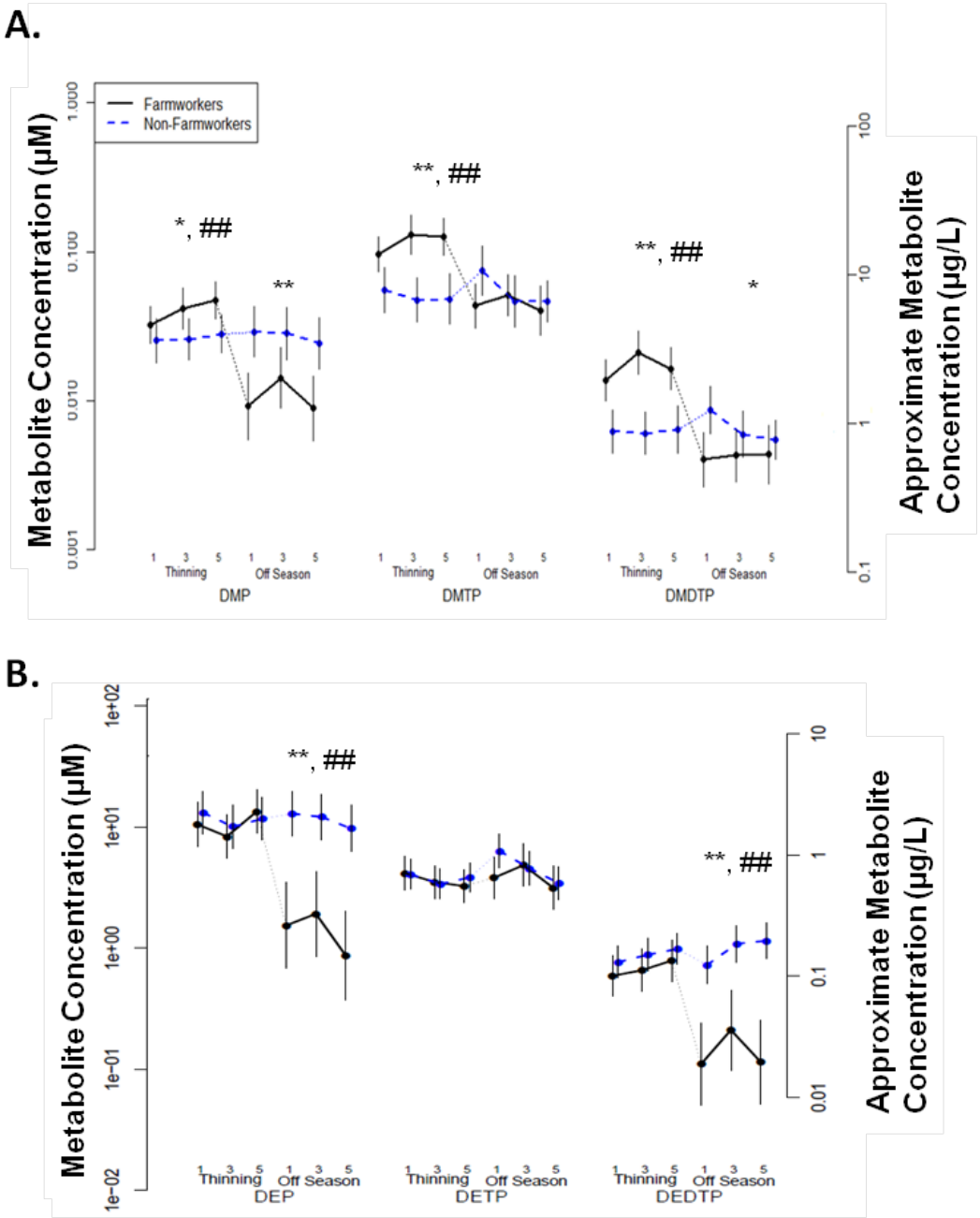


Figure 2-5 Profiles of A.) dimethyl and B.) diethyl DAP urinary metabolites in child participants. Urine was collected from farmworker and non-farmworker children in the same manner as for adults (see Fig 2-4 and methods). Data are represented as geometric means and 95% confidence intervals and the vertical axes are approximate concentrations (µg/L) and molar (µM) values in log-scale. * symbols indicate significant differences between farmworker and non-farmworker children in the same season, while # indicate significant differences between the thinning and off season values for farmworker children. *, #: $p < 0.05$; **, ##: $p < 0.001$.

Figure 2-6 Distributions of DMTP Measured in Urine Samples Collected from Farmworker and Non-Farmworker Households in Relation to NHANES Data

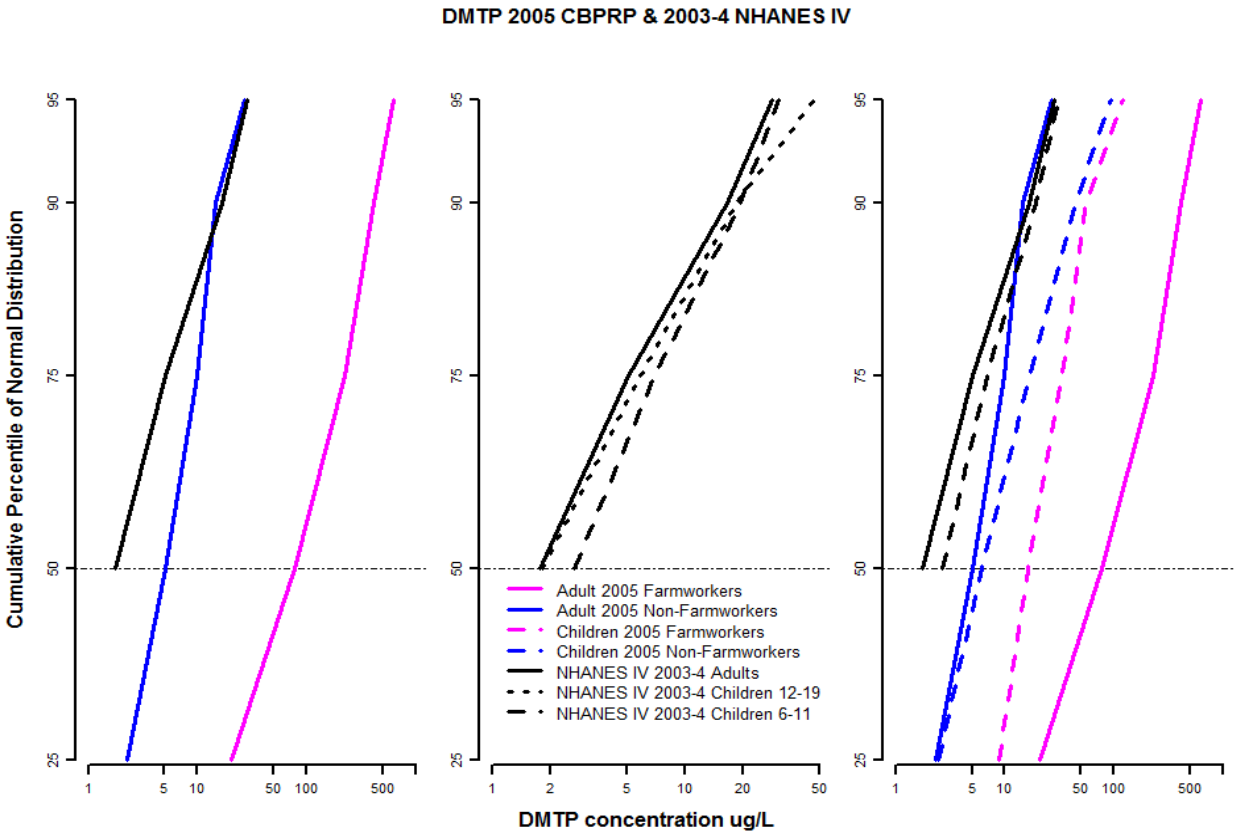


Figure 2-6. Distributions of DMTP measured in urine samples collected from farmworker and non-farmworker households in relation to NHANES data. Urinary DMTP values obtained from farmworker and non-farmworker households were modeled against data available for the general population (NHANES 2003-2004; http://www.cdc.gov/nchs/nhanes/nhanes2003-2004/lab03_04.htm).

Figure 2-7 Urinary Dimethyl Dialkylphosphates are Correlated with Azinphos Methyl Concentrations in Blood Sera

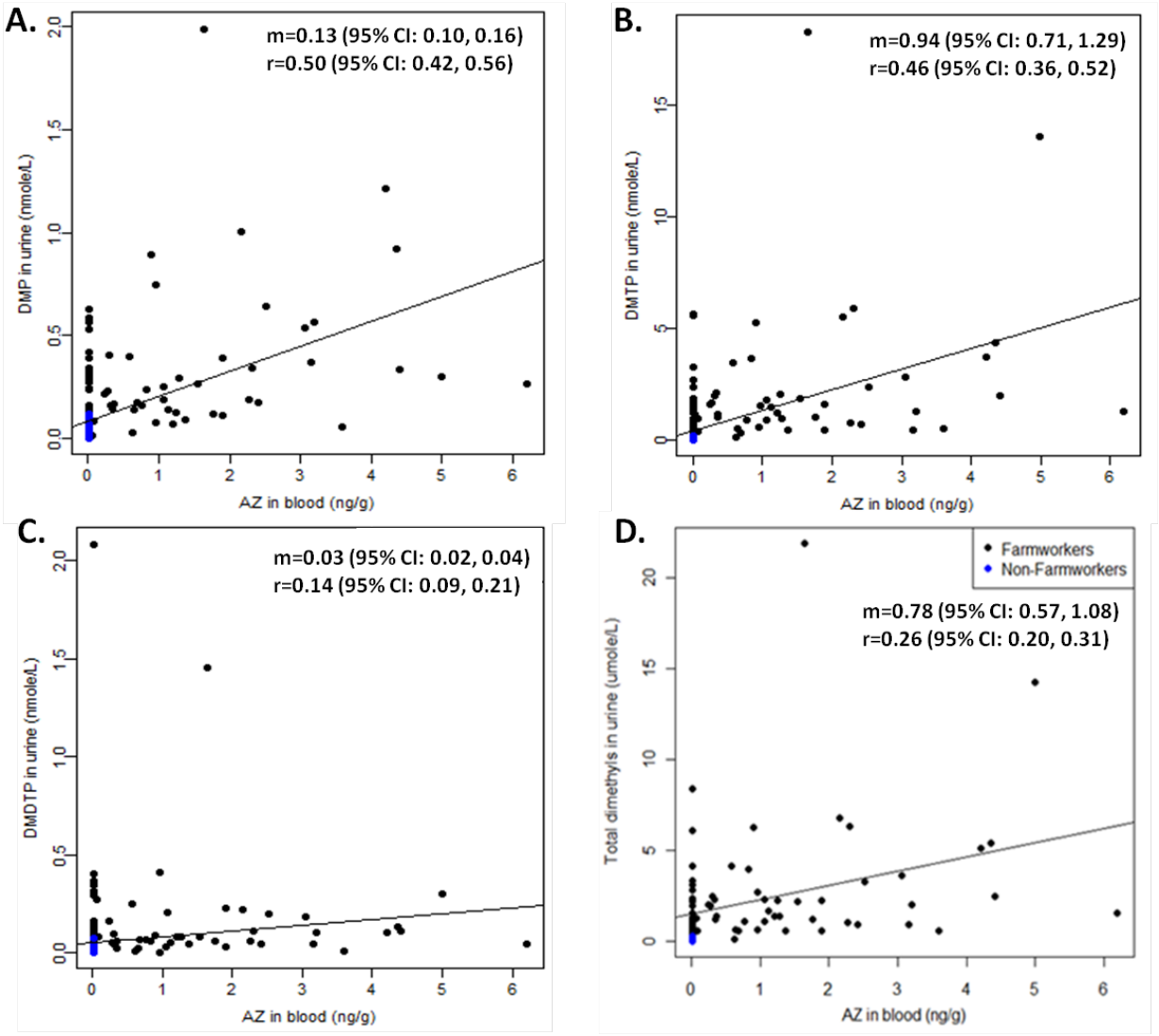


Figure 2-7 Urinary dimethyl dialkylphosphates are correlated with azinphos methyl concentrations in blood sera. Linear regression analysis was performed between azinphos methyl blood concentrations and urinary concentrations of A.) DMP, B.) DMTP, C.) DMDTP, and D.) total dimethyl metabolites. The associations are represented by their slope (m) and correlation (r). The values for the slope are in (nmol DAP/L urine)/ng AZ/g serum for DMP, DMTP, and DMDTP and (µmol DAP/L urine)/ng AZ/g serum for total dimethyls. Data points in black are farmworker data points (N = 99) and those in blue represent non-farmworker samples (N = 95).

Figure 2-8 Frequency Distributions of Cholinesterase Inhibition

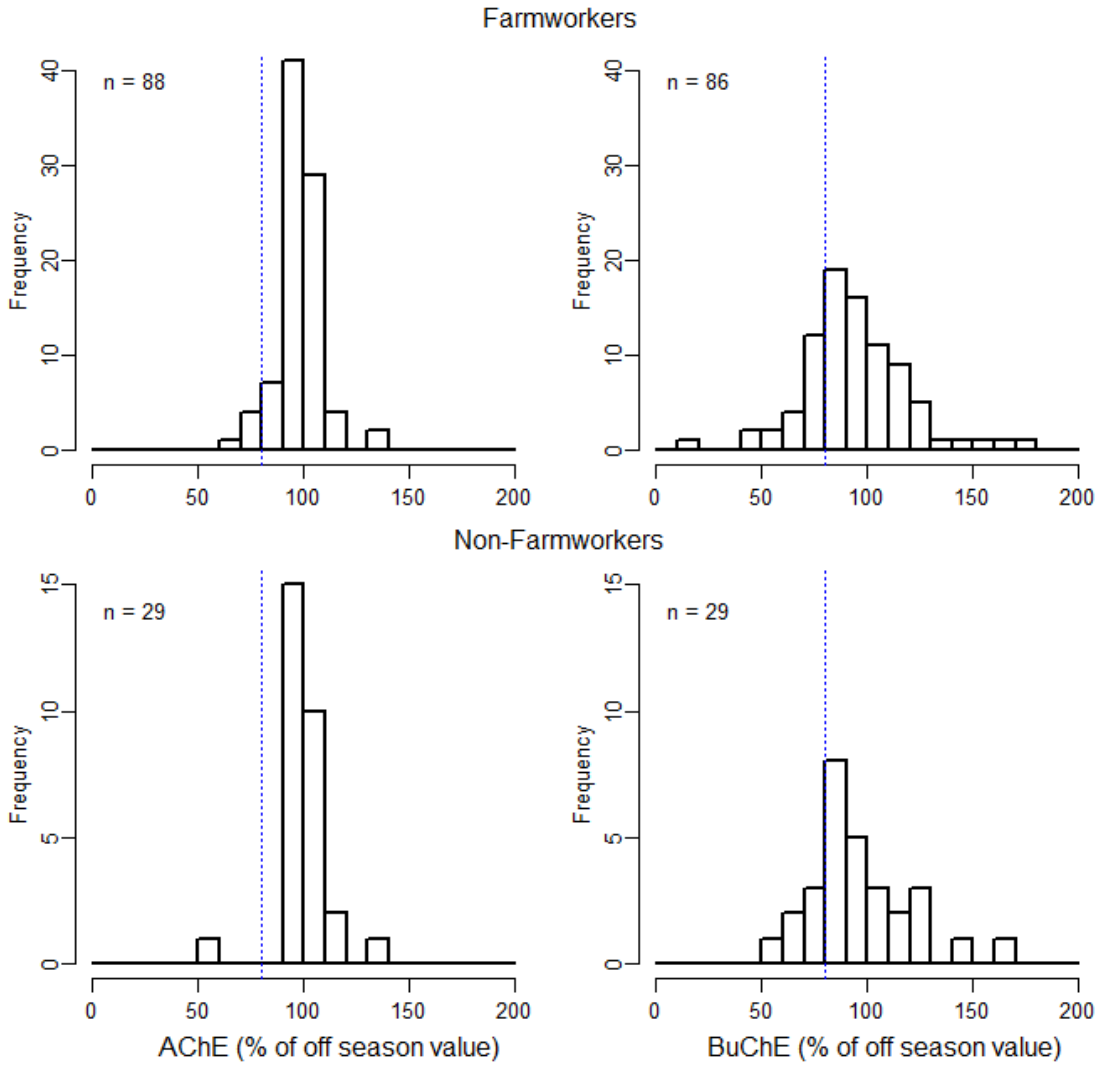


Figure 2-8 Frequency distributions of ChE inhibition. RBC AChE and plasma BuChE activity were determined from blood samples taken from farmworkers and non-farmworkers during the thinning and off (baseline) seasons. Levels of enzyme activity relative to the baseline (off season) were determined for the thinning season. The dashed vertical line designates 80% of baseline activity or 20% inhibition, the first action level for the WSDLI Cholinesterase Monitoring Program. Most study participants had low levels of ChE inhibition but a greater number of farmworkers had cholinesterase inhibition levels above 20%: 21 farmworkers had BuChE inhibition above 20% and 5 had 20% or greater AChE inhibition compared to 6 and 1 non-farmworkers, respectively. Three farmworkers had greater than 20% inhibition for both cholinesterase enzymes. AChE and BuChE inhibition were not significantly correlated ($p > 0.05$).

Figure 2-9 Cholinesterase Inhibition in Relation to Azinphos Methyl and Chlorpyrifos in Blood Sera During the Thinning Season

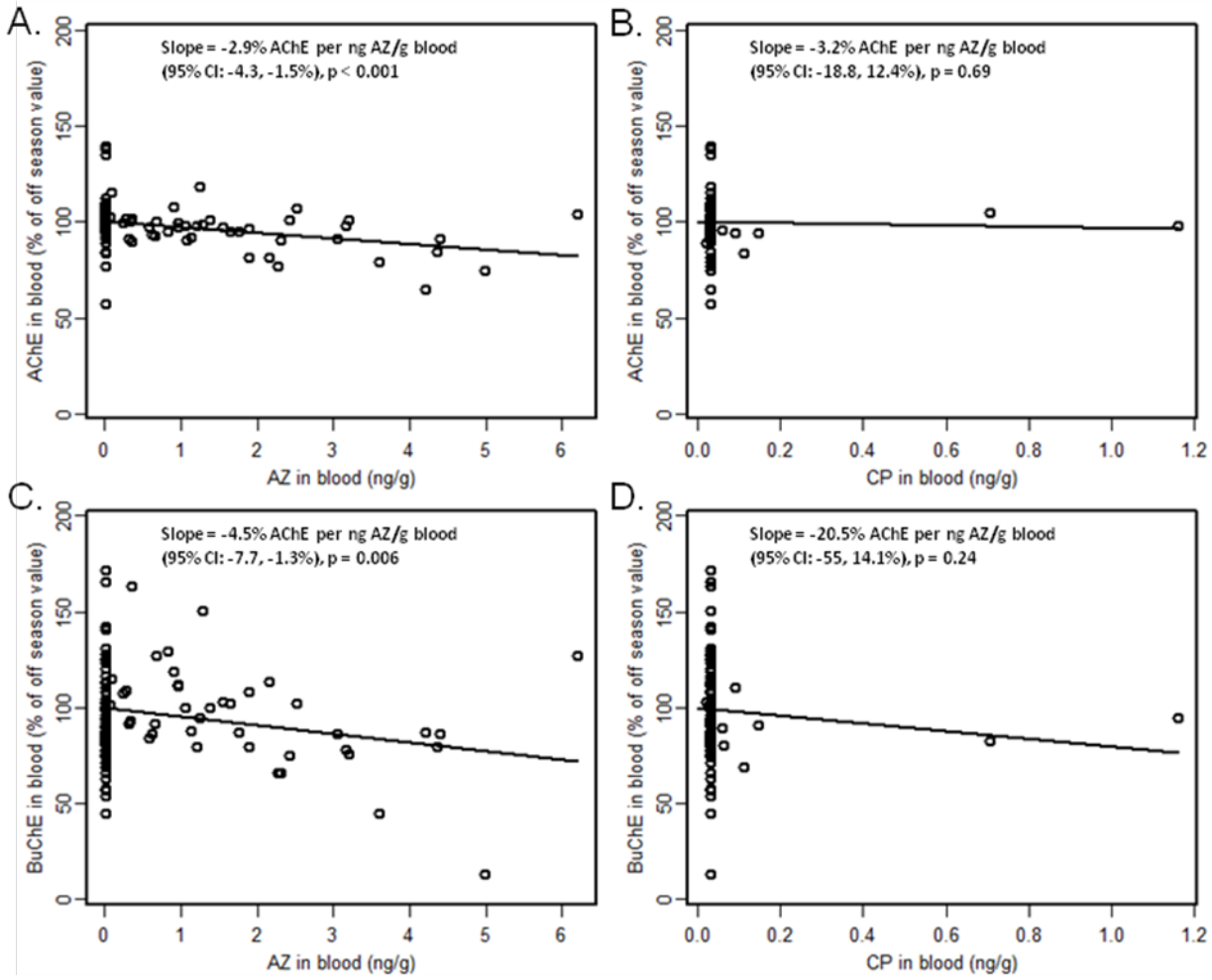


Figure 2-9. ChE Inhibition in relation to AZ and CP in blood sera during the thinning season. The association between blood AZ and activity of A.) AChE and C.) BuChE and between CP and inhibition of B.) AChE and D.) BuChE were evaluated via linear regression for farmworkers (N = 99) and non-farmworkers combined (N=95). Slopes of the regression lines were determined assuming an intercept of 100% of baseline activity. There was a significant relationship between blood AZ and AChE, indicating that AChE activity decreased by 2.9% per ng AZ/g serum (95% CI: -4.3, -1.5%), as well as between AZ and BuChE inhibition, inhibition levels increasing 4.5% per ng AZ/g serum (95% CI: -7.7, -1.3%). CP did not have significant associations with either AChE or BuChE activity as evidenced by the confidence intervals containing zero.

Table 2-1 Summary Statistics for Dimethyl Urinary Metabolites in Farmworkers and Non-Farmworker Adults

		Farmworkers					Non-Farmworkers				
	DMP	No.	Median (µg/L)	Range (µg/L)	No. < LOD	(%)	No.	Median (µg/L)	Range (µg/L)	No. < LOD	(%)
Thinning Season	**Day 1**	98	16.89	<LOD-267.54	6	(6)	95	2.32	<LOD-41.59	15	(16)
	Day 3	96	23.22	<LOD-374.04	7	(7)	92	3.11	<LOD-74.17	22	(24)
	Day 5	99	11.72	<LOD-291.03	8	(8)	94	2.62	<LOD-64.51	20	(21)
	DMTP										
	Day 1	98	141.19	<LOD-2850.37	1	(1)	95	4.96	<LOD-112.02	1	(1)
	Day 3	96	62.96	<LOD-1871.73	2	(2)	92	5.72	<LOD-383.09	1	(1)
	Day 5	99	43.13	1.30-1575.71	0	(0)	94	4.18	<LOD-420.13	2	(2)
	DMDTP										
	Day 1	98	6.96	<LOD-726.25	1	(1)	95	0.76	<LOD-41.27	10	(11)
	Day 3	96	6.47	<LOD-269.83	3	(3)	92	0.97	<LOD-44.97	11	(12)
	Day 5	99	4.62	<LOD-252.49	1	(1)	94	0.85	<LOD-57.07	16	(17)
Off Season	**DMP**										
	Day 1	91	0.51	<LOD-116.34	42	(46)	87	3.62	<LOD-77.06	18	(21)
	Day 3	91	1.36	<LOD-46.85	38	(42)	87	3.00	<LOD-53.65	17	(20)
	Day 5	91	1.72	<LOD-180.73	41	(45)	87	4.44	<LOD-34.83	20	(23)
	DMTP										
	Day 1	91	6.59	<LOD-825.99	1	(1)	87	5.50	0.43-268.12	0	(0)
	Day 3	91	6.90	<LOD-207.87	1	(1)	87	5.18	<LOD-286.03	1	(1)
	Day 5	91	5.44	<LOD-562.72	1	(1)	87	6.18	<LOD-161.31	1	(1)
	DMDTP										
	Day 1	91	0.63	<LOD-179.89	26	(29)	87	0.77	<LOD-24.61	9	(10)
	Day 3	91	0.83	<LOD-49.89	21	(23)	87	0.64	<LOD-116.86	8	(9)
	Day 5	91	0.68	<LOD-178.42	26	(29)	87	0.87	<LOD-32.04	11	(13)

LOD - Limit of Detection

LOD for DMP - 0.6 µg/L, DMTP - 0.2 µg/L, DMDTP - 0.1 µg/L

Table 2-2 Summary Statistics for Diethyl Urinary Metabolites in Farmworkers and Non-Farmworker Adults

		Farmworkers					Non-Farmworkers				
	DEP	No.	Median (µg/L)	Range (µg/L)	No. < LOD	(%)	No.	Median (µg/L)	Range (µg/L)	No. < LOD	(%)
Thinning Season	**Day 1**	98	2.58	<LOD-171.13	33	(34)	95	2.65	<LOD-31.21	24	(25)
	Day 3	96	3.63	<LOD-38.35	24	(25)	92	2.65	<LOD-21.07	24	(26)
	Day 5	99	4.10	<LOD-28.53	21	(21)	94	3.46	<LOD-35.43	21	(22)
	DETP										
	Day 1	98	1.12	<LOD-44.84	11	(11)	95	0.56	<LOD-8.34	11	(12)
	Day 3	96	0.94	<LOD-15.81	14	(15)	92	0.73	<LOD-18.55	14	(15)
	Day 5	99	0.91	<LOD-12.12	7	(7)	94	0.62	<LOD-13.51	11	(12)
	DEDTP										
	Day 1	98	0.14	<LOD-60.72	36	(37)	95	0.16	<LOD-5.67	30	(32)
	Day 3	96	0.12	<LOD-20.12	38	(40)	92	0.22	<LOD-8.91	30	(33)
	Day 5	99	0.12	<LOD-8.22	36	(36)	94	0.18	<LOD-6.34	35	(37)
Off Season	**DEP**										
	Day 1	91	<LOD	<LOD-27.52	49	(54)	87	2.94	<LOD-36.99	20	(23)
	Day 3	91	0.56	<LOD-30.02	45	(49)	87	2.95	<LOD-54.8	23	(26)
	Day 5	91	<LOD	<LOD-33.2	54	(59)	87	3.28	<LOD-58.93	21	(24)
	DETP										
	Day 1	91	0.83	<LOD-14.11	18	(20)	87	0.74	<LOD-83.66	9	(10)
	Day 3	91	0.86	<LOD-23.09	17	(19)	87	0.80	<LOD-24.87	7	(8)
	Day 5	91	0.67	<LOD-35.37	23	(25)	87	0.62	<LOD-74.76	5	(6)
	DEDTP										
	Day 1	91	<LOD	<LOD-4.69	56	(62)	87	0.16	<LOD-5.94	34	(39)
	Day 3	91	<LOD	<LOD-12.52	55	(60)	87	0.21	<LOD-4.18	27	(31)
	Day 5	91	<LOD	<LOD-5.18	53	(58)	87	0.19	<LOD-5.48	30	(34)

LOD - Limit of Detection

LOD for DEP - 0.2 µg/L, DETP - 0.1 µg/L, DEDTP - 0.1 µg/L

Table 2-3 Summary Statistics for Dimethyl Urinary Metabolites in Farmworkers and Non-Farmworker Children

		Farmworker Children					Non-Farmworker Children				
	DMP	**No.**	**Median (µg/L)**	**Range (µg/L)**	**No. < LOD**	**(%)**	**No.**	**Median (µg/L)**	**Range (µg/L)**	**No. < LOD**	**(%)**
Thinning Season	**Day 1**	98	6.37	<LOD-111.63	10	(10)	93	3.77	<LOD-121.41	16	(17)
	Day 3	96	7.31	<LOD-403.00	13	(14)	91	4.50	<LOD-101.78	18	(20)
	Day 5	97	8.62	<LOD-292.51	12	(12)	93	4.72	<LOD-110.20	10	(11)
	DMTP										
	Day 1	98	16.97	0.11-759.38	0	(0)	93	6.41	0.68-352.44	0	(0)
	Day 3	96	17.57	0.25-1595.65	0	(0)	91	5.39	0.40-951.44	0	(0)
	Day 5	98	13.30	<LOD-733.01	1	(1)	93	6.87	<LOD-1057.85	2	(2)
	DMDTP										
	Day 1	98	1.85	<LOD-232.35	5	(5)	93	0.81	<LOD-25.21	12	(13)
	Day 3	96	2.65	<LOD-719.23	3	(3)	91	0.80	<LOD-71.52	13	(14)
	Day 5	98	2.14	<LOD-248.08	5	(5)	93	1.12	<LOD-74.07	13	(14)
Off Season	**DMP**										
	Day 1	91	1.75	<LOD-90.71	36	(40)	86	5.48	<LOD-75.78	16	(19)
	Day 3	91	2.83	<LOD-144.69	35	(38)	86	5.88	<LOD-55.73	16	(19)
	Day 5	91	1.71	<LOD-65.27	39	(43)	85	3.87	<LOD-113.68	17	(20)
	DMTP										
	Day 1	91	6.93	<LOD-361.38	3	(3)	86	8.77	<LOD-337.35	1	(1)
	Day 3	91	6.13	<LOD-138.32	2	(2)	86	7.13	<LOD-236.68	4	(5)
	Day 5	91	5.76	<LOD-379.95	4	(4)	85	6.44	<LOD-140.89	3	(4)
	DMDTP										
	Day 1	91	0.72	<LOD-125.92	25	(27)	86	1.00	<LOD-85.55	4	(5)
	Day 3	91	0.77	<LOD-48.45	24	(26)	86	0.80	<LOD-52.72	10	(12)
	Day 5	91	0.82	<LOD-75.85	22	(24)	85	0.85	<LOD-14.00	8	(9)

LOD - Limit of Detection

LOD for DMP - 0.6 µg/L, DMTP - 0.2 µg/L, DMDTP - 0.1 µg/L

Table 2-4 Summary Statistics for Diethyl Urinary Metabolites in Farmworkers and Non-Farmworker Children

		Farmworker Children					Non-Farmworker Children				
	DEP	No.	Median (µg/L)	Range (µg/L)	No. < LOD	(%)	No.	Median (µg/L)	Range (µg/L)	No. < LOD	(%)
Thinning Season	**Day 1**	98	3.04	<LOD-239.35	21	(21)	93	3.75	<LOD-779.39	17	(18)
	Day 3	96	2.51	<LOD-149.98	26	(27)	91	3.32	<LOD-460.67	23	(25)
	Day 5	97	4.08	<LOD-112.33	21	(22)	93	3.81	<LOD-270.61	20	(22)
	DETP										
	Day 1	98	0.86	<LOD-8.39	11	(11)	93	0.84	<LOD-6.34	10	(11)
	Day 3	96	0.66	<LOD-20.16	14	(15)	91	0.64	<LOD-19.03	9	(10)
	Day 5	98	0.69	<LOD-96.65	19	(19)	93	0.80	<LOD-25.48	13	(14)
	DEDTP										
	Day 1	98	0.08	<LOD-16.81	43	(44)	93	0.15	<LOD-11.11	36	(39)
	Day 3	96	0.10	<LOD-20.28	43	(45)	91	0.13	<LOD-8.07	31	(34)
	Day 5	98	0.10	<LOD-117.70	41	(42)	93	0.18	<LOD-29.77	30	(32)
Off Season	**DEP**										
	Day 1	91	0.91	<LOD-25.45	44	(48)	86	4.16	<LOD-51.84	19	(22)
	Day 3	91	0.87	<LOD-31.76	43	(47)	86	3.74	<LOD-49.00	18	(21)
	Day 5	91	<LOD	<LOD-28.23	51	(56)	85	3.24	<LOD-37.02	19	(22)
	DETP										
	Day 1	91	0.73	<LOD-81.54	15	(16)	86	1.13	<LOD-17.40	5	(6)
	Day 3	91	0.94	<LOD-37.62	15	(16)	86	0.64	<LOD-31.56	8	(9)
	Day 5	91	0.62	<LOD-62.56	22	(24)	86	0.63	<LOD-11.22	12	(14)
	DEDTP										
	Day 1	91	<LOD	<LOD-14.88	62	(68)	86	0.12	<LOD-17.73	37	(43)
	Day 3	91	<LOD	<LOD-5.54	55	(60)	86	0.24	<LOD-2.16	30	(35)
	Day 5	91	<LOD	<LOD-26.73	59	(65)	86	0.17	<LOD-6.12	27	(31)

LOD - Limit of Detection

LOD for DEP - 0.2 µg/L, DETP - 0.1 µg/L, DEDTP - 0.1 µg/L

Table 2-5 Regression of Cholinesterase Enzyme Inhibition against DAP Urinary Metabolites During the Thinning Season for Farmworkers (N = 99) and Non-Farmworkers (N = 95) Combined

	AChE Activity (% Off Season/µmol/L urine)			BuChE Activity (% Off Season/µmol/L urine)		
	Slope	**95% CI**	**p-value**	**Slope**	**95% CI**	**p-value**
Dimethyl Metabolites						
DMP	-15.5	(-27.2, -3.7)	0.010	-27.0	(-53.6, -0.4)	0.047
DMTP	-2.9	(-4.9, -0.9)	0.005	-6.3	(-10.8, -1.9)	0.006
DMDTP	-12.4	(-30.2, 5.4)	0.170	-49.5	(-88.4, -10.6)	0.013
Diethyl Metabolites						
DEP	-54.9	(-159.0, 49.2)	0.300	-140.4	(-472.2, 191.4)	0.410
DETP	-140.8	(-344.7, 63.1)	0.180	-438.9	(-990.4, 112.6)	0.120
DEDTP	-211.8	(-800.3, 376.8)	0.480	-1104.4	(-2,399.5, 190.6)	0.095

AChE – Acetylcholinesterase, BuChE - Butyrylcholinesterase

CHAPTER 3: Association Between PON1 Status and Blood Cholinesterase Activities in Farmworkers

Introduction:

Paraoxanase-1 (PON1) is a serum enzyme that is associated with the HDL fraction of cholesterol and plays a significant role in the detoxification of OP pesticides (Furlong et al. 2005). It acts by hydrolyzing the activated oxon intermediate of these compounds. Animal experiments utilizing supplementation with purified PON1 enzyme (Costa et al. 1990; Li et al. 1995) or knockout animals (Furlong et al. 1998; Shih et al. 1998; Lockridge et al. 2005) showed ChE activity was positively associated with the amount of PON1 present after exposure to either CP or CP oxon, with a greater effect present for CP oxon. PON1, therefore, plays an essential role in balance of activation and detoxification that determines the toxicity of certain OP pesticides.

Genetic variability in PON1 is associated with differences in metabolism of OP pesticide oxons. In particular, the position R192Q single nucleotide polymorphism located in the coding sequence affects the ability of PON1 to hydrolyze these oxon chemical species (Mackness et al. 1997). The impacts of this polymorphism on the hydrolysis of certain oxons has been related to differences in catalytic efficiency, with the $PON1_{R192}$ isoform having roughly two and ten times greater catalytic efficiency towards the oxons of chlorpyrifos (CP) and parathion, respectively, than the $PON1_{Q192}$ isoform (Li et al. 2000). This lesser ability to metabolize oxons has translated to higher AChE inhibition in mice humanized with the $hPON1_{Q192}$ allele compared to animals with the $hPON1_{R192}$ genotype (Cole et al. 2005). In a group of pesticide applicators, Hofmann et al. (2009) observed significant alterations in the level of BuChE inhibition based on PON1 genotype and phenotype. These studies suggest that PON1 genotype and phenotype, may be a significant indicators of susceptibility to OP pesticide-induced health effects in exposed human populations (Costa et al. 2003; Furlong et al. 2005).

However, to date, only the oxons of CP and diazinon have been demonstrated to be toxicologically relevant substrates of PON1 (Li et al. 2000). For example, azinphos methyl (AZ) did not generate different levels of brain ChE inhibition in PON1 wild-type (PON +/+) and knockout (PON1 -/-) mice, strong evidence that AZ oxon is not a substrate for PON1 (Costa et al. 1999). PON1 did not provide protection when malaoxon, the oxon form of malathion, was administered to PON1 +/+, PON1 -/-, $hPON1_{R192}$, or $hPON1_{Q192}$ mice (Jansen et al. 2009). Moser and Padilla (2011), while showing CP oxon detoxification, observed no detoxification of malaoxon, the oxon of malathion, by PON1 when incubating human recombinant AChE with rat liver homogenate. These data suggest that PON1 status is not an appropriate marker of susceptibility for all OP pesticides to which humans may be exposed.

As our CBPRP populations consisted of individuals who had occupational exposures to AZ, this provided us with an opportunity to examine whether or not PON1 was involved in the metabolism of AZ in humans. To do this, we tested whether PON1 status affected the ChE inhibition/OP pesticide exposure relationships established in Chapter 1 for adult participants.

Methods:

Sample Collection and Exposure Measurements

Urine, venous blood, and fingerstick samples were collected and concentrations of urinary metabolites and parent compound in blood and ChE inhibition were determined as described in Chapter 2

PON1 Genotyping and Phenotyping

Plasma samples were used for the genotyping and phenotyping assays, details of which can be found in Richter et al. (2009). PON1 status was determined based on a two-substrate enzyme

assay using diazoxon and paraoxon that reveals both PON1 plasma activity levels and functional Q192R polymorphism in the PON1 coding region. Arylesterase (AREase) activity was determined by monitoring the hydrolysis of phenyl acetate. AREase activity was stratified into three groups for analysis such that approximately equal numbers of individuals with ChE inhibition were in each group: high > 112 U/ml, moderate 91-112 U/ml, and low < 91 U/ml. $PON1_{192}$ genotypes and AREase activity were successfully characterized for 186 individuals.

As part of a larger panel of metabolizing genes being investigated, we genotyped 70 participants for the PON1 position 55 polymorphism using the Drug Metabolizing Enzyme Transporter (DMET) plus genotyping platform (Affymetrix) (Deeken 2009; Sissung et al. 2010). The individuals genotyped in this manner were selected based on having the most complete datasets for exposure parameters and ChE inhibition. DNA was isolated from buffy coat samples utilizing the Gentra Puregene DNA Isolation Kit (Qiagen) with a few modifications to the protocol. Briefly, buffy coat samples were lysed and incubated overnight with 50 µg proteinase K (Qiagen) to maximize cell lysis. The following day, samples were treated with 10 µg RNAse A (Qiagen) and DNA purification proceeded according to the manufacturer's specifications. DNA concentration and relative purity were measured with the Nanodrop D-1000 (Thermo Scientific) and the concentration was validated with the Picogreen-based Quant-iT Broad Range assay (Invitrogen).

Subjects were genotyped using 1 µg of DNA with the DMET Plus assay according to the manufacturer's instructions. After PCR amplification, the samples were fragmented, labeled, and hybridized to the DMET Plus arrays. The arrays were washed and stained at a GeneChip Fluidics Station 450 (Affymetrix) and scanned with a GeneChip Scanner 3000 7G (Affymetrix). Sample files (.cel and .arr) were generated with Affymetrix GeneChip Command Console

(AGCC). These files were then imported into DMET Console version 1.1 (Affymetrix) to evaluate call rates and to perform genotyping using the dynamic clustering algorithm with default settings. Genotypes generated were at the polymorphism level and translated genotype level (star nomenclature). All 70 individuals had successfully called genotypes for the $PON1_{55}$ polymorphism and 68 individuals were successfully genotyped for both the $PON1_{55}$ and $PON1_{192}$ polymorphisms.

Data Analysis

Parameters for the joint distributions of dimethyl DAP metabolites were estimated for a multivariate normal model using a Bayesian Markov chain Monte Carlo (MC-MC) method known as Gibbs sampling as described in Chapter 2. Differences in ChE inhibition between participants stratified by PON1 genotype and phenotype were determined by analysis of variance with post-hoc t-tests of each combination of $PON1_{192}$ genotype and AREase activity phenotype compared to the RR and high AREase group as the referent. Linear regression analyses were performed to determine whether slopes between ChE inhibition and AZ blood concentration or dimethyl metabolites concentration in urine were significantly altered when stratified by AREase activity or $PON1_{192}$ genotypes. Differences in ChE activity by these genotypes or phenotype were tested using ANOVA to determine whether the stratification with a separate slope for each genotype or phenotype significantly improved the fit compared to using a single slope. In the case of a significant ANOVA test ($p < 0.05$), post-hoc testing was used to determine which groups significantly differed from each other. A Bonferroni correction was used to adjust the post-hoc p-value for multiple testing. All statistical analyses were performed using R statistical software (http://www.r-project.org/).

Results:

To evaluate the impact PON1 variation in a population of agricultural workers largely exposed to azinphos methyl and other dimethyl OP pesticides (Chapter 2), we characterized the genotypes and phenotypes for PON1 in this population. Using plasma samples with a two substrate assays it was possible to determine PON1 status, including the $PON1_{192}$ genotype and plasma activity level (Fig 3-1). In this assay, the PON1 catalytic activities towards diazoxon and paraoxon were used to discriminate between the groups. A clear resolution with no overlap between the genotype groups was observed. The allele frequencies for the $PON1_{192}$ and $PON1_{55}$ positions were 192R = 0.51, 192Q = 0.49, 55L = 0.84, and 55M = 0.16. In Fig 3-1 the position 55 genotypes are represented by solid shapes. Due to the position 55 polymorphism not being associated with AREase activity (t-test between L/M and L/L groups, $p = 0.48$), further analyses of PON1 status only considered the variation at position-192 and AREase activity.

Table 3-1 lists the distributions of $PON1_{192}$ genotype and AREase, POase, and DZOase activity for farmworkers and non-farmworkers. ANOVA analyses showed significant differences in AREase activity between farmworkers and non-farmworkers ($p < 0.0001$) and in AREase ($p = 0.016$), POase ($p < 0.001$), and DZOase ($p < 0.001$) activity across $PON1_{192}$ genotypes. Post-hoc testing showed that mean AREase activity for non-farmworkers was significantly greater than farmworker values in each of the genotype groups. In addition, mean AREase activity decreased with increasing number of $PON1_{192R}$ alleles. Paraoxonase- and diazoxonase-specific activity differed significantly based on $PON1_{192}$ genotype, in accordance with the genotype separation observed in Fig 3-1.

Cholinesterase inhibition in relation to AREase activity and $PON1_{192}$ genotype were determined for AChE (Table 3-2) and BuChE (Table 3-3). AREase activity was separated into

three groups, low (< 91 Units [U]/ml), mid (91-112 U/ml) and high (>112 U/ml), such that approximately equal numbers of individuals fell into each group when evaluating ChE inhibition. For both enzymes, levels of inhibition for groups defined by PON1 genotype and phenotype were evaluated using t-tests with the reference group being the $PON1_{192}$ RR genotype/high AREase activity group. No significant differences in inhibition of either AChE (ANOVA, $p = 0.70$) or BuChE (ANOVA, $p = 0.72$) were observed based on AREase activity and $PON1_{192}$ genotype. Linear regression analyses were performed between ChE activity and AREase activity, modeled as a continuous variable, with stratification by $PON1_{192}$ genotype (Fig 3-2). Slopes for linear regressions between percent AChE activity vs. AREase activity were -0.198 %/U/ml (95% CI: -0.406, 0.010), 0.111 %/U/ml (95% CI: -0.011, 0.233), and -0.011 %/U/ml (95% CI: -0.083, 0.062) for R/R, Q/R, and Q/Q individuals, respectively. For BuChE, the slopes for R/R, Q/R, and Q/Q individuals were -0.187 %/U/ml (95% CI: -0.645, 0.271), 0.149 %/U/ml (95% CI: -0.154, 0.452), and 0.035 %/U/ml (95% CI: -0.164, 0.235), respectively. None of these slopes were significantly different from zero and no significant differences were observed based on $PON1_{192}$ genotype (ANOVA, $p > 0.05$).

Linear regression analyses between ChE activity and AZ were stratified by PON1 genotype or phenotype (Table 3-4) to determine if PON1 status affected these relationships. As was previously observed, AChE and BuChE inhibition were associated with AZ levels in blood without stratification by PON1 status: activity as percent of off season changed by -2.7 %/ng AZ/g serum (95% CI: -4.2, -1.2). Stratification by $PON1_{192}$ genotype showed that AChE activity was altered by -2.1 %/ng AZ/g serum (95% CI: -5.4, 1.3), -2.7 %/ng/g (95% CI: -4.6, -0.8), and -3.6 %/ng/g (95% CI: -7.4, 0.2) for Q/Q, Q/R, and R/R individuals respectively. When stratifying by AREase activity, the slopes were -4.7 % relative to off season/ng AZ/g serum (95% CI: -7.1, -

2.2) for those with low activity, -0.4 %/ng/g (95% CI: -2.9, 2.1) for those with mid activity, and -2.9 %/ng/g (95% CI: -5.6, -0.2) for those with high activity. No significant differences in the relationship between AChE inhibition and AZ were observed based on $PON1_{192}$ genotype or AREase activity (ANOVA, $p > 0.05$).

BuChE inhibition was also associated with AZ exposure (Table 3-4, Fig 3-3), with BuChE activity as percent of off season changed by -3.8 %/ng AZ/g serum (95% CI: -7.2, -0.5). $PON1_{192}$ genotype did not affect the relationship between BuChE inhibition and AZ exposure (ANOVA, $p = 0.583$): the slopes for Q/Q, Q/R, and R/R individuals were -6.0 %/ng AZ/g serum (95% CI: -13.6, 1.5), -2.5 %/ng/g (95% CI: -6.8, 1.7), and -6.4 %/ng/g (95% CI: -14.9, 2.2), respectively. However, significant modification of the BuChE inhibition/AZ relationship by AREase activity was observed (ANOVA, $p = 0.022$). The difference between AREase groups can be seen in Figure 3-3. The slope for the low AREase group (Fig 3-3B) was -9.4 %/ng/g (95% CI: -14.9, -4.0), that for the mid group (Fig 3-3C) was 1.5 %/ng/g (95% CI: -4.1, 7.1), and the value for the high group (Fig 3-3D) was by -3.1 %/ng/g (95% CI: -9.3, 3.0). Post-hoc analysis showed significant differences ($p = 0.019$) in BuChE inhibition with AZ exposure between the low and mid AREase groups, whereas the high AREase group was not different from either group.

We have also observed significant relationships between ChE inhibition and levels of dimethyl-substituted urinary metabolites (Chapter 2). We examined the impact of PON1 status on the relationship between ChE inhibition and total dimethyl metabolites, DMP, DMTP, and DMDTP. For inhibition of AChE, significant increases were observed with levels of total dimethyls, DMP, and DMTP in the urine (slope $p < 0.05$) (Table 3-5). No impacts on these relationships were observed based on $PON1_{192}$ genotype or AREase activity (ANOVA, $p >$

0.05). For inhibition of BuChE in relation to the urinary metabolites (Table 3-6), significant associations were observed for total dimethyls, DMTP, and DMDTP (slope $p < 0.05$). No modification by $PON1_{192}$ genotype was observed but significant impacts by AREase activity were observed for DMTP and total dimethyls (ANOVA, $p = 0.019$ and 0.028, respectively). Similar to the observations for BuChE inhibition and AZ, post-hoc analysis showed that for both total dimethyls and DMTP the low and mid AREase groups had significantly different slopes ($p < 0.05$). However, the high AREase group did not differ from either the low or mid group.

Discussion:

This research continues our examination of how exposures to OP pesticides affect the health of farmworkers living in the Yakima Valley agricultural region of Washington State. In Chapter 2, we reported associations between levels of ChE inhibition and concentrations of AZ and urinary OP pesticide metabolites. We genotyped study participants using a two-substrate assay and determined PON1 phenotype by monitoring the hydrolysis of phenyl acetate (Fig. 3-1) (Richter et al. 2009). As expected for independent regulation of each allele, a greater variability was seen in the heterozygous Q/R group compared to the two homozygous groups. $PON1_{192}$ allele frequencies were R = 0.51 and Q = 0.49. These values are similar to other published data for Hispanic or Mexican populations as other researchers have observed frequencies of the 192R allele between 0.41-0.54 (Davies et al. 1996; Richter and Furlong 1999; Rojas-Garcia et al. 2005; Holland et al. 2006; Perez-Herrera et al. 2008; Huen et al. 2010).

We observed significant differences in AREase activity based on farmworker status ($p < 0.001$) and $PON1_{192}$ genotype ($p = 0.016$), with AREase activity being lower for farmworkers than non-farmworkers and increasing with the number of 192Q alleles (Table 3-1). In the

CHAMACOS study, higher AREase activity in women with the 192Q/Q genotype was observed during pregnancy, birth, and when the child was an infant but not seven years later (Huen et al. 2010; Harley et al. 2011). Brophy et al. (2001) observed higher AREase activity with increasing number of 192Q alleles in a population of white individuals. In humanized mice, AREase activity in $hPON1_{192Q}$ mice was greater than that of $hPON1_{192R}$ mice (Jansen et al. 2009). The opposite trend, with AREase activity and the number of $PON1_{192R}$ alleles being positively associated, was observed in a population of Mexican farmworkers (Perez-Herrera et al. 2008). Still others have found no association between $PON1_{192}$ genotype and AREase activity (Davies et al. 1996; Furlong et al. 2006; Holland et al. 2006). These observation may be due to the position 192 polymorphism being in linkage disequilibrium with other loci in the promoter region of the PON1 gene in some populations (Brophy et al. 2001) but is not currently understood. With regards to the difference in AREase activity between farmworkers and non-farmworkers, Singh et al. (2011) observed lower AREase activity in a group of pesticide sprayers compared to controls, although adjustment for age, BMI, smoking, duration of exposure, and alcohol use accounted for this difference. The difference in AREase activity between farmworkers and non-farmworkers in the current study may be explained by similar factors or others as yet unidentified and warrants further investigation.

For the work presented here, we sought to replicate and expand on the observations of Hofmann et al. (2009) who found significant differences in BuChE inhibition in pesticide handlers based on PON1 status. Information gleaned from surveys showed that the individuals in the Hofmann study handled CP, carbaryl, and AZ most frequently. Their analyses showed that $PON1_{192}$ genotype significantly affected BuChE inhibition. Furthermore, those with the Q/Q $PON1_{192}$ genotype and low PON1 activity, as represented by AREase activity, were at

significantly higher risk of having greater than 20% BuChE inhibition. These observations lend support to the utility of PON1 status as a marker of susceptibility for persons exposed to certain OP pesticides and their oxons.

In order to validate *in vivo* findings of AZ not being a substrate for PON1 (Costa et al. 1999), we tested the hypothesis that PON1 status altered the relationships observed between ChE inhibition and markers of exposure in this population of agricultural workers. AChE and BuChE activity represented as percent of the off season value were stratified by $PON1_{192}$ genotype (Table 3-3) and AREase activity (Table 3-4). No differences in ChE activity were observed based on PON1 genotype or phenotype, either individually or in combination. We then compared ChE activity to AREase activity, modeled as a continuous variable, for each $PON1_{192}$ genotype group (Fig 3-2). No significant association between AREase activity and ChE inhibition and no modification by PON1 genotype was observed. Finally, we examined the impact of PON1 status on the associations between ChE inhibition and markers of OP pesticide exposure that we observed in Chapter 2. We found no differences in AChE inhibition per unit exposure biomarker by $PON1_{192}$ genotype or AREase activity (Tables 3-5 and 3-6). Additionally, we did not observe any modification of the BuChE inhibition/exposure biomarker relationship by $PON1_{192}$ genotype. However, significant modification of the BuChE inhibition with levels of AZ (Fig 3-3), total dimethyls, and DMTP (Table 3-6) by AREase activity, and post-hoc testing showed that the low activity group had significantly different regression slopes than the mid group for each of these comparisons.

Closer examination of these results showed that two individuals were potential outliers driving the ANOVA results: one with 4.99 ng AZ/g blood and 13% BuChE activity relative to the off season and the other with 6.19 ng AZ/g blood and 127% BuChE activity relative to the

off season. Both of these individuals are plotted in Figure 3-3, with the former falling in the mid AREase group (Fig 3-3C) and the latter in the low AREase group (Fig 3-3D). When these two individuals were removed from the regression analysis, AREase activity no longer modified the BuChE inhibition/AZ relationship (ANOVA, p-value = 0.059). When this process was repeated for DMTP with two potential outliers, the BuChE inhibition/AZ relationship was no longer modified by AREase activity after their removal (ANOVA, p-value = 0.283). These observations, combined with the fact that AREase activity did not impact BuChE inhibition in an activity-dependent manner, supports the lack of ability for PON1 to detoxify AZ and affect ChE inhibition after exposures occur.

These observations support the *in vivo* data generated with PON1 -/- mice exposed to AZ (Costa et al. 1999) and is the first study demonstrating such a lack of an effect in humans. Other epidemiological studies have demonstrated an effect of PON1 status on cholinesterase inhibition (Akgur et al. 1999; Sirivarasai et al. 2007; Hofmann et al. 2009). Others have found differences in oxidative stress or DNA damage by PON1 status after exposure to OPs (Lee et al. 2007; Perez-Herrera et al. 2008; Rojas-García et al. 2009; Satyender Singh et al. 2011a). In most of these cases, the OP pesticides to which the human populations were exposed to consisted of known PON1 substrates, including CP. Therefore the utility of PON1 status as a marker of susceptibility for OP pesticides is contingent on having accurate exposure assessments consisting of the identity of individual OP pesticides to which people are exposed.

For AZ and other OP pesticides not detoxified by PON1, carboxylesterases (CESs) likely play an important role in mediating their toxicity. A group of β-esterases like AChE, CES enzymes are expressed in the human liver, kidney, and blood tissues and can act on OP pesticides by hydrolyzing them or by reducing their availability to interact with AChE through

reversibly binding in the active site (Jokanovic 2009). Pre-or co-treatment of animals with CES inhibitors increased the toxicity of administered OP pesticides (Dettbarn et al. 1999; Tang and Chambers 1999; Barata et al. 2004; Jansen et al. 2009). The ability of hepatic CESs to detoxify malathion or its oxon has been demonstrated (Buratti and Testai 2006; Moser and Padilla 2011). To our knowledge, no research has been performed regarding the ability of mammalian CES enzymes to metabolize AZ. However, resistance to AZ in insect species has been associated with higher CES activity (Anguiano et al. 2008; Soleño et al. 2008) and may therefore be involved in the detoxification of AZ in humans. Characterizations of human CES enzymes have provided measures of genetic (Marsh et al. 2004) and phenotypic variability (Hosokawa et al. 1995; Jewell et al. 2007) across individuals and populations. The impact of this variability on the metabolism of OP pesticides should be characterized to determine if CES enzymes can modify their toxicity in humans and whether they could be used as markers of susceptibility for OP pesticides that are not substrates for PON1.

In order for genetic or phenotypic information to be of value as markers of susceptibility, accurate exposure assessments need to be performed to determine which OP pesticides contribute to the exposures. This can be accomplished by measuring the amounts of compound in blood tissues or metabolites in urine. Since the dialkyl phosphate metabolites don't provide information on the specific OP pesticide to which an individual was exposed, their utility will not be as great as that for the specific OP pesticide metabolites. Exposure assessment will be particularly important in cases where exposures to multiple OP pesticides occur: CP oxon, diazoxon, and paraoxon are capable of lowering the activity of CES enzymes such that the toxicity of subsequently administered malaoxon was increased in mice (Jansen et al. 2009). In particular, PON1 -/- mice given pretreatment with CP oxon or diazoxon had significantly greater

AChE inhibition after malaoxon administration compared to PON +/+ likely due to the lack of active or available CES to interact with the malaoxon. This suggests that the toxicity of an OP pesticide that is not a substrate for PON1 can be affected by PON1 genotype when exposures to PON1 substrates occur.

Further monitoring of human populations exposed to OP pesticides should aim to accurately assess the individual OPs to which people are exposed. In our studies, we have evaluated exposure by measuring the levels of parent compound in the blood or amounts of metabolites excreted in the urine. Quantifying exposures using such biomonitoring is more accurate than relying on occupational status and is less likely to result in exposure misclassification (Watson and Mutti 2004). The value of genotypic and phenotypic information will increase when combined with this type of quantitative exposure assessment since researchers will be able to conduct dose-response relationships incorporating key metabolic information. Genotyping and phenotyping assays will continue to elucidate the mechanisms by which OP pesticides contribute to cholinesterase inhibition and other health impacts in exposed human populations.

Figure 3-1 Inference of Paraoxonase-1 Status by a Two-Substrate Assay

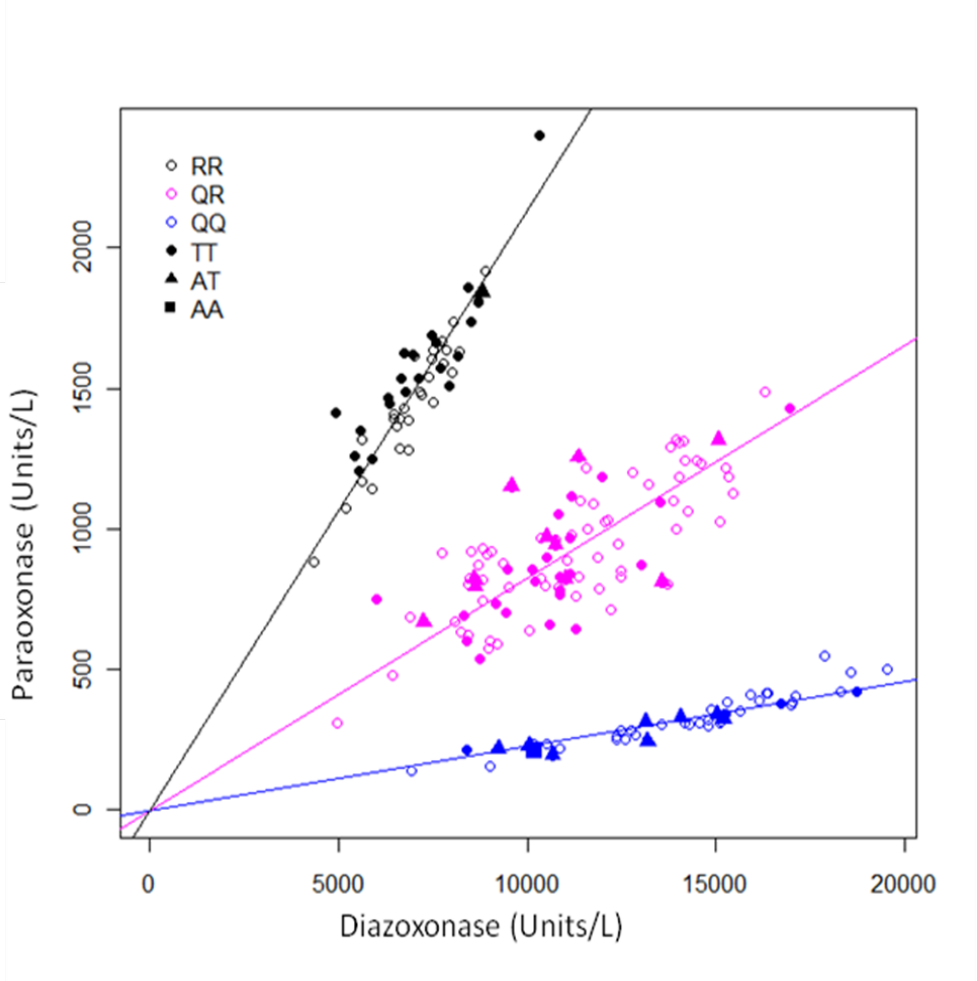


Figure 3-1. A two-substrate assay infers PON1 status. Plasma samples were used to determine the diazoxonase and paraoxonase-specific activities. The samples resolved according to the position 192 polymorphisms and serum protein abundance. The $PON1_{55}$ polymorphisms are represented by filled-in symbols and were characterized with the Affymetrix DMET Plus genotyping arrays as described in the Methods section.

Figure 3-2 Cholinesterase Inhibition in relation to Paraoxonase-1 Phenotype and Genotype

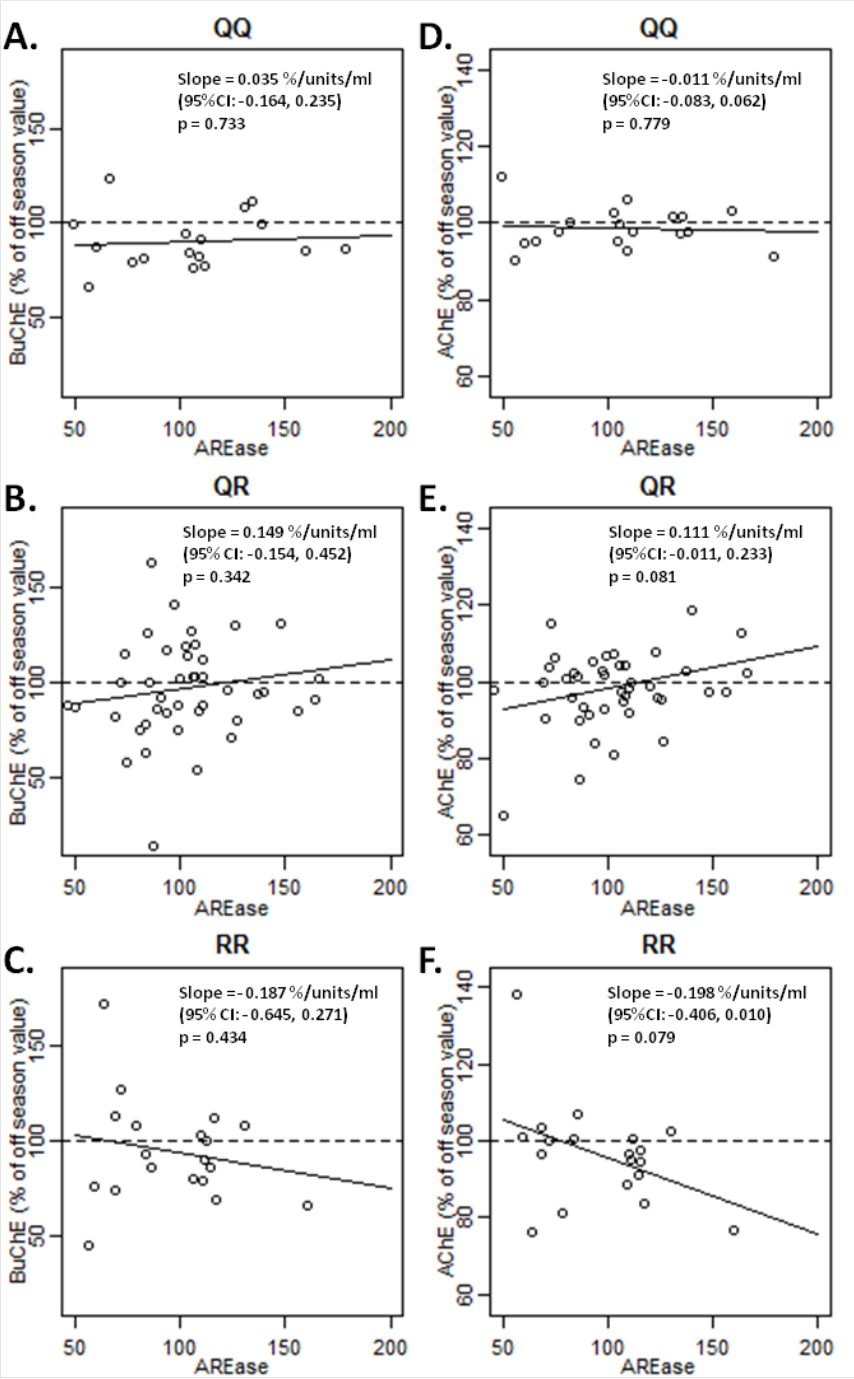


Figure 3-2 BuChE (A-C) and AChE (D-F) inhibition in relation to AREase activity and position 192 genotype. Linear regression analysis found no statistically significant differences in the relationship between ChE inhibition and AREase activity by $PON1_{192}$ genotypes (ANOVA, $p < 0.05$).

Figure 3-3 Effect of Paraoxonase-1 Phenotype on BuChE inhibition After Exposure to Azinphos Methyl

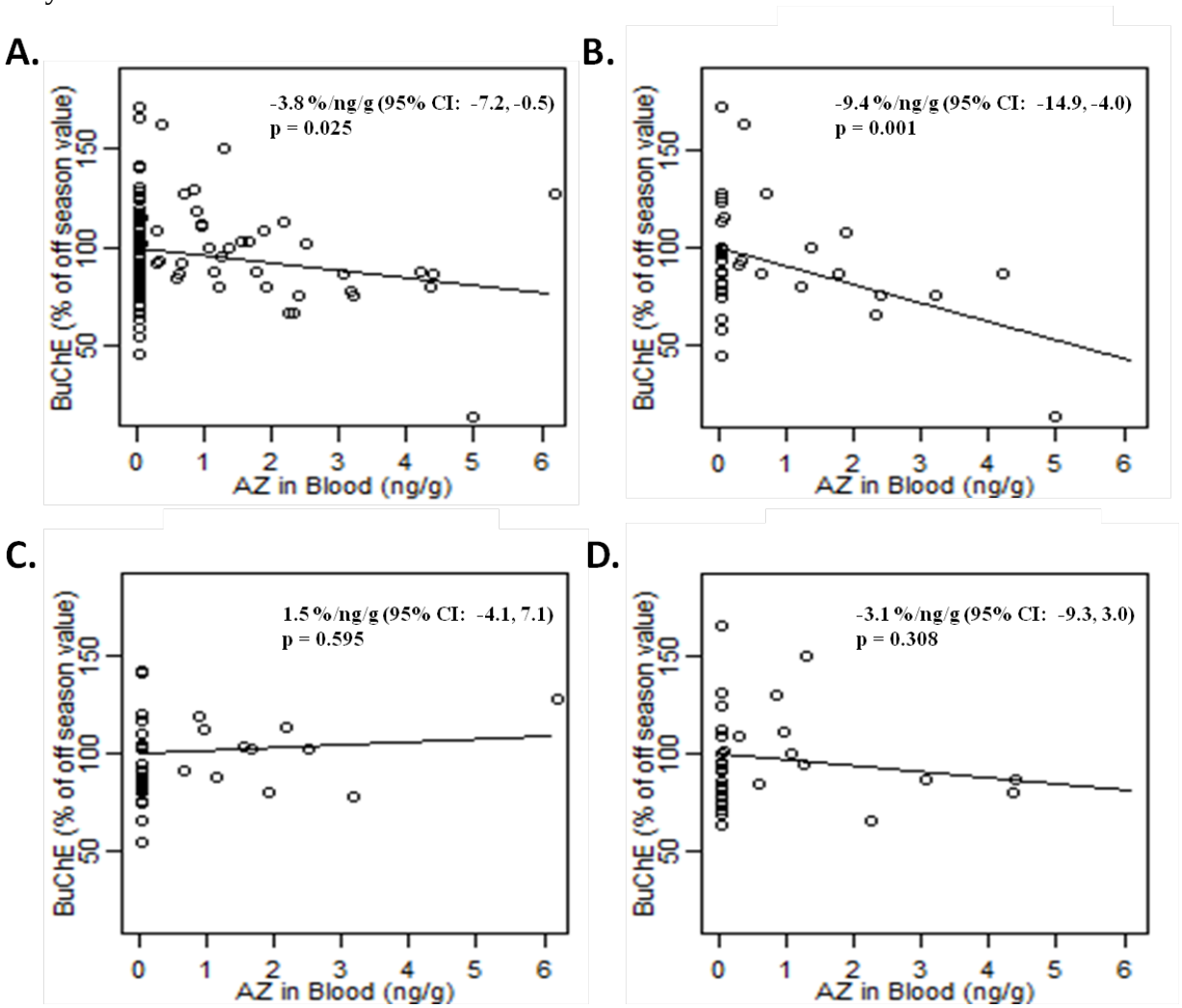


Figure 3-3. Effect of PON1 Phenotype on BuChE activity/AZ relationship. BuChE activity during the thinning season, as a percentage of the off season baseline value, was regressed against azinphos methyl (AZ) in blood samples for A.) all PON1 phenotypes, B.) low AREase, C.) mid AREase, and D.) high AREase. Significant non-zero slopes were seen for all phenotypes combined (A.) and low AREase (B.) and a significant effect by AREase activity across the groups was observed (ANOVA, p = 0.022).

Table 3-1

			Plasma Enzyme Activity[1]					
			AREase (U/ml)*#		DZOase (U/L)**		POase (U/L)**	
Occupational Status	$PON1_{192}$ Genotype	N	Mean	(St Dev)	Mean	(St Dev)	Mean	(St Dev)
Farmworker	QQ	21	106.9	(35.4)	13833.8	(2949.5)	310.9	(88.3)
	QR	46	103.0	(26.6)	11038.9	(2486.4)	887.8	(249.5)
	RR	25	93.6	(27.2)	7009.5	(1262.8)	1516.2	(257.2)
	All	92	101.4	(29.1)	10581.9	(3382.5)	926.8	(483.4)
Non-Farmworker	QQ	26	128.5	(36.6)	14034.4	(3137.3)	319.9	(94.9)
	QR	44	126.8	(29.3)	11059.2	(2448.4)	953.4	(209.0)
	RR	24	111.0	(25.1)	7172.1	(1116.7)	1520.1	(245.1)
	All	94	123.2	(31.1)	10889.7	(3474.3)	922.9	(481.3)
All	QQ	47	118.8	(37.3)	13944.8	(3023.5)	315.9	(91.1)
	QR	90	114.6	(30.3)	11048.8	(2454.0)	919.8	(231.6)
	RR	49	102.1	(27.4)	7089.2	(1183.9)	1518.1	(248.8)
	All	186	112.4	(32.0)	10737.5	(3423.4)	924.8	(481.0)

1. Plasma enzyme activity was evaluated by monitoring the metabolism of phenyl acetate (AREase), diazoxon (DZOase), and paraoxon (POase) as described in the methods.

ANOVA tests were used to determine significant differences based on genotype (* $p < 0.05$, ** $p < 0.001$) or occupational status (# $p < 0.001$).

Table 3-2 Mean (SD) AChE Activity as Percentage of Off Season in Farmworkers by PON1 Genotype and Phenotype

$PON1_{192}$ genotype	AREase Activity*		
	High	Mid	Low
RR	n = 7	n = 3	n = 9
	92.4 (4.2)	93.5 (6.3)	100.5 (3.7)
	reference	p = 0.89	p = 0.15
QR	n = 11	n = 17	n = 16
	101.2 (3.3)	100.3 (2.7)	95.2 (2.7)
	p = 0.10	p = 0.12	p = 0.57
QQ	n = 6	n = 6	n = 6
	98.8 (4.5)	99.1 (4.5)	98.4 (4.5)
	p = 0.30	p = 0.28	p = 0.33

No significant differences observed by PON1 genotype or phenotype by ANOVA (p = 0.70). *Based on AREase activity: high: > 112 U/ml, mid: 91-112 U/ml, low: < 91 U/ml. P values show the result of a t-test performed for each cell compared to the reference cell (RR genotype and high PON1 activity).

Table 3-3 Mean (SD) BuChE Activity as Percentage of Off Season in Farmworkers by PON1 Genotype and Phenotype

$PON1_{192}$ genotype	AREase Activity*		
	High	Mid	Low
RR	n = 6	n = 4	n = 9
	90.4 (10.3)	88.1 (12.6)	99.3 (8.4)
	reference	p = 0.89	p = 0.50
QR	n = 11	n = 17	n = 15
	101.9 (7.6)	101.8 (6.1)	88.2 (6.5)
	p = 0.37	p = 0.35	p = 0.86
QQ	n = 5	n = 6	n = 6
	98.2 (11.3)	84.3 (10.3)	89.4 (10.3)
	p = 0.61	p = 0.68	p = 0.95

No significant difference by observed by PON1 genotype or phenotype by ANOVA (p = 0.72). *Based on AREase activity: high: > 112 U/ml, mid: 91-112 U/ml, low: < 91 U/ml. P values show the result of a t-test performed for each cell compared to the reference cell (RR genotype and high PON1 activity).

Table 3-4 Cholinesterase Activity as Percentage of Off Season in Relation to AZ Exposure Stratified by PON1 Genotype and Phenotype

Enzyme	Stratified by	N	Slope (%/ng/g serum)	95% CI	slope p-value	ANOVA p-value
AChE	All	108	-2.7	(-4.2, -1.2)	<0.001	
	Genotype					0.826
	Q/Q	25	-2.1	(-5.4, 1.3)	0.219	
	Q/R	57	-2.7	(-4.6, -0.8)	0.005	
	R/R	26	-3.6	(-7.4, 0.2)	0.059	
	Phenotype					0.054
	Low AREase	36	-4.7	(-7.1, -2.2)	<0.001	
	Mid AREase	36	-0.4	(-2.9, 2.1)	0.732	
	High AREase	36	-2.9	(-5.6, -0.2)	0.035	
BuChE	All	106	-3.8	(-7.2, -0.5)	0.025	
	Genotype					0.583
	Q/Q	25	-6	(-13.6, 1.5)	0.112	
	Q/R	56	-2.5	(-6.8, 1.7)	0.240	
	R/R	25	-6.4	(-14.9, 2.2)	0.138	
	Phenotype					0.022
	Low AREase	35	-9.4	(-14.9, -4)	0.001	*
	Mid AREase	38	1.5	(-4.1, 7.1)	0.595	*
	High AREase	33	-3.1	(-9.3, 3)	0.308	

*: Post-hoc testing showed significant differences between the Low and Mid AREase groups (p = 0.019)

Table 3-5 AChE as Percentage of Off Season in Relation to Dimethyl DAP Urinary Metabolites Stratified by PON1 Genotype and Phenotype

Urinary Metabolite	Stratified by	N	Slope (%/µM/L)	95% CI	slope p-value	ANOVA p-value
Total dimethyls	All	109	-2.2	(-3.8, -0.6)	0.008	
	Genotype					0.701
	Q/Q	26	-1.0	(-4.7, 2.7)	0.590	
	Q/R	57	-2.3	(-4.3, -0.4)	0.015	
	R/R	26	-3.6	(-9.3, 2.0)	0.197	
	Phenotype					0.190
	Low AREase	36	-3.9	(-6.5, -1.2)	0.004	
	Mid AREase	36	-0.7	(-3.0, 1.7)	0.573	
	High AREase	37	-2.7	(-6.8, 1.3)	0.175	
DMP	All	109	-15.2	(-27.1, -3.2)	0.013	
	Genotype					0.668
	Q/Q	26	-5.5	(-31.2, 20.2)	0.668	
	Q/R	57	-17.1	(-31.7, -2.5)	0.021	
	R/R	26	-22.9	(-59.9, 14.1)	0.218	
	Phenotype					0.150
	Low AREase	36	-30.6	(-52.8, -8.3)	0.007	
	Mid AREase	36	-4.1	(-20.8, 12.7)	0.629	
	High AREase	37	-20.6	(-46.2, 4.9)	0.109	
DMTP	All	109	-2.8	(-4.8, -0.8)	0.006	
	Genotype					0.759
	Q/Q	26	-1.5	(-6.3, 3.4)	0.544	
	Q/R	57	-3	(-5.3, -0.6)	0.013	
	R/R	26	-4.6	(-11.8, 2.6)	0.207	
	Phenotype					0.193
	Low AREase	36	-4.8	(-8.0, -1.7)	0.003	
	Mid AREase	36	-0.9	(-3.9, 2.1)	0.560	
	High AREase	37	-3.3	(-8.4, 1.8)	0.200	
DMDTP	All	109	-12.1	(-30.3, 6.0)	0.184	
	Genotype					0.488
	Q/Q	26	-1.7	(-29.0, 25.5)	0.901	
	Q/R	57	-17.1	(-43.8, 9.6)	0.204	
	R/R	26	-37.8	(-98.0, 22.5)	0.213	
	Phenotype					0.748
	Low AREase	36	-12.1	(-39.0, 14.7)	0.368	
	Mid AREase	36	-6.4	(-35.4, 22.5)	0.658	
	High AREase	37	-28	(-76.6, 20.6)	0.251	

Table 3-6 BuChE as Percentage of Off Season in Relation to Dimethyl DAP Urinary Metabolites Stratified by PON1 Genotype and Phenotype

Urinary Metabolite	Stratified by	N	Slope (%/μM/L)	95% CI	slope p-value	ANOVA p-value
Total dimethyls	All	107	-4.9	(-8.4, -1.3)	0.007	
	Genotype					0.324
	Q/Q	26	-6.6	(-14.7, 1.6)	0.109	
	Q/R	56	-3.4	(-7.6, 0.7)	0.103	
	R/R	25	-12.4	(-24.1, -0.6)	0.037	
	Phenotype					0.028
	Low AREase	35	-10.9	(-16.6, -5.2)	<0.001	*
	Mid AREase	38	-0.7	(-5.7, 4.3)	0.777	*
	High AREase	34	-3.1	(-11.8, 5.7)	0.482	
DMP	All	107	-25.8	(-52.4, 0.8)	0.055	
	Genotype					0.301
	Q/Q	26	-33.4	(-90.5, 23.7)	0.245	
	Q/R	56	-14.1	(-46.6, 18.4)	0.387	
	R/R	25	-78.9	(-157.0, -0.8)	0.046	
	Phenotype					0.250
	Low AREase	35	-60.7	(-111.4, -10.1)	0.018	
	Mid AREase	38	-8.3	(-45.3, 28.7)	0.656	
	High AREase	34	-23.1	(-80.8, 34.5)	0.424	
DMTP	All	107	-6.2	(-10.6, -1.8)	0.006	
	Genotype					0.319
	Q/Q	26	-9.3	(-20.0, 1.5)	0.087	
	Q/R	56	-4.4	(-9.6, 0.7)	0.087	
	R/R	25	-15.5	(-30.5, -0.4)	0.043	
	Phenotype					0.019
	Low AREase	35	-13.7	(-20.6, -6.9)	<0.001	*
	Mid AREase	38	-0.6	(-6.9, 5.8)	0.861	*
	High AREase	34	-3.5	(-14.7, 7.6)	0.531	
DMDTP	All	107	-48.5	(-87.4, -9.5)	0.014	
	Genotype					0.304
	Q/Q	26	-29.2	(-87.6, 29.2)	0.320	
	Q/R	56	-48.5	(-106.0,9.0)	0.094	
	R/R	25	-135.7	(-260.1,-11.4)	0.031	
	Phenotype					0.677
	Low AREase	35	-65.9	(-123.9, -7.9)	0.025	
	Mid AREase	38	-28.4	(-90.2, 33.3)	0.360	
	High AREase	34	-49.4	(-154.0, 55.2)	0.348	

*: Post-hoc testing showed significant differences between the Low and Mid AREase groups ($p < 0.05$)

CHAPTER 4: CYP450 Genotype Modifies Cholinesterase Inhibition in Farmworkers Occupationally Exposed to Organophosphate Pesticides

Introduction:

For many xenobiotics to be toxicologically active, they must first be metabolized in the body to produce a reactive intermediate. In the case of OP pesticides, much of this metabolism is mediated by the cytochrome P-450 (CYP) family of enzymes. A number of CYPs catalyze the desulfuration reaction that activates the parent OP pesticide compound to the oxon form (Buratti et al. 2002). The CYPs are also capable of facilitating a dearylation of the parent compound, producing the nontoxic dialkyl phosphates (DAPs) and specific metabolites that are excreted into the urine (Hodgson 2003). The major CYP enzymes catalyzing these reactions include CYP1A2, CYP2B6, CYP2C19, CYP2D6, and CYP3A4 (Buratti et al. 2003; Sams et al. 2004; Mutch and Williams 2006) (See Appendix 1 for a review of the *in vitro* literature). Given the central role of CYPs in the metabolism of OP pesticides, an understanding of these processes is essential for estimating risk resulting from exposures in human populations.

The genes encoding the CYPs are highly polymorphic and this genetic variation can translate to differences in phenotype for a given enzyme (Zhou et al. 2009). These differences in phenotype can impact how OP pesticides are metabolized by CYPs *in vitro* (Dai et al. 2001; Tang et al. 2001). Genetic variation in CYPs can lead to different levels of oxon activation and ultimately the amount of ChE inhibition resulting from this metabolism, variant genotypes for CYPs can potentially confer sensitivity to individuals exposed to OP compounds and make them more susceptible to OP pesticide-induced health impacts.

Few data are available regarding the impact of CYP genetic variability on ChE inhibition. To that end, we tested whether the associations between exposure biomarkers and ChE inhibition discussed in Chapter 2 differed based on the genotypes for several CYPs identified from the *in*

vitro OP pesticide metabolism literature. The impact of genetic variation in glutathione S-transferase (GSTs) was also evaluated in this manner. While there is little evidence to suggest a role as markers of susceptibility for OP pesticides (Eaton 2005), more recently conjugates of OP pesticides have been produced with GSTs *in vitro* (Fujioka and Casida 2007) so we included the GSTs in our analysis to determine if they modified ChE inhibition in our study population. We utilized a multiplexed genotyping platform, namely the Affymetrix Drug Metabolizing Enzyme and Transporter (DMET) arrays (Deeken 2009; Sissung et al. 2010) to interrogate the genetic variants present in our study population and determined whether those individuals with functional variant forms of the CYPs had differing levels of ChE inhibition.

Methods:

Sample Collection

Urine, venous blood, and fingerstick samples were collected and concentrations of urinary metabolites and parent compound in blood and ChE inhibition were determined as described in Chapter 2

Genotyping of Metabolic Enzymes

We genotyped 70 participants, 52 farmworkers and 18 non-farmworkers, for variation in a number of CYP genes, including CYP1A1, 1A2, 1B1, 2B6, 3D6, 2C8, 2C9, 2C19, 3A4, 3A5, and 3A7. We also determined the genotypes for glutathione S-transferase (GST) M1, P1, and T1. The individuals genotyped in this manner were selected based on having the most complete datasets for exposure parameters and ChE inhibition. DNA was isolated from buffy coat samples utilizing the Gentra Puregene DNA Isolation Kit (Qiagen) with a few modifications to

the protocol. Briefly, buffy coat samples were lysed and incubated overnight with 50 µg proteinase K (Qiagen) to maximize cell lysis. The following day, samples were treated with 10 µg RNAse H (Qiagen) and DNA purification proceeded according to the manufacturer's specifications. DNA concentration and relative purity were measured with the Nanodrop D-1000 (Thermo Scientific) and the concentration was validated with the Picogreen-based Quant-iT Broad Range assay (Invitrogen).

Subjects were genotyped using 1 µg of DNA with the DMET Plus assay according to the manufacturer's instructions. After PCR amplification, the samples were fragmented, labeled, and hybridized to the DMET Plus arrays. The arrays were washed and stained at a GeneChip Fluidics Station 450 (Affymetrix) and scanned with a GeneChip Scanner 3000 7G (Affymetrix). Sample files (.cel and .arr) were generated with Affymetrix GeneChip Command Console (AGCC). These files were then imported into DMET Console version 1.1 (Affymetrix) to evaluate call rates and to perform genotyping using the dynamic clustering algorithm with default settings. CYP genotypes were then converted to the star nomenclature using the genotype translation algorithm. In the case of CYP3A5, the *3C, *6, and *7 alleles produce proteins with decreased to no activity. As such, genotypes for CYP3A5 were separated into groupings of *1A/*1A, *1A/*3C and *3C/*3C as well as *1A/*1A, *1A/*3C, and *3C/X, where X = *3, *6, or *7 alleles to examine the impacts of reduced activity for this CYP.

Data Analysis

Parameters for the joint distributions of dimethyl DAP metabolites were estimated for a multivariate normal model using a Bayesian Markov chain Monte Carlo (MC-MC) method known as Gibbs sampling as described in Chapter 2. Linear regression analyses were performed to determine whether slopes between ChE inhibition and AZ blood concentration or dimethyl

metabolites concentration in urine were significantly altered when stratified by enzyme genotype. Differences in ChE activity by genotypes for a given enzyme were tested using ANOVA to determine whether the stratification with a separate slope for each genotype significantly improved the fit compared to using a single slope. In the case of a significant ANOVA test ($p < 0.10$), post-hoc testing was used to determine which groups significantly differed from each other. A Bonferroni correction was used to adjust the post-hoc p-value for multiple testing. Significant differences were noted where corrected post-hoc p-value < 0.05. All statistical analyses were performed using R statistical software (http://www.r-project.org/).

Results:

To evaluate the impact of genetic variation in xenobiotic metabolizing enzymes on the relationship between markers of exposure and ChE inhibition presented in Chapter 2, we characterized the genotypes of CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C19, CYP2D6, CYP3A5, CYP3A7, GSTM1, GSTP1, and GSTT1in our study population utilizing the Affymetrix DMET multiplexed genotyping platform. Allele frequencies for these genes are presented in Tables 4-1 and 4-2, for CYPs and GSTs, respectively, along with the biological impacts of these variants (Hayes and Strange 2000; Zhou et al. 2009).

These genotypes were incorporated into our linear models to determine if ChE activity is altered by genetic variation in enzymes involved in OP pesticide metabolism. We investigated whether these genotypes impacted the relationships between levels of AZ in blood and ChE activity observed in Chapter 2. The results of these analyses with CYP genotypes are presented in Table 4-3 for AChE and Table 4-4 for BuChE. Only CYP1B1 modified the amount of AChE activity compared to the off season per unit AZ exposure (Table 4-3): post-hoc tests showed that the CYP1B1 *1/*1 group had less inhibition/unit AZ exposure than the *1/*3 individuals ($p =$

0.009). For BuChE, CYP1B1 and CYP2B6 were found to modify the amount of BuChE activity after adjustment for AZ exposure (Table 4-4): CYP1B1 *1/*1 individuals had lower levels of BuChE inhibition than those with the CYP1B1 *1/*3 genotype ($p = 0.009$) and CYP2B6 *1/*1 individuals with the had lower BuChE activity than those with the CYP2B6 *6/*6 genotype ($p = 0.013$). The effect of genetic variation in GSTs on the relationship between AChE (Table 4-5) or BuChE activity (Table 4-6) and AZ were also evaluated but no significant associations were observed.

An association between ChE activity and dimethyl DAP metabolites was previously observed (Chapter 2). The impact of CYP and GST variants on these relationships was evaluated. Significant modifications of the AChE activity/dimethyl DAPs relationships were observed for all CYPs with the exception of combinations CYP1A2 *1F and *1L allele combinations (Table 4-7). When considering genetic variation in CYP1B1, CYP2B6, and both groupings of CYP3A5, we observed decreased AChE activity compared to the off season with decreasing number of *1 alleles. This was also observed for CYP1A1 and CYP2D6 when comparing the *1/*1 group to the heterozygote group. Variation in CYP3A7 had the opposite effect, with AChE activity increasing being negatively associated with the number of *1 alleles. This trend was also observed when comparing CYP2A6 *1/*1 and *1/*9 individuals. For CYP2C19 (*1 vs. *17 alleles), AChE activity/unit metabolite was lower in *1/*1 individuals than heterozygote individuals. The two members of the CYP1A2 *1A/*1A group had significantly greater AChE inhibition/unit metabolite than either of the *1A/*1F and *1F/*1F groups. Only for DMDTP was there a significant impact of CYP2C19 (*1 vs. *2 alleles) on AChE activity, with the *2/*2 genotype being associated with lower activity/μmol DMDTP/L urine than the *1/*1 genotype ($p = 0.028$).

Significant effects were also observed when considering CYP genotype and the BuChE activity/dimethyl metabolite relationships (Table 4-8). A negative association between the number of wild-type alleles and BuChE activity adjusted for dimethyl DAP concentrations for CYP1A2 (*1A vs. *1F), CYP1B1, CYP2B6, and CYP2C19 (*1 vs. *17): for each of these CYPs, BuChE activity was significantly higher in those homozygous for the variant allele, or heterozygous in the case of CYP2C19 (*1 vs. *17), compared to those with the wild-type/wild-type genotype. For CYP2A6, no differences were observed between the *1/*1 and *1/*9 genotype groups, and the *9/*9 group was different from the other two but only one person possessed this genotype. Comparing CYP2C19 (*1 vs. *2) genotypes, the *1/*1 group had lower BuChE activity than the *1/*2 group when DMP and DMDTP were used as the exposure metric. A similar trend was observed for CYP2D6: those with the *1/*2 genotype had lower activity/unit DMP or DMDTP compared to the *2/*2 genotype group. Significantly lower BuChE activity was observed in the CYP3A5 *3C/*3C or *3C/X groups compared to individuals with the *1A/*3C genotype when using DMTP as the exposure metric ($p < 0.01$). A similar finding was observed for DMP when considering the CYP3A5 grouping with the *3, *6, and *7 alleles ($p = 0.028$). For CYP3A7 and either DMP or DMTP, differences were observed between the *1/*1 and *1/*2 groups, with the latter having higher BuChE activity. Significant modification of BuChE inhibition was observed for CYP1A2 (*1F vs. *1L) with *1L/*1L individuals having higher BuChE inhibition/µmol DMDTP/L than those with the *1F/*1F genotype. No significant results were observed for CYP1A1 genotype and BuChE activity.

The BuChE activity/dimethyl metabolite relationships were impacted by genetic variation in GSTs. For AChE (Table 4-9), GSTM1 *A/*A individuals had higher activity in terms of DMTP concentration than *B/*B individuals ($p = 0.049$). In addition, those with the

GSTP1 *A/*A genotype possessed lower activity/unit DMP than did persons with the *B/*B genotype ($p = 0.018$). A similar observation was made between GSTM1 genotype and BuChE activity (Table 4-10): when considering OP pesticide exposure in terms of DMTP concentration, GSTM1 *A/*A individuals had higher activity than *B/*B individuals ($p = 0.032$). Using DMDTP as the exposure metric, a significant difference in BuChE activity was observed between GSTM1 *0/*0 individuals and GSTM1 *B/*B individuals ($p = 0.017$), with the latter having lower activity per unit DMDTP. Finally, those with the GSTP1 *A/*A genotype had lower levels of BuChE inhibition than did GSTP1 *B/*B individuals ($p = 0.025$).

Discussion:

In Chapter 2 we demonstrated that farmworkers performing work not directly involving pesticides have appreciable exposures to OP pesticides based on parent compound in the blood and urinary metabolites. Furthermore, those exposures, expressed as concentrations of AZ in blood or dimethyl metabolites in urine, were associated with cholinesterase inhibition in a dose dependent manner. We tested whether these associations were modified by genetic variation in CYP or GST genes. Genotyping was accomplished with the Affymetrix DMET assay (Deeken 2009). Allele frequencies for genes of interest were determined (Table 4-1). The frequencies observed in our population are representative of what has been previously published for Hispanic populations. Reported allele frequency of the CYP2D6 *2 allele were 0.18-0.23 for (Mendoza et al. 2001; Luo et al. 2005) which corresponds with our frequency of 0.17. We observed a frequency of 0.10 for the CYP2C19 *2 allele, which is in agreement with published values (Aguilar et al. 2008; Dorado et al. 2011). The frequency of the *3 allele of CYP3A5 was 0.67 in our study population. This value is slightly lower than the values of 0.70-0.84 published

by others (Blanco et al. 2002; Langaee et al. 2007). Previously reported frequencies of the GSTT1 and GSTM1 null (*0) alleles were 0.10-0.13 (Nelson et al. 1995; Kelsey et al. 1997) and 0.45 (Kelsey et al. 1997), respectively. These are similar to the 0.13 and 0.49 values observed in our population. These results demonstrate a high degree of similarity between the CBPRP study participants and other Hispanic populations.

We incorporated the genotypic information into the regression models relating cholinesterase inhibition and OP pesticide exposure. Significant modification was observed in the ChE inhibition/AZ relationship by genotypes of CYP1B1 for AChE and of CYP1B1 and CYP2B6 for BuChE (Tables 4-3 & 4-4). This association is likely due to correlations between AZ and the dimethyl metabolites (Chapter 2) and AZ oxon and it is these metrics that are more closely related to ChE inhibition than the parent compound itself. No significant effects by GST genotype were observed for the ChE inhibition/AZ relationship (Tables 4-5 & 4-6) suggesting a lack of metabolism of the AZ parent compound by GSTs.

The effect of CYP genetic variation on the ChE inhibition/dimethyl DAP relationships was also investigated. For several CYP genes, a small number of individuals possessed one of the homozygous genotypes. In order to avoid making conclusions from small sample sizes, only comparisons between groups with five or more individuals will be considered when discussing potential biological significance. In the case of AChE, decreases in the levels of activity in terms of the off season baseline value were seen with increasing numbers of variant alleles of CYP1B1, CYP3A5 and was suggested for CYP1A1 and CYP2D6 (Table 4-7). In contrast, AChE activity increased with the number of variant alleles for CYP2C19 (*1 vs. *17) and CYP3A7. Little evidence was found to suggest impacts by CYP1A2, CYP2B6, or CYP2C19 (*1 vs. *2) genotype.

Results also showed a genotypic impact of CYP genetic variation on BuChE activity (Table 4-8). Our data suggests that BuChE activity decreased with an increasing number of variant alleles for CYP3A5 when DMTP was used as the exposure metric. In contrast, BuChE activity increased with the number of CYP1B1 variant alleles. This same pattern was suggested for CYP3A7 as the wild-type homozygotes had lower activity relative to the off season than the heterozygotes when considering DMP and DMTP as the exposure metric. For both groupings of CYP2C19, the wild-type homozygotes had lower BuChE activity than the heterozygous group. Little to no evidence for an impact by CYP1A1, CYP1A2, CYP2A6, CYP2B6, or CYP2D6 genotype on BuChE activity/unit metabolite was observed.

These data suggest that possessing variant alleles for certain CYP enzymes alters the level of ChE inhibition in this population, likely by altering the amount of desulfuration or dearylation reactions that mediate the amount of oxon formed. Examining the findings across AChE and BuChE, it appears as though CYP3A5 variants consistently increased ChE inhibition whereas the CYP2C19 *17 variant decreases ChE inhibition. The CYP3A5 variant alleles possess an early stop codon from a splice defect, causing them to have little to no activity *in vivo* (Table 4-1). As the level of inhibition is higher in those homozygous for variant alleles compared to the wild-type homozygotes, this suggests that the functional form of CYP3A5 preferentially catalyzes the dearylation reaction over the desulfuration reaction. In the case of CYP2C19, the *17 allele encodes a protein with higher activity than the wild-type. The lower level of ChE inhibition observed for the *1/*17 group over the *1/*1 group may be a result of more dearylation products being formed over the oxon in those individuals. More data are needed in order to confirm these observations.

There are limited data available on the effects of CYP genetic variation on the metabolism of OP pesticides. *In vitro* studies have demonstrated differences in oxon formation for variants of CYP2C19 (Tang et al. 2001), CYP3A4 (Dai et al. 2001), and CYP2B6 (Crane et al. 2012). Studies of CYP genetic variation in human populations exposed to pesticides have been performed. An association was observed between CYP2E1 and oxidative damage in agricultural workers exposed to OP and other pesticides (da Silva et al. 2008). No association between DNA damage assessed via the comet assay and CYP1A1 genotype was observed in pesticide sprayers living in Ecuador (Paz-y-Mino et al. 2004). Profiles of CYP genotypes and DNA damage in an Indian population of pesticide sprayers suggest a potential affect by CYP2D6 (Satyender Singh et al. 2011b) and CYP2C9 (Singh et al. 2012) genotype. While these findings are interesting, none of these epidemiological studies use quantitative measures to evaluate the degree of exposure, instead relying on occupational status to define their exposure groups. The present study has increased power to indentify gene x environment interactions because the degree to which individuals were exposed was determined. This study and more like it will help identify candidate CYP genotypes that can be used as markers of susceptibility.

Genetic variation in GST enzymes was also incorporated into our models relating dimethyl DAP metabolites and ChE inhibition. Elevated AChE inhibition/µmol DMTP/L was observed for GSTM1 *B/*B individuals over *A/*A individuals (Table 4-9). For GSTP1, higher inhibition/per unit DMP was observed for the *A/*A group compared to the *B/*B group. In the case of BuChE inhibition, significant differences were observed between GSTM1 genotype groups and exposures measured via DMTP or DMDTP (Table 4-10). When using DMDTP as the exposure metric, higher BuChE inhibition was found for the GSTP1 *B/*B individuals

compared to the *A/*A group. We found no results to suggest an impact by GSTT1 genotype for either enzyme.

The significance of the findings with GST genetic variation are not currently understood. There is little evidence to suggest that GSTs can serve a role as markers of susceptibility for OP pesticides (Eaton 2005). However GST-mediated conjugation of GSH to OP pesticides has been demonstrated *in vitro* (Fujioka and Casida 2007). One study of pesticide applicators has shown associations between GST polymorphisms and oxidative damage (S. Singh et al. 2011), but these findings may be a result of GSTs role as an oxidative response enzyme more than one which is involved in the metabolism of OP pesticides. Further experimentation with *in vivo* systems should elucidate any role GSTs have in detoxifying OP pesticides.

Overall these findings are strongly suggestive of a role for CYP3A5 and CYP2C19 as markers of susceptibility for populations exposed to AZ. This work improves on other studies examining the impact of CYP genetic variation on health impacts caused by OP pesticides by including quantitative measures of exposure and, thus, is able to identify any gene-environment interactions present in this population of farmworkers. We believe these results translate to other agricultural populations exposed to OP pesticides and call for more investigation of how genetic variability can impact the toxicity of different OP pesticides and pesticide classes.

Table 4-1. Genotype Frequencies for CYP-450s in a subset of CBPRP Study Population (N=70)

Gene	Allele	Freq	Effect*
CYP1A1	*1	0.62	Wild-Type
	*2C	0.34	2455A>G (I462V); normal *in vitro* activity
	Other	0.04	
CYP1A2	*1A	0.18	Wild-Type
	*1F	0.47	-163C>A; higher inducibility *in vivo*
	*1L	0.34	(predicted haplotype): -3860G>A; -2467delT; -163C>A; 5347T>C
	Other	0.01	
CYP1B1	*1	0.62	Wild-Type
	*3	0.28	4326C>G (L432V)
	Other	0.10	
CYP2A6	*1	0.88	Wild-Type
	*9	0.10	-48T>G; reduced expression and catalytic activity *in vitro*
	Other	0.02	
CYP2B6	*1	0.61	Wild-Type
	*6	0.33	516G>T; 785A>G; Lower activity *in vitro* and *in vivo*
	Other	0.07	
CYP2C19	*1	0.79	Wild-Type
	*2	0.10	681G>A (I331V, splicing defect); no activity *in vivo*
	*17	0.12	99C>T; 991A>G (I331V); increased activity *in vivo* and *in vitro*
CYP2D6	*1	0.57	Wild-type
	*2	0.17	R296C, S486T; normal activity
	*4	0.19	1846G>A(splicing defect); no enzyme activity
	Other	0.07	
CYP3A5	*1A	0.30	Wild-Type
	*3C	0.67	6986A>G (splicing defect); decreased activity *in vivo* and *in vitro*
	*6	0.01	14690G>A (splicing defect); decreased activity in vitro
	*7	0.02	346frameshift
CYP3A7	*1	0.66	Wild-type
	*2	0.30	1226C>G (T409R); increased activity *in vitro*
	other	0.04	

*Genotypic Effect from Zhou et al. (2009) and http://www.cypalleles.ki.se/

Table 4-2. Genotype Frequencies for GSTs in a subset of CBPRP Study Population (N=70)

Gene	Allele	Freq	Effect*
GSTM1	*0	0.49	Gene deletion; no activity
	*A	0.25	Wild-Type
	*B	0.26	519G>C (K173N)
GSTP1	*A	0.47	Wild-Type
	*B	0.47	313A>G (I105V)
	*C	0.06	313A>G (I105V), 341C>T (A114V)
GSTT1	*0	0.19	Gene deletion; no activity
	*A	0.81	Wild-Type

*Genotypic Effect from Hayes and Strange (2000)

Table 4-3 Impact of CYP450 Genotype on the Relationship Between AChE Activity and AZ in Blood

Gene	Genotype	N	slope (% off season value/ ng AZ/g)	95% CI	ANOVA p-value$
CYP1A1	All	60	-2.36	(-4.38, -0.34)	0.872
	*1/*1	25	-0.67	(-4.75, 3.41)	
	*1/*2C	27	-3.17	(-5.58, -0.77)	
	*2C/*2C	8	1.54	(-8.43, 11.52)	
CYP1A2	All	28	-0.13	(-4.74, 4.48)	0.830
	*1A/*1A	2	-240.99	(-1229.07, 747.09)	
	*1A/*1F	17	0.52	(-5.27, 6.31)	
	*1F/*1F	9	-1.47	(-9.83, 6.89)	
CYP1A2	All	35	-0.84	(-3.38, 1.7)	0.951
	*1F/*1F	9	-1.47	(-8.92, 5.97)	
	*1F/*1L	16	-0.96	(-3.87, 1.94)	
	*1L/*1L	10	1.54	(-8.10, 11.19)	
CYP1B1	All	51	-2.51	(-4.48, -0.53)	0.049
	*1/*1	24	-0.40	(-2.99, 2.18)	a
	*1/*3	22	-4.64	(-7.51, -1.78)	a
	*3/*3	5	-10.70	(-22.96, 1.55)	
CYP2A6	All	60	-1.63	(-3.68, 0.43)	0.249
	*1/*1	48	-3.03	(-5.66, -0.4)	
	*1/*9	11	0.74	(-2.82, 4.3)	
	*9/*9	1	-0.68	(-8.41, 7.05)	
CYP2B6	All	56	-2.61	(-4.74, -0.47)	0.256
	*1/*1	22	-0.64	(-4.05, 2.76)	
	*1/*6	30	-3.80	(-6.51, -1.09)	
	*6/*6	4	-33.97	(-104.09, 36.14)	
CYP2C19	All	51	-1.69	(-3.76, 0.39)	0.954
	*1/*1	35	-1.71	(-3.90, 0.49)	
	*1/*17	16	-1.48	(-8.70, 5.74)	
CYP2C19	All	46	-1.64	(-3.75, 0.47)	1.000
	*1/*1	35	-1.71	(-3.96, 0.55)	
	*1/*2	10	-2.65	(-23.50, 18.19)	
	*2/*2	1	-0.68	(-8.54, 7.17)	
CYP2D6	All	37	-0.20	(-2.99, 2.59)	1.000
	*1/*1	22	0.10	(-2.91, 3.1)	
	*1/*2	12	-2.53	(-10.85, 5.8)	
	*2/*2	3	309.64	(-428.37, 1047.64)	

Table 4-3 Continued

Gene	Genotype	N	slope (% off season value/ ng AZ/g)	95% CI	ANOVA p-value$
CYP3A5	All	60	-2.35	(-4.24, -0.46)	0.358
	*1A/*1A	8	2.53	(-2.96, 8.02)	
	*1A/*3C	23	-3.55	(-7.30, 0.2)	
	*3C/*3C	29	-2.77	(-5.11, -0.43)	
CYP3A5	All	63	-2.35	(-4.35, -0.35)	0.432
	*1A/*1A	8	2.53	(-3.30, 8.35)	
	*1A/*3C	23	-3.55	(-7.53, 0.43)	
	*3C/*X#	32	-2.77	(-5.25, -0.28)	
CYP3A7	All	56	-3.26	(-5.71, -0.81)	0.206
	*1/*1	28	-4.98	(-8.21, -1.76)	
	*1/*2	20	-3.23	(-7.67, 1.2)	
	*2/*2	8	2.53	(-3.41, 8.46)	

- X = *3C, *6, or *7

$ - Genotype groups for a given enzyme with the same letters are significantly different from each other (post-hoc test, $p < 0.05$)

Table 4-4. Impact of CYP450 Genotype on the Relationship Between BuChE Activity and AZ in Blood

Gene	Genotype	N	slope (% off season value/ ng AZ/g)	95% CI	ANOVA p-value$
CYP1A1	All	60	-3.94	(-7.70, -0.18)	0.946
	*1/*1	25	-4.42	(-12.20, 3.36)	
	*1/*2C	27	-3.62	(-8.14, 0.9)	
	*2C/*2C	8	-6.61	(-25.35, 12.13)	
CYP1A2	All	28	-2.69	(-11.05, 5.68)	0.742
	*1A/*1A	2	-1123.81	(-2828.55, 580.93)	
	*1A/*1F	17	-0.87	(-10.86, 9.12)	
	*1F/*1F	9	-6.81	(-22.04, 8.41)	
CYP1A2	All	35	-1.73	(-5.53, 2.06)	0.951
	*1F/*1F	9	-6.81	(-18.25, 4.62)	
	*1F/*1L	16	-0.59	(-4.82, 3.65)	
	*1L/*1L	10	-6.71	(-20.75, 7.33)	
CYP1B1	All	51	-4.01	(-7.87, -0.16)	0.021
	*1/*1	24	0.49	(-4.38, 5.35)	a
	*1/*3	22	-10.40	(-15.84, -4.97)	a
	*3/*3	5	9.84	(-13.22, 32.91)	
CYP2A6	All	60	-4.04	(-8.07, -0.01)	0.149
	*1/*1	48	-7.35	(-12.42, -2.28)	
	*1/*9	11	2.59	(-4.22, 9.39)	
	*9/*9	1	-7.09	(-21.89, 7.7)	
CYP2B6	All	56	-3.76	(-7.90, 0.39)	0.009
	*1/*1	22	1.40	(-4.75, 7.55)	a
	*1/*6	30	-7.21	(-12.06, -2.37)	
	*6/*6	4	168.97	(43.60, 294.34)	a
CYP2C19	All	51	-4.06	(-8.43, 0.31)	0.786
	*1/*1	35	-4.65	(-9.23, -0.06)	
	*1/*17	16	2.27	(-12.76, 17.3)	
CYP2C19	All	46	-4.58	(-8.90, -0.25)	1.000
	*1/*1	35	-4.65	(-9.20, -0.09)	
	*1/*2	10	19.25	(-22.61, 61.11)	
	*2/*2	1	-7.09	(-22.86, 8.68)	
CYP2D6	All	37	-0.31	(-4.76, 4.15)	1.000
	*1/*1	22	0.48	(-4.33, 5.29)	
	*1/*2	12	-6.26	(-19.48, 6.96)	
	*2/*2	3	118.77	(-1052.86, 1290.41)	

Table 4-4 Continued

Gene	Genotype	N	slope (% off season value/ ng AZ/g)	95% CI	ANOVA p-value$
CYP3A5	All	60	-3.86	(-7.52, -0.2)	0.994
	*1A/*1A	8	-4.32	(-15.25, 6.6)	
	*1A/*3C	23	-4.01	(-11.58, 3.56)	
	*3C/*3C	29	-3.72	(-8.37, 0.93)	
CYP3A5	All	63	-3.87	(-7.65, -0.09)	0.995
	*1A/*1A	8	-4.32	(-15.59, 6.94)	
	*1A/*3C	23	-4.01	(-11.82, 3.8)	
	*3C/*X#	32	-3.74	(-8.54, 1.06)	
CYP3A7	All	56	-6.05	(-10.38, -1.71)	0.446
	*1/*1	28	-8.56	(-14.43, -2.68)	
	*1/*2	20	-2.27	(-10.34, 5.81)	
	*2/*2	8	-4.32	(-15.13, 6.48)	

- X = *3C, *6, or *7

$ - Genotype groups for a given enzyme with the same letters are significantly different from each other (post-hoc test, $p < 0.05$)

Table 4-5 Impact of GST Variants on the AChE Activity/AZ relationship

Gene	Genotype	N	slope (% off season value/ ng AZ/g)	95% CI	ANOVA p-value
GSTM1	All	63	-3.31	(-5.52, -1.09)	1.000
	*0/*0	31	-2.94	(-6.30, 0.43)	
	*A/*A	15	0.34	(-7.63, 8.3)	
	*B/*B	17	-4.24	(-7.45, -1.02)	
GSTP1	All	57	-2.36	(-4.27, -0.46)	0.402
	*A/*A	16	-6.44	(-11.53, -1.35)	
	*A/*B	27	-1.55	(-3.63, 0.53)	
	*B/*B	14	-4.62	(-13.29, 4.04)	
GSTT1	All	64	-2.36	(-4.32, -0.4)	0.181
	*0/*0	11	-6.00	(-10.56, -1.43)	
	*A/*A	53	-1.57	(-3.70, 0.57)	

Table 4-6 Impact of GST Variants on the BuChE Activity/AZ Relationship

Gene	Genotype	N	slope (% off season value/ ng AZ/g)	95% CI	ANOVA p-value
GSTM1	All	63	-6.58	(-10.65, -2.5)	1.000
	*0/*0	31	-5.62	(-11.86, 0.63)	
	*A/*A	15	-1.38	(-16.00, 13.24)	
	*B/*B	17	-8.28	(-14.18, -2.38)	
GSTP1	All	57	-3.93	(-7.63, -0.24)	0.576
	*A/*A	16	-0.55	(-10.85, 9.76)	
	*A/*B	27	-4.83	(-8.94, -0.73)	
	*B/*B	14	2.32	(-14.75, 19.4)	
GSTT1	All	64	-3.94	(-7.65, -0.23)	0.580
	*0/*0	11	-1.68	(-10.47, 7.12)	
	*A/*A	53	-4.44	(-8.56, -0.31)	

Table 4-7 Alteration of the AChE Activity/Dimethyl DAP Relationship by CYP450 Genotype

Gene	Urinary Metabolite	Genotype	N	Slope (% off season value / µmol/L)	95% CI	ANOVA p-value$
CYP1A1	DMP	All	60	-8.84	(-16.61, -2.62)	0.001
		*1/*1	25	5.16	(-2.33, 20.9)	a
		*1/*2C	27	-18.88	(-35.58, -5.96)	a, b
		*2C/*2C	8	8.04	(-8.51, 26.63)	b
	DMTP	All	60	-1.73	(-3.19, -0.56)	0.098
		*1/*1	25	-0.30	(-1.31, 2.09)	
		*1/*2C	27	-2.62	(-5.45, -0.75)	
		*2C/*2C	8	1.70	(-1.57, 5.46)	
	DMDTP	All	60	-6.86	(-17.85, -1.59)	0.082
		*1/*1	25	6.47	(-4.01, 32.66)	a
		*1/*2C	27	-10.09	(-32.54, -2.23)	a
		*2C/*2C	8	-4.31	(-29.92, 37.02)	
CYP1A2	DMP	All	28	8.17	(0.63, 24.58)	<0.001
		*1A/*1A	2	-311.99	(-730.30, -108.79)	a, b
		*1A/*1F	17	8.67	(1.15, 27.41)	a
		*1F/*1F	9	8.52	(-7.71, 51.25)	b
	DMTP	All	28	1.02	(-0.39, 4.02)	<0.001
		*1A/*1A	2	-120.46	(-248.10, -51.18)	a, b
		*1A/*1F	17	1.13	(-0.15, 4.05)	a
		*1F/*1F	9	1.07	(-1.99, 11.92)	b
	DMDTP	All	28	9.09	(-1.17, 36.41)	<0.001
		*1A/*1A	2	-951.31	(-1909.72, -431.68)	a, b
		*1A/*1F	17	6.82	(-1.70, 31.97)	a
		*1F/*1F	9	29.04	(-15.23, 152.41)	b
CYP1A2	DMP	All	35	-4.41	(-10.57, 1.18)	0.162
		*1F/*1F	9	8.52	(-7.71, 51.25)	
		*1F/*1L	16	-10.99	(-23.39, -2.92)	
		*1L/*1L	10	4.31	(-8.11, 16.94)	
	DMTP	All	35	-0.71	(-1.85, 0.24)	0.203
		*1F/*1F	9	1.07	(-1.99, 11.92)	
		*1F/*1L	16	-1.34	(-3.19, -0.26)	
		*1L/*1L	10	1.17	(-1.62, 3.8)	
	DMDTP	All	35	-6.77	(-17.47, -0.09)	0.203
		*1F/*1F	9	29.04	(-15.23, 152.41)	
		*1F/*1L	16	-10.08	(-32.99, -2.62)	
		*1L/*1L	10	-6.85	(-24.96, 16.22)	

Table 4-7 Continued

Gene	Urinary Metabolite	Genotype	N	Slope (% off season value / µmol/L)	95% CI	ANOVA p-value$
CYP1B1	DMP	All	51	-12.63	(-22.08, -4.01)	<0.001
		*1/*1	24	-3.82	(-10.58, 1.34)	a, b
		*1/*3	22	-28.63	(-45.29, -10.81)	a
		*3/*3	5	-105.16	(-238.33, -35.01)	b
	DMTP	All	51	-2.74	(-4.71, -1.07)	<0.001
		*1/*1	24	-0.75	(-2.41, 0.4)	a
		*1/*3	22	-4.51	(-8.62, -1.64)	
		*3/*3	5	-21.40	(-59.79, -5.66)	a
	DMDTP	All	51	-10.37	(-28.49, -1.68)	<0.001
		*1/*1	24	-5.08	(-16.40, -0.58)	a
		*1/*3	22	-46.66	(-92.30, 2.65)	b
		*3/*3	5	-313.42	(-762.89, -87.55)	a, b
CYP2A6	DMP	All	60	-4.67	(-10.55, -0.02)	<0.001
		*1/*1	48	-8.30	(-16.31, -2.52)	a
		*1/*9	11	12.91	(2.00, 38.04)	a, b
		*9/*9	1	-11.62	(-36.93, -3.58)	b
	DMTP	All	60	-1.33	(-2.44, -0.39)	<0.001
		*1/*1	48	-1.75	(-3.16, -0.56)	a
		*1/*9	11	1.78	(0.04, 5.14)	a, b
		*9/*9	1	-5.71	(-19.47, -1.67)	b
	DMDTP	All	60	-5.53	(-14.89, -1.12)	<0.001
		*1/*1	48	-7.29	(-19.08, -1.77)	a,b
		*1/*9	11	27.43	(3.85, 95.36)	a, c
		*9/*9	1	-51.85	(-159.45, -16.46)	b, c
CYP2B6	DMP	All	56	-11.32	(-20.01, -3.23)	0.046
		*1/*1	22	-6.64	(-15.88, -0.76)	
		*1/*6	30	-12.89	(-24.45, -2.08)	
		*6/*6	4	-44.67	(-119.74, -10.74)	
	DMTP	All	56	-2.48	(-4.23, -1)	0.021
		*1/*1	22	-1.24	(-3.14, -0.13)	a
		*1/*6	30	-2.84	(-5.10, -1.02)	
		*6/*6	4	-12.12	(-36.81, -3.78)	a
	DMDTP	All	56	-8.79	(-23.34, -1.46)	<0.001
		*1/*1	22	-1.87	(-11.02, 0.38)	a
		*1/*6	30	-19.12	(-40.82, -3.63)	b
		*6/*6	4	-152.72	(-488.49, -39.76)	a, b

Table 4-7 Continued

Gene	Urinary Metabolite	Genotype	N	Slope (% off season value / µmol/L)	95% CI	ANOVA p-value$
CYP2C19	DMP	All	51	-4.46	(-10.62, 0.69)	0.020
		*1/*1	35	-7.81	(-15.44, -2.29)	
		*1/*17	16	17.58	(1.51, 51.27)	
	DMTP	All	51	-1.30	(-2.41, -0.37)	0.006
		*1/*1	35	-1.85	(-3.20, -0.64)	
		*1/*17	16	3.41	(0.59, 9.42)	
	DMDTP	All	51	-5.31	(-15.11, -0.81)	0.024
		*1/*1	35	-6.89	(-18.29, -1.58)	
		*1/*17	16	78.89	(4.32, 178.69)	
CYP2C19	DMP	All	46	-7.52	(-14.40, -2.45)	0.459
		*1/*1	35	-7.81	(-15.44, -2.29)	
		*1/*2	10	-6.01	(-23.26, 2.16)	
		*2/*2	1	-11.62	(-36.93, -3.58)	
	DMTP	All	46	-1.83	(-3.14, -0.64)	0.188
		*1/*1	35	-1.85	(-3.20, -0.64)	
		*1/*2	10	-1.01	(-6.16, 3.15)	
		*2/*2	1	-5.71	(-19.47, -1.67)	
	DMDTP	All	46	-6.81	(-17.27, -1.7)	0.028
		*1/*1	35	-6.89	(-18.29, -1.58)	a
		*1/*2	10	-6.62	(-27.24, 9.67)	
		*2/*2	1	-51.85	(-159.45, -16.46)	a
CYP2D6	DMP	All	60	-8.84	(-16.61, -2.62)	0.001
		*1/*1	25	5.16	(-2.33, 20.9)	a
		*1/*2C	27	-18.88	(-35.58, -5.96)	a, b
		*2C/*2C	8	8.04	(-8.51, 26.63)	b
	DMTP	All	60	-1.73	(-3.19, -0.56)	0.098
		*1/*1	25	-0.30	(-1.31, 2.09)	
		*1/*2C	27	-2.62	(-5.45, -0.75)	
		*2C/*2C	8	1.70	(-1.57, 5.46)	
	DMDTP	All	60	-6.86	(-17.85, -1.59)	0.082
		*1/*1	25	6.47	(-4.01, 32.66)	a
		*1/*2C	27	-10.09	(-32.54, -2.23)	a
		*2C/*2C	8	-4.31	(-29.92, 37.02)	
CYP3A5	DMP	All	60	-9.51	(-17.49, -3.12)	<0.001
		*1A/*1A	8	35.96	(9.12, 92.25)	a, b
		*1A/*3C	23	-7.56	(-16.96, -1.57)	a
		*3C/*3C	29	-23.70	(-38.59, -9.83)	b

Table 4-7 Continued

Gene	Urinary Metabolite	Genotype	N	Slope (% off season value / µmol/L)	95% CI	ANOVA p-value$
CYP3A5	DMTP	All	60	-1.79	(-3.32, -0.56)	<0.001
		*1A/*1A	8	9.34	(2.93, 22.3)	a, b
		*1A/*3C	23	-1.18	(-2.84, -0.27)	a
		*3C/*3C	29	-4.44	(-8.18, -1.61)	b
	DMDTP	All	60	-7.48	(-19.62, -1.8)	<0.001
		*1A/*1A	8	98.04	(32.21, 216.58)	a, b
		*1A/*3C	23	-8.46	(-24.71, -2.48)	a, c
		*3C/*3C	29	-14.95	(-42.90, -2.34)	b, c
CYP3A5	DMP	All	63	-8.56	(-16.20, -2.46)	<0.001
		*1A/*1A	8	35.96	(9.12, 92.25)	a, b
		*1A/*3C	23	-7.56	(-16.96, -1.57)	a
		*3C/*X	32	-20.89	(-34.11, -8.06)	b
	DMTP	All	63	-1.70	(-3.13, -0.55)	<0.001
		*1A/*1A	8	9.34	(2.93, 22.3)	a, b
		*1A/*3C	23	-1.18	(-2.84, -0.27)	a
		*3C/*X	32	-3.92	(-7.03, -1.49)	b
	DMDTP	All	63	-6.70	(-17.63, -1.53)	<0.001
		*1A/*1A	8	98.04	(32.21, 216.58)	a, b
		*1A/*3C	23	-8.46	(-24.71, -2.48)	a
		*3C/*X	32	-12.97	(-36.06, -2.1)	b
CYP3A7	DMP	All	56	-8.17	(-16.30, -1.92)	<0.001
		*1/*1	28	-25.54	(-42.36, -10.34)	a, b
		*1/*2	20	-3.37	(-10.81, 4.04)	a, c
		*2/*2	8	35.96	(9.12, 92.25)	b, c
	DMTP	All	56	-1.67	(-3.26, -0.48)	<0.001
		*1/*1	28	-4.59	(-8.69, -1.63)	a, b
		*1/*2	20	-0.72	(-2.16, 0.12)	a, c
		*2/*2	8	9.34	(2.93, 22.3)	b, c
	DMDTP	All	56	-6.13	(-17.20, -1.2)	<0.001
		*1/*1	28	-14.78	(-44.06, -2.28)	a
		*1/*2	20	-4.62	(-14.63, 3.61)	b
		*2/*2	8	98.04	(32.21, 216.58)	a, b

- X = *3C, *6, or *7

$ - Genotype groups for a given enzyme/DAP combination with the same letters are significantly different from each other (post-hoc test, $p < 0.05$)

Table 4-8 Alteration of the BuChE Activity/Dimethyl DAP Relationship by CYP450 Genotype

Gene	Urinary Metabolite	Genotype	N	Slope (% off season value / µmol/L)	95% CI	ANOVA p-value$
CYP1A1	DMP	All	60	-20.16	(-41.46, -5.28)	0.706
		*1/*1	25	-20.24	(-52.36, 5.81)	
		*1/*2C	27	-24.32	(-54.99, -5.27)	
		*2C/*2C	8	-12.17	(-66.75, 11.49)	
	DMTP	All	60	-4.84	(-8.83, -1.27)	0.706
		*1/*1	25	-6.39	(-12.00, -1.8)	
		*1/*2C	27	-5.32	(-10.52, -1.06)	
		*2C/*2C	8	-0.71	(-13.41, 2.99)	
	DMDTP	All	60	-32.51	(-72.46, -9.44)	0.706
		*1/*1	25	-38.16	(-92.61, 2.56)	
		*1/*2C	27	-30.23	(-84.43, -7.32)	
		*2C/*2C	8	-75.45	(-159.11, -21.58)	
CYP1A2	DMP	All	28	-1.93	(-26.10, 23.27)	<0.001
		*1A/*1A	2	-1222.85	(-3219.54, -327.11)	a, b
		*1A/*1F	17	-7.27	(-40.37, 22.4)	a
		*1F/*1F	9	24.67	(-20.27, 79.2)	b
	DMTP	All	28	-1.97	(-6.72, 4.27)	<0.001
		*1A/*1A	2	-495.74	(-1106.94, -163.13)	a, b
		*1A/*1F	17	-4.25	(-10.44, 2.69)	a
		*1F/*1F	9	9.66	(-1.13, 24.55)	b
	DMDTP	All	28	-19.37	(-59.52, 16.73)	<0.001
		*1A/*1A	2	-4186.38	(-8840.39, -1500.71)	a, b
		*1A/*1F	17	-26.79	(-75.91, 13.01)	a
		*1F/*1F	9	47.97	(-41.22, 182.94)	b
CYP1A2	DMP	All	35	-16.10	(-37.99, -1.66)	0.203
		*1F/*1F	9	24.67	(-20.27, 79.2)	
		*1F/*1L	16	-16.35	(-43.28, -1.88)	
		*1L/*1L	10	-36.44	(-91.87, 2.61)	
	DMTP	All	35	-2.38	(-6.42, -0.2)	0.130
		*1F/*1F	9	9.66	(-1.13, 24.55)	
		*1F/*1L	16	-2.84	(-7.36, -0.33)	
		*1L/*1L	10	-5.15	(-19.72, 1.32)	
	DMDTP	All	35	-33.21	(-81.03, -5.07)	0.030
		*1F/*1F	9	47.97	(-41.22, 182.94)	a
		*1F/*1L	16	-14.72	(-66.85, -0.96)	
		*1L/*1L	10	-101.68	(-189.41, -42.84)	a

Table 4-8 Continued

Gene	Urinary Metabolite	Genotype	N	Slope (% off season value / µmol/L)	95% CI	ANOVA p-value$
CYP1B1	DMP	All	51	-24.44	(-48.62, -6.82)	<0.001
		*1/*1	24	-17.88	(-38.73, -0.09)	a
		*1/*3	22	-62.01	(-117.84, -17.63)	b
		*3/*3	5	245.16	(80.07, 494.36)	a, b
	DMTP	All	51	-7.07	(-11.55, -2.87)	<0.001
		*1/*1	24	-3.85	(-8.77, 0.02)	a
		*1/*3	22	-12.59	(-24.60, -4.86)	b
		*3/*3	5	61.33	(21.26, 140.64)	a, b
	DMDTP	All	51	-48.07	(-99.45, -13.49)	<0.001
		*1/*1	24	-37.50	(-79.29, -10.9)	a
		*1/*3	22	-192.43	(-310.04, -76.1)	b
		*3/*3	5	816.52	(316.15, 1619.36)	a, b
CYP2A6	DMP	All	60	-18.67	(-40.38, -3.84)	0.009
		*1/*1	48	-21.15	(-47.79, -4.09)	a
		*1/*9	11	-6.36	(-44.38, 13.5)	b
		*9/*9	1	-121.01	(-384.63, -37.29)	a, b
	DMTP	All	60	-4.72	(-8.72, -1.16)	<0.001
		*1/*1	48	-5.60	(-10.31, -1.33)	a
		*1/*9	11	0.44	(-5.87, 3.8)	b
		*9/*9	1	-59.46	(-202.74, -17.43)	a, b
	DMDTP	All	60	-31.78	(-72.13, -9.15)	<0.001
		*1/*1	48	-30.14	(-72.67, -8.25)	a
		*1/*9	11	-57.56	(-147.52, -5.23)	b
		*9/*9	1	-539.98	(-1660.54, -171.37)	a, b
CYP2B6	DMP	All	56	-23.37	(-47.06, -6.25)	0.046
		*1/*1	22	-12.61	(-32.90, 12.71)	
		*1/*6	30	-32.28	(-68.32, -8.66)	
		*6/*6	4	205.53	(-2.71, 450.36)	
	DMTP	All	56	-6.59	(-10.66, -2.75)	0.004
		*1/*1	22	-3.06	(-7.21, 1.47)	a
		*1/*6	30	-8.40	(-14.58, -3.32)	b
		*6/*6	4	57.30	(13.42, 138)	a, b
	DMDTP	All	56	-46.40	(-93.11, -13.96)	0.021
		*1/*1	22	-26.27	(-58.28, -8.36)	
		*1/*6	30	-89.12	(-158.90, -34.35)	a
		*6/*6	4	545.36	(15.71, 1254.64)	a

Table 4-8 Continued

Gene	Urinary Metabolite	Genotype	N	Slope (% off season value / µmol/L)	95% CI	ANOVA p-value$
CYP2C19	DMP	All	51	-21.17	(-45.21, -4.56)	<0.001
		*1/*1	35	-31.77	(-63.28, -8.43)	
		*1/*17	16	44.36	(14.43, 104.32)	
	DMTP	All	51	-4.80	(-8.83, -1.16)	<0.001
		*1/*1	35	-6.21	(-11.42, -1.6)	
		*1/*17	16	7.00	(1.70, 19.4)	
	DMDTP	All	51	-38.34	(-88.38, -10.37)	0.024
		*1/*1	35	130.57	(13.43, 300.69)	
		*1/*17	16	-4.46	(-10.62, 0.69)	
CYP2C19	DMP	All	46	-27.88	(-55.98, -7.53)	<0.001
		*1/*1	35	-31.77	(-63.28, -8.43)	a
		*1/*2	10	29.75	(4.77, 78.8)	a, b
		*2/*2	1	-121.01	(-384.63, -37.29)	b
	DMTP	All	46	-6.08	(-11.07, -1.61)	0.002
		*1/*1	35	-6.21	(-11.42, -1.6)	a
		*1/*2	10	6.55	(-5.50, 23.57)	b
		*2/*2	1	-59.46	(-202.74, -17.43)	a, b
	DMDTP	All	46	-34.67	(-78.92, -9.9)	<0.001
		*1/*1	35	-38.34	(-88.38, -10.37)	a, b
		*1/*2	10	37.31	(-2.82, 117.23)	a, c
		*2/*2	1	-539.98	(-1660.54, -171.37)	b, c
CYP2D6	DMP	All	37	-9.07	(-26.48, 2.43)	0.007
		*1/*1	22	2.69	(-18.82, 22.38)	
		*1/*2	12	-22.60	(-62.65, -2.82)	a
		*2/*2	3	11.88	(-2.08, 76.88)	a
	DMTP	All	37	-1.23	(-4.83, 0.49)	0.027
		*1/*1	22	0.91	(-4.16, 5.27)	
		*1/*2	12	-2.71	(-10.48, -0.22)	
		*2/*2	3	0.26	(-0.55, 6.75)	
	DMDTP	All	37	-21.51	(-51.56, -5.8)	0.027
		*1/*1	22	-25.05	(-74.91, 14.92)	
		*1/*2	12	-22.25	(-60.77, -5.3)	a
		*2/*2	3	-0.74	(-7.03, 36.02)	a
CYP3A5	DMP	All	60	-16.31	(-35.36, -3.45)	0.041
		*1A/*1A	8	-15.13	(-41.40, 8.75)	
		*1A/*3C	23	-1.89	(-18.53, 12.35)	
		*3C/*3C	29	-38.14	(-75.50, -11.31)	

Table 4-8 Continued

Gene	Urinary Metabolite	Genotype	N	Slope (% off season value / µmol/L)	95% CI	ANOVA p-value$
CYP3A5	DMTP	All	60	-4.38	(-8.18, -1.02)	0.006
		*1A/*1A	8	-3.33	(-9.91, 0.96)	
		*1A/*3C	23	-0.56	(-3.49, 1.65)	a
		*3C/*3C	29	-10.37	(-17.92, -4.33)	a
	DMDTP	All	60	-28.88	(-66.84, -8.15)	0.045
		*1A/*1A	8	-20.08	(-84.85, 31.97)	
		*1A/*3C	23	-4.44	(-33.44, 8.96)	
		*3C/*3C	29	-51.67	(-123.20, -12.91)	
CYP3A5	DMP	All	63	-18.45	(-38.79, -4.42)	0.019
		*1A/*1A	8	-15.13	(-41.40, 8.75)	
		*1A/*3C	23	-1.89	(-18.53, 12.35)	a
		*3C/*X	32	-43.26	(-83.23, -14.18)	a
	DMTP	All	63	-4.64	(-8.50, -1.2)	0.004
		*1A/*1A	8	-3.33	(-9.91, 0.96)	
		*1A/*3C	23	-0.56	(-3.49, 1.65)	a
		*3C/*X	32	-10.43	(-17.90, -4.52)	a
	DMDTP	All	63	-31.87	(-71.37, -9.29)	0.036
		*1A/*1A	8	-20.08	(-84.85, 31.97)	
		*1A/*3C	23	-4.44	(-33.44, 8.96)	
		*3C/*X	32	-56.10	(-127.95, -14.38)	
CYP3A7	DMP	All	56	-17.46	(-38.77, -3.93)	0.019
		*1/*1	28	-43.80	(-84.21, -14.21)	a
		*1/*2	20	0.17	(-16.65, 15.69)	a
		*2/*2	8	-15.13	(-41.40, 8.75)	
	DMTP	All	56	-4.52	(-8.63, -0.99)	0.001
		*1/*1	28	-11.09	(-19.45, -4.56)	a
		*1/*2	20	0.03	(-2.59, 2.62)	a
		*2/*2	8	-3.33	(-9.91, 0.96)	
	DMDTP	All	56	-30.40	(-70.91, -8.5)	0.084
		*1/*1	28	-52.63	(-127.34, -13.16)	
		*1/*2	20	-6.99	(-41.30, 7.26)	
		*2/*2	8	-20.08	(-84.85, 31.97)	

- X = *3C, *6, or *7

$ - Genotype groups for a given enzyme/DAP combination with the same letters are significantly different from each other (post-hoc test, $p < 0.05$)

Table 4-9 The AChE Activity/Dimethyl Metabolite Relationship is Modified by GST Genotype

Gene	Metabolite	Genotype	N	Slope (% off season value / µmol/L)	95% CI	ANOVA p-value$
GSTM1	DMP	All	63	-9.01	(-16.87, -2.73)	0.067
		*0/*0	31	-8.07	(-16.32, -2.12)	
		*A/*A	15	1.92	(-7.15, 8.02)	
		*B/*B	17	-19.24	(-35.18, -2.41)	
	DMTP	All	63	-1.75	(-3.22, -0.57)	0.065
		*0/*0	31	-1.47	(-3.15, -0.42)	
		*A/*A	15	0.28	(-1.52, 1.47)	a
		*B/*B	17	-3.47	(-6.59, -1.01)	a
	DMDTP	All	63	-6.97	(-18.07, -1.63)	0.193
		*0/*0	31	-4.60	(-15.19, -0.54)	
		*A/*A	15	-7.38	(-21.87, 3.88)	
		*B/*B	17	-21.37	(-54.34, 0.73)	
GSTP1	DMP	All	57	-9.64	(-17.78, -3.14)	0.036
		*A/*A	16	-22.63	(-42.23, -6.41)	a
		*A/*B	27	-8.72	(-18.25, -2.12)	
		*B/*B	14	3.92	(-6.77, 14.1)	a
	DMTP	All	57	-1.83	(-3.39, -0.57)	0.114
		*A/*A	16	-3.44	(-8.06, 0.11)	
		*A/*B	27	-2.05	(-3.80, -0.62)	
		*B/*B	14	0.84	(-1.59, 2.79)	
	DMDTP	All	57	-7.63	(-20.13, -1.83)	0.114
		*A/*A	16	-5.84	(-34.35, -0.48)	
		*A/*B	27	-13.88	(-33.08, -3.49)	
		*B/*B	14	4.74	(-8.22, 30.7)	
GSTT1	DMP	All	64	-8.68	(-16.33, -2.53)	0.283
		*0/*0	11	-19.51	(-47.14, -4.18)	
		*A/*A	53	-4.20	(-11.15, 2.27)	
	DMTP	All	64	-1.72	(-3.14, -0.56)	0.514
		*0/*0	11	-2.69	(-9.12, -0.46)	
		*A/*A	53	-1.53	(-2.64, -0.3)	
	DMDTP	All	64	-6.85	(-17.83, -1.58)	0.473
		*0/*0	11	-14.94	(-59.77, -3.45)	
		*A/*A	53	-4.67	(-16.19, 0.91)	

$ - Genotype groups for a given enzyme/DAP combination with the same letters are significantly different from each other (post-hoc test, $p < 0.05$)

Table 4-10 The BuChE Activity/Dimethyl Metabolite Relationship is Modified by GST Genotype

Gene	Metabolite	Genotype	N	Slope (% off season value / µmol/L)	95% CI	ANOVA p-value$
GSTM1	DMP	All	63	-20.35	(-41.81, -5.41)	0.067
		*0/*0	31	-20.33	(-42.61, -4.09)	
		*A/*A	15	9.50	(-21.59, 40.13)	
		*B/*B	17	-47.53	(-102.44, -11.59)	
	DMTP	All	63	-4.89	(-8.89, -1.3)	0.054
		*0/*0	31	-3.32	(-7.72, -0.49)	
		*A/*A	15	0.62	(-5.50, 7.62)	a
		*B/*B	17	-11.07	(-19.71, -4.5)	a
	DMDTP	All	63	-32.95	(-73.86, -9.49)	0.035
		*0/*0	31	-19.22	(-50.82, -4.65)	a
		*A/*A	15	-22.38	(-80.07, 31.51)	
		*B/*B	17	-127.33	(-228.50, -51.57)	a
GSTP1	DMP	All	57	-16.23	(-35.98, -3.21)	0.114
		*A/*A	16	3.92	(-11.18, 39.85)	
		*A/*B	27	-25.68	(-59.18, -4.76)	
		*B/*B	14	-23.34	(-67.12, 6.96)	
	DMTP	All	57	-4.45	(-8.31, -1.02)	0.114
		*A/*A	16	-0.29	(-3.23, 8.48)	
		*A/*B	27	-6.04	(-12.11, -1.22)	
		*B/*B	14	-2.48	(-13.02, 2.06)	
	DMDTP	All	57	-29.00	(-68.13, -8.07)	0.041
		*A/*A	16	-10.01	(-24.44, 17.32)	a
		*A/*B	27	-42.09	(-113.85, -6)	
		*B/*B	14	-92.75	(-167.56, -33.88)	a
GSTT1	DMP	All	64	-18.75	(-39.12, -4.67)	0.090
		*0/*0	11	-2.28	(-16.62, 15.85)	
		*A/*A	53	-30.22	(-57.17, -9.33)	
	DMTP	All	64	-4.69	(-8.55, -1.23)	0.063
		*0/*0	11	-0.47	(-3.92, 1.49)	
		*A/*A	53	-6.99	(-11.36, -2.96)	
	DMDTP	All	64	-3.84	(-35.49, 11.95)	0.047
		*0/*0	11	-48.88	(-98.85, -14.23)	
		*A/*A	53	-8.68	(-16.33, -2.53)	

$ - Genotype groups for a given enzyme/DAP combination with the same letters are significantly different from each other (post-hoc test, $p < 0.05$)

CHAPTER 5: Optimization of RNA Isolation from Exfoliated Buccal Epithelial Cells

Introduction:

Biological sampling is an integral component of molecular epidemiology studies. Typically tissues or body fluids are collected based on their ability to function as surrogates for internal tissues (Rockett et al. 2004) or for their ability to be collected non-invasively (Esteban and Castano 2009). Buccal epithelial cells collected from the oral cavity with cytological brushes or mouthwashes are an efficient noninvasive manner to collect biological material (Salama et al. 1999) and have the potential to encourage higher response rates in molecular epidemiology studies than taking blood samples (Hansen et al. 2007). Recently these samples have been used as a source of RNA for gene expression analyses. This has included both RT-PCR (Spivack et al. 2004; Kumar et al. 2005b) and microarray analysis (Sridhar et al. 2008; Kupfer et al. 2010) completed in order to determine alterations in gene expression as a result of exposure to tobacco smoke (Spivack et al. 2004; Sridhar et al. 2008; Kupfer et al. 2010), clinical radiation (Narayan et al. 2008), or environmental contaminants (Liu et al. 2007).

In order for buccal sampling to be useful for these types of analyses, the RNA isolation procedure needs to be optimized to produce samples of sufficient quantity and quality. Spivack et al. (2004) utilized a standard column purification protocol (Qiagen RNEasy kit) incorporating the use of poly-C RNA as a carrier to isolate RNA from buccal cells collected with brushes. With the addition of 1 µg of poly-C RNA, the authors were able to obtain yielded 0.75 to 1.5 µg per brush, suggesting that carrier RNA makes up most of their sample. Despite this they and others using their protocol (Kumar et al. 2005a; Kupfer et al. 2010) have been able to isolate RNA and identify biological signals in their subjects. In the case of Liu et al. (2007), samples were collected after brushing with a toothbrush and rinsing with saline. RNA was then isolated

using Trizol reagent and samples were cleaned up with RNEasy columns. Another protocol developed by Spira et al. (2004) involves the collection of buccal cells with a "plastic tool that is concave with serrated edges" that they designed and RNA isolation using Trizol. While the collection of the samples may cause some discomfort, they successfully demonstrated its use in profiling gene expression on microarrays after serially collecting the samples (Sridhar et al. 2008).

To improve the isolation of RNA from exfoliated buccal cells, we compared two protocols, one using Trizol reagent to lyse the cells and the other using the lysis buffer included along with the RNEasy columns, in order to maximize the RNA yields. Doing so will identify an optimal RNA extraction method that will improve the utility of these samples.

Methods:

Buccal samples were collected from laboratory personnel on a volunteer basis. Volunteers refrained from eating or drinking for one hour prior to donating samples. After rinsing their mouths twice with water, volunteers applied a sterile Catch-All Sample Collection Swab (Epicentre Biotechnologies) to the inside surface of the cheek with firm pressure and rotated the swab twenty times before repeating on the other cheek surface. After both cheek surfaces had been sampled, the swabs were plunged into a sufficient volume of RNAlater (Ambion) to completely cover the swab and stored at -20 °C. Since there is a great amount of inter- and intra-person variability in the amount of buccal cells obtained from human subjects (Moore et al. 2001; Osswald et al. 2003) we have pooled buccal cells collected from several laboratory members and aliquoted the cells into known amounts to reduce the variability of RNA yields. Once enough samples had been collected, they were pooled together, counted using a

hemocytometer after a five minute incubation in Trypan Blue (Sigma), and aliquoted into units of desirable cell number. A subset of sample had cell counts taken to ascertain the inter- and intra-person variability in cell number obtained from these volunteers. Once aliquoted, samples were stored at -20 °C until nucleic acid extraction took place. Mouse embryonic fibroblasts (MEF) generated using previously published methods (Yu et al. 2008) and aliquoted into 500,000 cell units were used as controls.

RNA isolation was performed using one of two methods: either Trizol reagent (Invitrogen) in combination with RNEasy (Qiagen) columns (Trizol method) or a modified protocol based on the Fibrous Tissue RNA extraction kit (Qiagen) (see Figure 5-1 for a diagram depiction of each protocol). During each round of isolation, cells were rinsed and pelleted in CMF-PBS and a negative control consisting of 1 ml of RNAlater was included to determine the background contribution from the reagents used for each protocol. Each protocol was performed three times, independently, with two buccal samples for each isolation, with a total of six samples for each protocol.

For the Trizol method, pelleted cells were lysed in Trizol reagent (Invitrogen) and, after the addition of chloroform, the aqueous phase containing the RNA was separated from the organic phase. Ethanol (70%) was added to the aqueous phase and the combined solution was transferred to an RNneasy Mini spin column (Wong et al. 2004). After an initial centrifugation step to allow the RNA to bind to the column, they were washed twice with Buffer RW1, with a DNase (Qiagen) digestion step between washes, and RPE. After the final wash step, the columns were centrifuged to remove residual ethanol from the buffers and the RNA was eluted with 25 µl RNAse-free water.

The Fibrous Tissue Mini protocol incorporates a proteinase K digestion into the cell lysis procedure, a step included in the Gentra Puregene (Qiagen) protocol commonly used to isolate DNA from buccal cells (Neuhaus et al. 2004; Paynter et al. 2006; Woo et al. 2007b). We tested whether the addition of this digestion increased yields of RNA from buccal cells. RNA isolation with the Fibrous Tissue method involved lysing pelleted cells with Buffer RLT (Qiagen) containing β-mercaptoethanol and incubated with 400 µg proteinase K (Qiagen) for 20 mins at 55 °C. The cell debris was pelleted and the supernatant transferred to an RNEasy Mini spin column after addition of 70% EtOH. After an initial centrifugation step to allow the RNA to bind to the column, they were washed twice with Buffer RW1, with a DNase digestion step according to the manufacturer's protocol being performed between washes, and two washes with Buffer RPE. After the final wash step, the columns were centrifuged to remove residual ethanol from the buffers and the RNA was eluted with 25 µl RNAse-free water. To determine the effect of the DNAse digestion on nucleic acid yields for buccal samples, we compared the effect of adding no DNAse to the column with applying the recommended 27 Kunitz units (KU) to the sample. In the case of MEF samples, we compared the impact of adding 0, 13, or 27 KU of DNAse to the column.

Further modifications were made to optimize the Fibrous Tissue protocol for use with RNEasy MinElute spin columns. After the proteinase K digestion and cell debris pelleting, 100 % EtOH was added to the sample. After the DNAse digestion step, samples were washed once with Buffer RW1, Buffer RPE, and then with 80% EtOH. Prior to elution of the RNA, the RNAse-free water was heated to 55 °C. When placed on the membrane, the solution was incubated on the column for 5 minutes. This method was referred to as the Fibrous Tissue Micro protocol. To determine if further sample homogenization was required when using proteinase K,

samples were placed on Qiashredder columns (Qiagen) and centrifuged according to the manufacturer's specifications.

RNA concentrations and relative purity were evaluated on the Nanodrop ND-1000 (Thermo Scientific). RNA integrity was determined on the Agilent 2100 Bioanalyzer with the picochip assay. The nanochip platform was used for the cell culture positive controls. RNA Integrity Numbers (RIN) (Schroeder et al. 2006) were obtained in order to have quantitative metrics for RNA quality.

All statistical analyses were performed using the Analysis ToolPak in Microsoft Excel 2007. Comparisons of nucleic acid yield and quality obtained from different protocols were made using two sample t-tests assuming unequal variance. Significant differences resulted if the p-value was less than 0.05.

Results:

Initially we attempted to isolate RNA from buccal swabs isolated from single individuals but variation in the number of cells obtained per swab precluded the comparison of efficiency of different isolation methods. As seen in Table 5-1, there is a high degree of inter- and intra-person variability in the number of cells obtained from these swabs. By serially collecting samples, combining them into a common pool, and then aliquoting them out into units containing a desired number of cells we were able to reduce the sample variation to the point that we could judge the RNA yield generated with the different protocols.

We set out to compare the yields of RNA obtained using the Trizol reagent and the Fibrous Tissue protocol. We found that the Fibrous Tissue method provided more RNA than the Trizol method when using 400,000 ($p < 0.05$) or 800,000 cells ($p < 0.005$) after adjusting for

the negative control values (Fig 5-2). When using 400,000 cells, the mean ± st dev. RNA yields for the Fibrous Tissue derived samples was 187 ± 65 ng while the Trizol protocol produced 103 ± 28 ng. With 800,000 cells, the Fibrous Tissue and Trizol methods yielded 196 ± 66 ng and 62 ± 45 ng, respectively. In fact, there were four cases, two for each cell number, where the samples generated with the Trizol protocol had concentrations less than the negative controls while this was the case for only one sample with 400,000 cells under the Fibrous Tissue protocol. Relative purities based on absorbance ratios of 260/280 for proteins and 260/230 for organic solvents showed no statistical differences between the two protocols (data not shown).

RNA integrity of the samples was evaluated with the Agilent 2100 Bioanalyzer. Representative electropherograms for the buccal and MEF samples are presented in Figure 5-3. RNA degradation is clear in all the buccal samples regardless of the method used to isolate it. One the other hand, both the Trizol and Fibrous Tissue methods are capable of isolating high quality RNA: the profiles of both MEF RNA samples generated by either method exhibited high quality, with the two ribosomal RNA peaks being sharp and clearly visible. This observation supports the idea that the cells collected using swabs do not have intact RNA regardless of isolation protocol.

A typical inclusion in RNA isolation protocols is a DNA digestion in order to generate a final solution that is free of DNA contamination that can impact downstream gene expression results. At this point no publication using buccal RNA has utilized a DNAse step in their protocol. As such we examined whether its inclusion in the Fibrous Tissue method affected the absorbance values in the RNA solution. As can be seen in Figure 5-4, the inclusion of a DNAse step during the RNA isolation procedure significantly decreased the concentrations of nucleic acids as measured by absorbance at 260 nm for buccal samples (Fig 5-4A) and for MEF controls

samples (Fig 5-4B). With approximately 790,000 cells, 1.7 µg on average were obtained from buccal samples without the DNAse digestion compared to 230 ng when it was included ($p < 0.001$). Using ~530,000 MEF cells, there was a clear downward trend of nucleic acid yield with increasing amounts of DNase (ANOVA $p < 0.001$). Post hoc t-tests showed that the yields from the sets of samples generated using 13 and 27 KU were significantly lower from those samples that were not subjected to DNAse digestion ($p < 0.005$ for both sets). This suggests that significant DNA contamination is present in samples generated with the Fibrous Tissue protocol when a DNAse step is not included. When viewed on a Bioanalyzer electropherogram, rounded peaks, rather than jagged ones, are present in the buccal sample not treated with DNAse and additional or wider peaks are observed for the MEF samples (Fig 5-5). These observations indicate that DNA contamination could be a potential problem for buccal-derived RNA if steps to prevent co-isolation of DNA are not taken.

After applying the modifications discussed in the methods, we optimized the Fibrous Tissue protocol for use with the MinElute columns as mentioned in the methods. We then investigated whether the proteinase K incubation increases the yield over using the Qiagen columns without it as was done by Spivack et al. (2004). With 500,000 cells we found that without the proteinase K, little to no RNA was isolated from these cells (Fig. 5-6): RNA yields with the proteinase K were 112 ng $\pm$ 42 ng compared to 17 ng $\pm$ 16 ng ($p < 0.005$). In fact most of the samples generated without proteinase K had lower absorbance values than the negative control did. We next set out to determine if a homogenization step, accomplished with the Qiashredder, in combination with proteinase K digestion increased RNA yields from 500,000. After adjustment for negative control values, there was no significant difference between the

groups (data not shown; $p > 0.05$) suggesting that homogenization with the proteinase K digestion is not required.

Discussion:

Presented here are efforts to identify an optimal method to isolate RNA from noninvasively obtained buccal epithelial cells. Our first observation was that there was a great amount of inter- and intra-person variability in the amount of buccal cells obtained (Table 5-1). This has been previously reported for mouthwashes (Osswald et al. 2003) or swabs (Moore et al. 2001). This is likely due to differences in the technique, such as pressure used when collecting with swabs, or differences in the rate of shedding buccal epithelial cells. Since we wanted to discriminate the ability of two different isolation methods given the same input of cellular material, a common pool of samples was generated and used to compare the different extraction techniques in order to eliminate inter- and intra-person variability in the quantity or quality of buccal samples. Doing so enabled us to make proper comparisons between protocols when evaluating the quantity and quality of RNA isolated from buccal cells.

In our hands, the Fibrous Tissue protocol provided the highest yields of RNA from buccal cells collected with cytological swabs compared to using the Trizol reagent in conjunction with RNEasy columns. Furthermore, we determined that digestion with proteinase K is essential to isolating RNA from buccal cells. This observation may be due to 1.) additional lysis and breakdown of the cell membranes that occurs with the additional digestion, 2.) breakdown of components that may obstruct the column thereby preventing efficient binding of the RNA to the column, 3.) or a combination of these. This method allows for the isolation of RNA without the need for carrier RNA as has been performed by Spivack et al. (2004). The addition of carrier

makes it difficult to measure the amount of biological RNA in the sample. To perform proper comparisons of gene expression between groups, similar amounts of biological RNA need to be used for microarrays or RT-PCR. This will now be possible with the Fibrous Tissue protocols described here.

To obtain high purity RNA solutions, we also found that a DNAse treatment was required when isolating RNA from buccal samples with the Fibrous Tissue protocol. This treatment has not been performed in any publications utilizing buccal RNA to date and raises some concern over the potential DNA contamination present in some of the samples being reported on. For example, Kupfer et al. (2010) reported on gene expression differences in smokers and non-smokers using buccal-derived RNA. In their Bioanalyzer traces, regular rounded peaks similar to the ones we observed in our buccal RNA samples that were not treated with DNAse are present for all of their buccal samples. Care should be taken to avoid DNA contamination, especially when utilizing RNA amplification as DNA targets may be amplified instead of RNA.

Regardless of the isolation method used, the integrity of the buccal RNA was very low, consistent with previous studies. Buccal samples obtained using swabs (Klaassen et al. 1998; Spivack et al. 2004) or mouthwashes (Osswald et al. 2003; Michalczyk et al. 2004) were found to consist largely of nonviable cells which, along with the presence of digestive enzymes in the oral cavity, led to substantial degradation of the RNA transcripts in these cells. Other researchers have commented on the degraded nature of buccal RNA. When examined on agarose gels, RNA from buccal cells was significantly degraded, as gauged by barely visible bands for the 28s and 18s ribosomal subunits, compared to cell culture controls (Fall-Dickson et al. 2007). Likewise, visualization of 18s on northern plots showed that exfoliated buccal cells had high levels of degradation compared to cultured keratinocytes (Klaassen et al. 1998). Care

needs to be taken when using these samples for gene expression analysis to ensure that the degraded nature of buccal RNA does not produce false positive results.

Although some level of transcript degradation does not preclude their use in gene expression assays (Schoor et al. 2003; Xiang et al. 2003; Mengual et al. 2006), loss of RNA integrity does affect the results of gene expression assays and high-quality results are dependent on the use of intact RNA transcripts (Auer et al. 2003; Imbeaud et al. 2005; Fleige et al. 2006; Thompson et al. 2007; Ravo et al. 2008). Xiang et al. (2003) demonstrated that degraded RNA could be successfully amplified when the reverse transcription (RT) step included random primers rather than the customary oligo-dT primers. This strategy has been adopted for several amplification strategies. One of these, the whole transcriptome (WT) Ovation methodology produced by Nugen, utilizes both oligo-dT and random primers during the RT step to generate cDNA for isothermal linear amplification (Gill and Ghaemi 2008; Pioch et al. 2008). The final amplified product is single-stranded cDNA (sscDNA) that has been shown to provide higher hybridization specificity on microarrays than cRNA (Barker et al. 2005; Eklund et al. 2006). When compared to other amplification strategies, these methods have high sensitivity and can reproducibly detect differentially expressed genes (Viale et al. 2007; Clement-Ziza et al. 2009). In addition, Nugen Ovation methodology has been used successfully to evaluate gene expression patterns of formalin-fixed, paraffin-embedded (FFPE) tumor samples (which have levels of RNA degradation equivalent to that observed in our buccal samples) and the results demonstrated a high correlation with matched fresh-frozen tissues (Scicchitano et al. 2006; Lassmann et al. 2009; Linton et al. 2009). Similar strategies could prove useful when characterizing gene expression in other samples possessing degraded RNA, including buccal swabs.

Figure 5-1 Diagram of Protocols Used to Optimize RNA Isolation from Buccal Cells

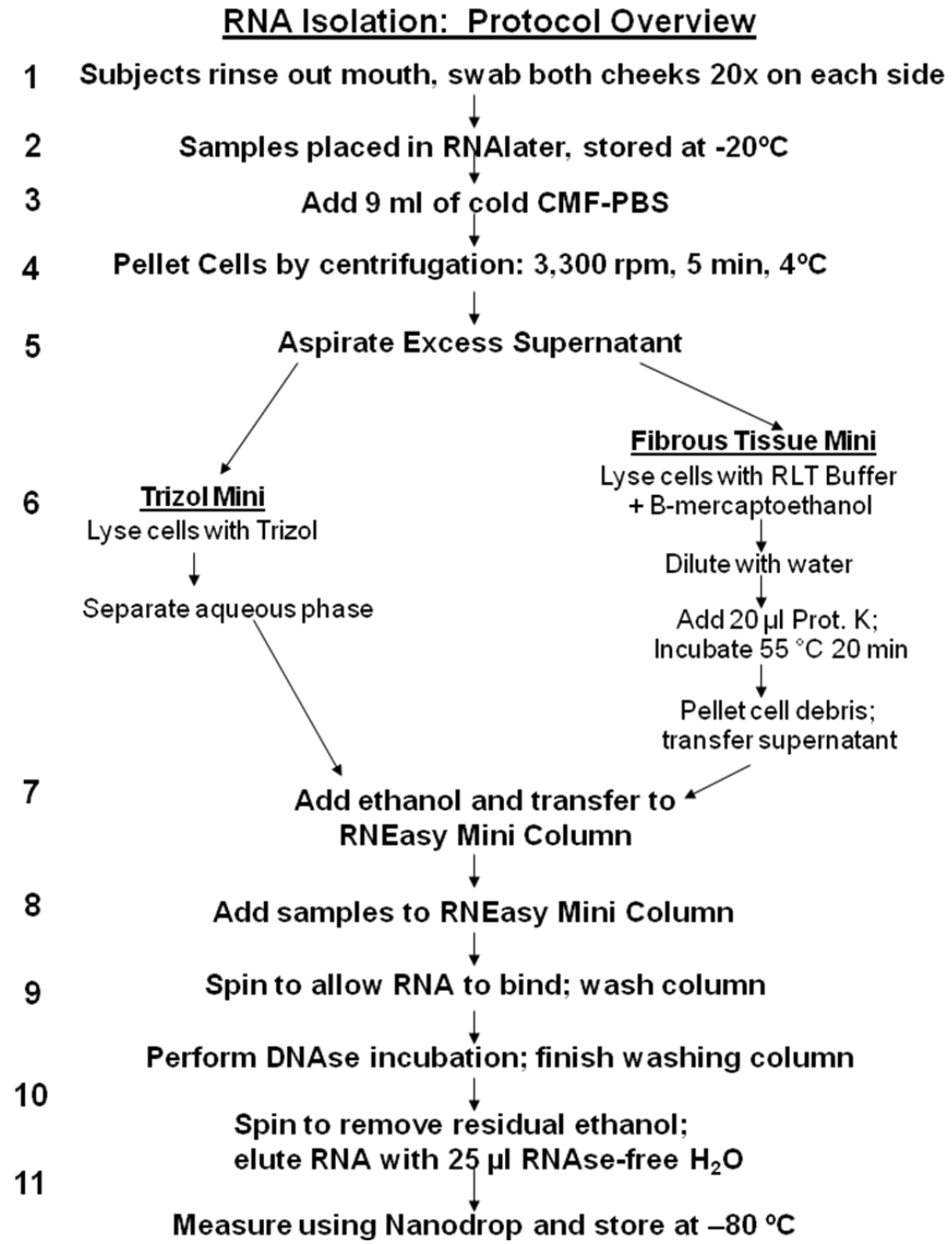


Fig 5-1 Flow diagram depicting details of the Fibrous Tissue Mini and Trizol Mini protocols.

Figure 5-2. Yield of RNA from Buccal Cells Using Two Protocols

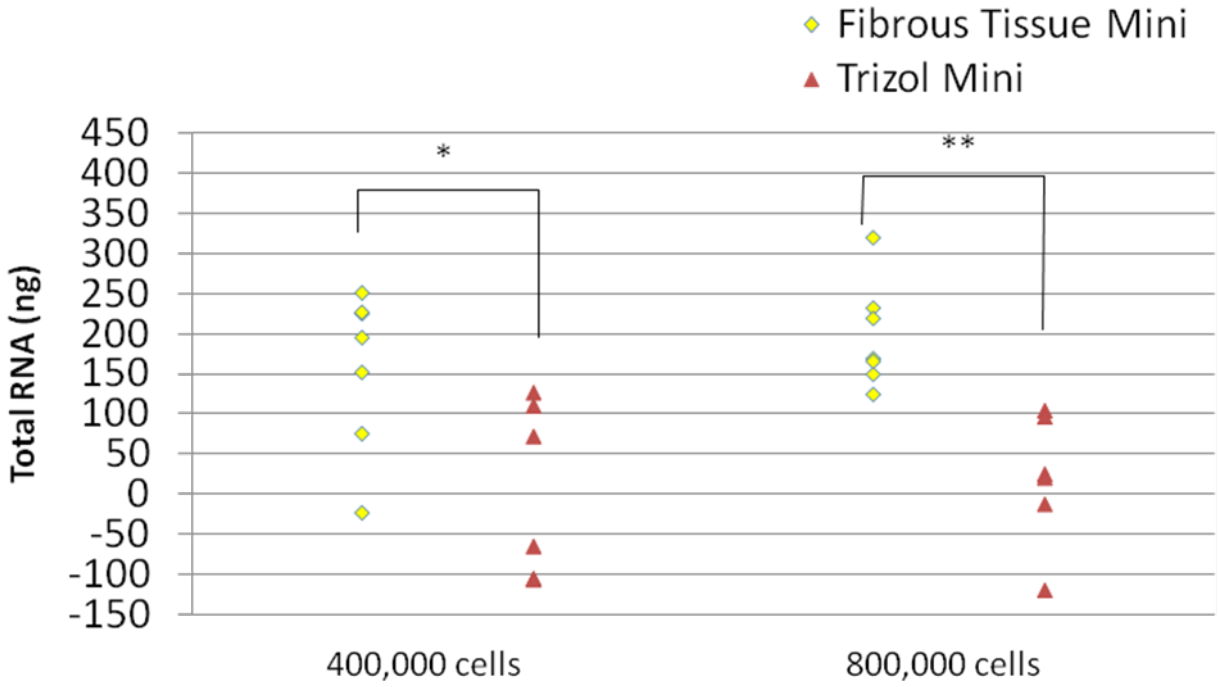


Fig 5-2 Yields of RNA from buccal cells using two protocols. RNA was isolated from buccal cells using either the Fibrous Tissue Mini or Trizol Mini protocol. The concentration of RNA was determined using a Nanodrop 1000 spectrophotometer. Yields were calculated by subtracting off the value of the concurrent negative control sample as background and multiplying by the volume of the elution buffer used. *: p-value < 0.05, **: p-value < 0.005 (two-sample t-test assuming unequal variance).

Figure 5-3. Comparison of RNA Integrity from Samples Isolated with Two Protocols

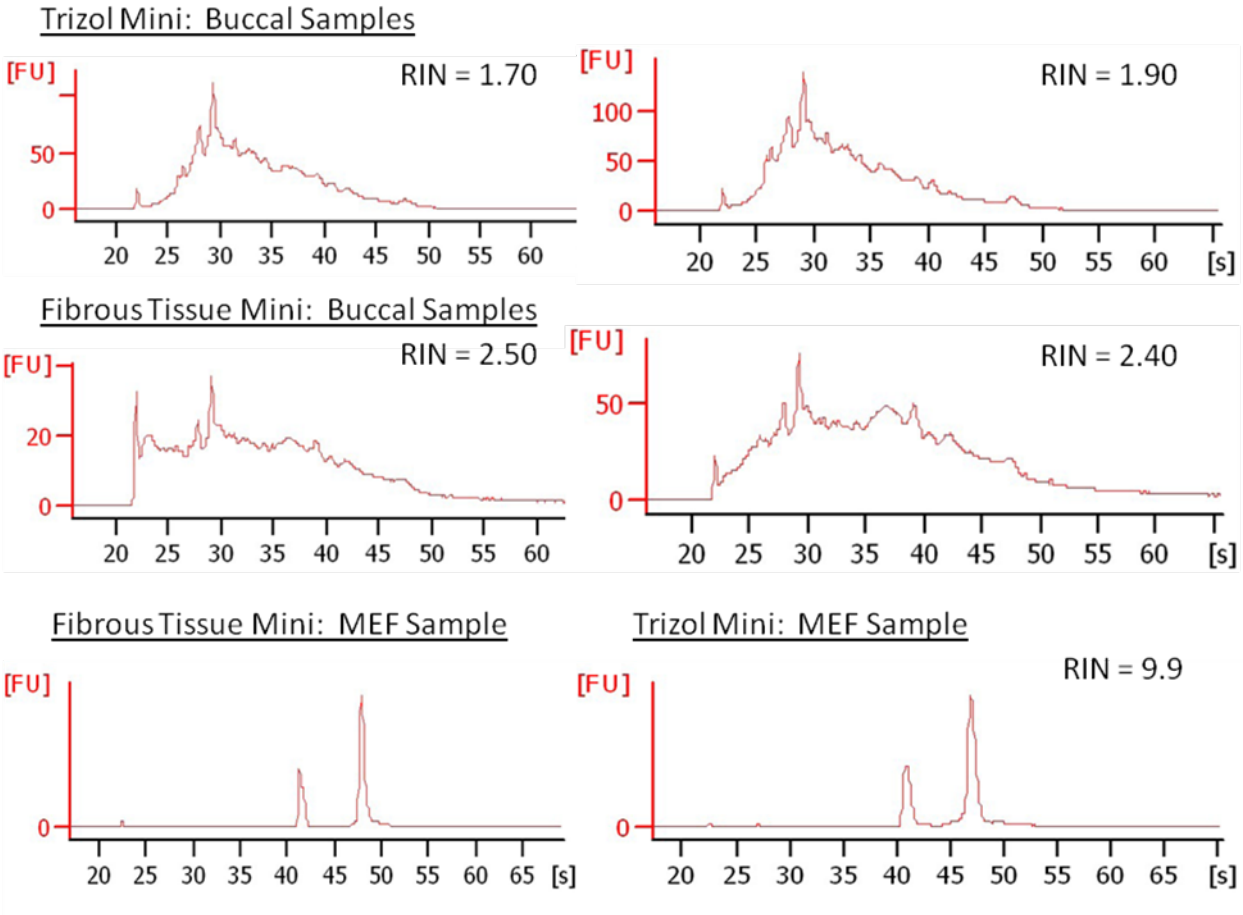
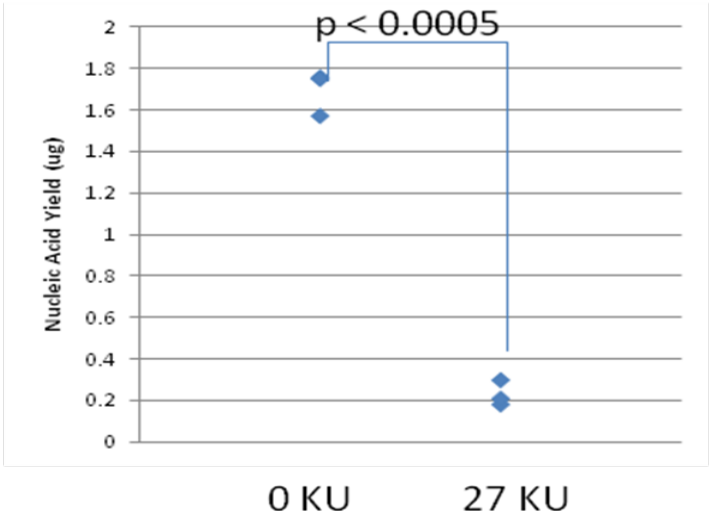


Fig. 5-3 Integrity of buccal RNA isolated with the Fibrous Tissue Mini and Trizol Mini protocols. RNA was isolated from buccal samples as described in the Methods section. Significant degradation of buccal RNA is evident for samples generated with either protocol. Mouse Embryonic Fibroblast (MEF) cells were used as controls and both protocols generated high quality RNA from these samples, as evident from the clear, sharp peaks corresponding to the 18s and 28s ribosomal subunits. Representative traces are presented.

Figure 5-4 Impact of DNAse Digestion During RNA Isolation with the Fibrous Tissue Protocol

A



B

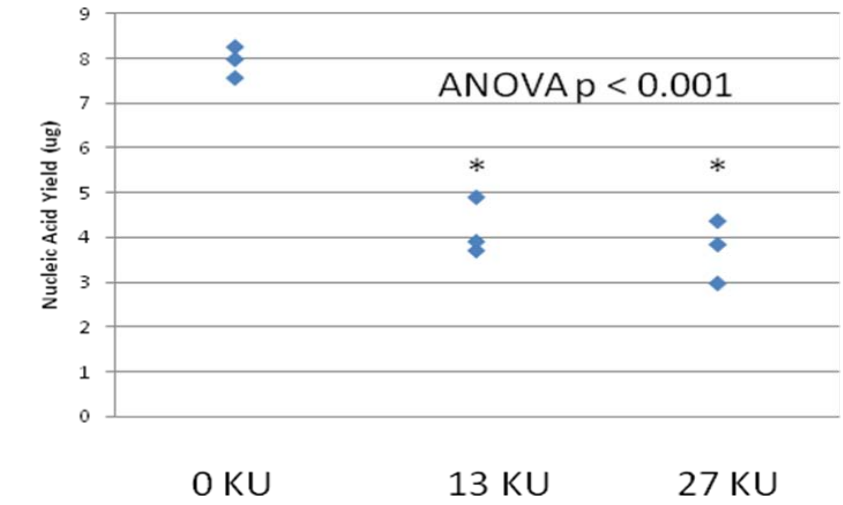


Fig 5-4 Effect of DNAse on Buccal RNA Isolation. A.) RNA was isolated from 790,000 buccal cells using the Fibrous Tissue Mini protocol with and without an on-column DNAse digestion. Without a DNAse treatment there is significantly more nucleic acid being isolated than when it is included ($p < 0.0005$). This suggests that there is significant levels of DNA co-isolated when using the Fibrous Tissue Mini protocol. B.) 530,000 MEFs were used for RNA isolation with 0, 13, or 27 KU of DNAse used to remove any DNA contamination. An ANOVA analysis indicated significant differences between the treatments ($p < 0.001$) and post-hoc t-tests showed that the samples generated with 0 KU DNAse treatment had significantly higher yields of nucleic acid than those generated with either 13 or 27 KU DNAse. * - $p < 0.005$ (t-test).

Figure 5-5 Integrity of Buccal RNA isolated with the Fibrous Tissue Method With and Without DNAse Digestion.

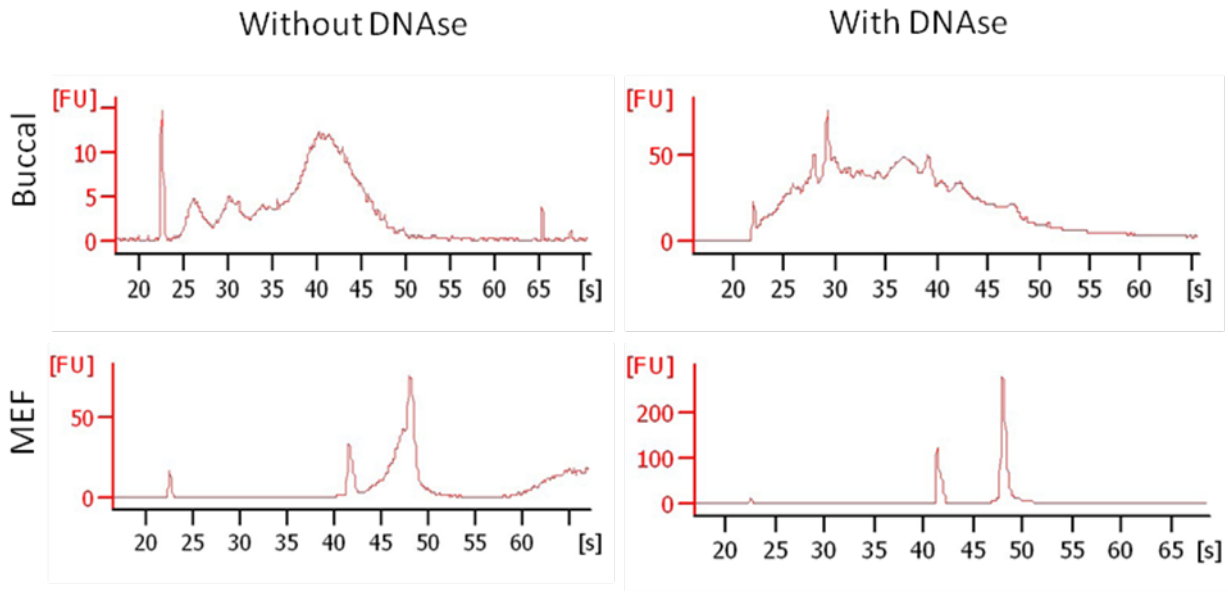


Fig. 5-5 Evidence of DNA contamination in Bioanalyzer electropherograms. Buccal and MEF RNA was isolated using the Fibrous Tissue Mini protocol with and without a DNAse digestion. When the DNAse was not included, rounded peaks were observed in the buccal samples and wider peaks were seen for the MEF samples. These were not present when a DNAse digestion was included in the RNA isolation. Representative traces are presented.

Figure 5-6. RNA Yields from Buccal Samples With and Without a Proteinase K Digestion.

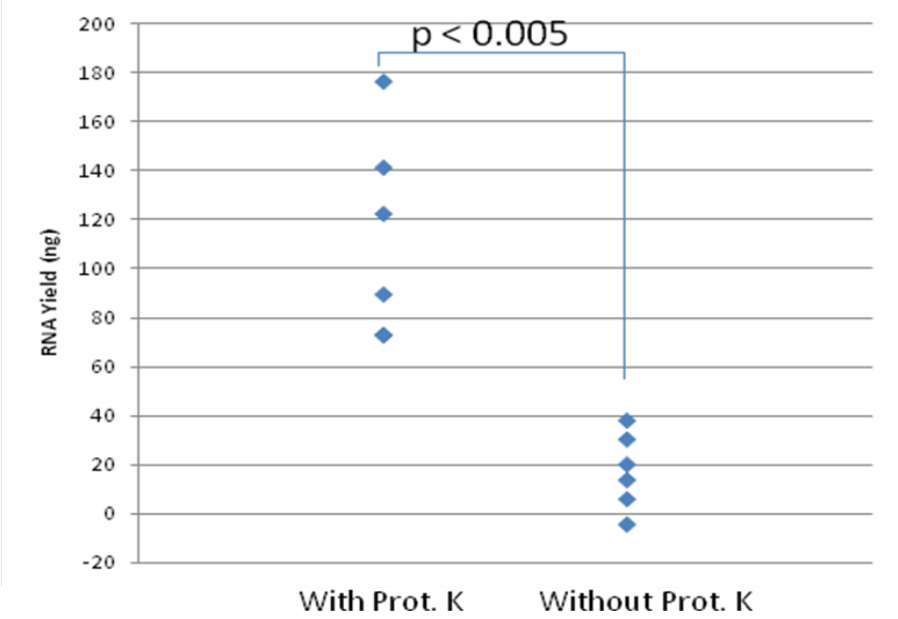


Fig. 5-6 The importance of proteinase K on RNA isolation from buccal cells. RNA was isolated from 500,000 buccal cells using the Fibrous Tissue Micro protocol with or without a proteinase K digestion. The inclusion of the proteinase K digestion significantly increased RNA yields ($p < 0.005$).

Table 5-1. Inter- and Intra-Person Variability in the Number of Buccal Cells Collected with Cytological Brush

Subject #	N	Range	Avg Number of Cells	St Dev.	CV (%)
1	8	480,000-1,060,000	744,688	220,286	30
2	10	230,000-810,000	518,667	186,015	36
3	3	280,000-662,500	530,833	217,318	41
5	2	150,000-352,000	251,250	143,189	57
7	1	190,000	190,000	0	0
8	3	190,000-300,000	240,000	55,678	23
10	1	340,000	340,000	0	0

CHAPTER 6: Significance of Research Findings and Implications for Risk Assessments of Organophosphate Pesticides

For this work, we sought to test the hypothesis that 1.) markers of OP pesticide exposure are associated with cholinesterase markers of effect, and 2.) these relationships are modified by genetic variation in metabolic pathways responsible for the activation and detoxification of OP pesticides. In the preceding chapters, we have demonstrated associations between ChE inhibition and levels of AZ in the blood or dimethyl urinary metabolites during the thinning agricultural season (Chapter 2). We then showed that PON1 status, that is, genotype and phenotype, did not alter the level of ChE inhibition, agreeing with predictions from *in vivo* studies of AZ (Chapter 3). Subsequently, we tested whether genetic variability in CYP450 or GST enzymes affected the ChE inhibition/exposure relationship and found significant results for CYP2C19 and CYP3A5 (Chapter 4). We then presented an optimized protocol for the isolation of RNA from buccal swabs to increase the utility of the non-invasively obtained samples (Chapter 5). Based on these results, we reject both H_{O1} and H_{O2} and accept H_{A1} and H_{A2}.

H_{O1} (rejected): Exposure metrics for OP pesticides are not associated with cholinesterase inhibition

H_{A1} (accepted): OP Pesticide Exposure metrics are associated with inhibition of cholinesterase enzymes

H_{O2} (rejected): Cholinesterase inhibition resulting from OP pesticide exposure is not modified by genetic variation in pathways responsible for the metabolism of OP pesticides.

H_{A2} (accepted): Genetic variation in OP pesticide metabolic pathways alter cholinesterase inhibition after exposure to OP pesticides

This work is of scientific value since few studies have examined associations between urinary biomarkers for OP pesticides and ChE inhibition (Sudakin and Stone 2011). Furthermore, no data to our knowledge is available on how genetic variation in CYP450s, a class of enzymes responsible for key reactions in the metabolism of OP pesticides, affects ChE inhibition. Results presented here suggest that CYP2C19 and CYP3A5 could be potential markers of susceptibility in populations exposed to AZ. Our work also shows that PON1 status can be used as a marker of susceptibility for certain OP pesticides (Furlong 2007), but can't be universally applied to all members of this chemical class since only a few have been demonstrated to be PON1 substrates. For the toxicity of compounds to be mediated, genetic underpinnings of vulnerability need to be understood.

This information has implications for the current cumulative risk assessment model for OP pesticides promulgated by the US Environmental Protection Agency (USEPA) (USEPA 2002b, 2006). Under this model, the common mode of action for OP pesticides, AChE inhibition, is used as the basis to sum the toxicological potential of a mixture of or cumulative exposure to OP pesticides. This is done by incorporating relative potency factors (RPFs) that describes the ability of an individual pesticide to inhibit AChE compared to the reference compound, methamidophos. The combined exposure or mixture is then thought of as a single unit and its potential to cause harm to human health is determined.

While this approach is relatively straightforward, it underestimates the complexity of biological reactions leading up to the AChE inhibition used as the common mode of action. For each OP pesticide/CYP450 combination, equal, more, or lesser amounts of oxon product, which goes on to inhibit AChE, can be produced relative to the detoxification products generated

through the dearylation or dealkylation pathway. This has been demonstrated for metabolism of CP vs. diazinon by CYP2C19 (Foxenberg et al. 2011): a greater fraction of the parent compound is converted to TCP than oxon for CP but equivalent amounts of oxon and non-toxic metabolites are formed for diazinon. Once converted to the oxon form, OP pesticides have differential abilities to be detoxified by PON1, with only the toxicity of CP and diazinon being reduced by the activity of this enzyme (Li et al. 2000). Complicating matters is the observation that genetic variation for PON1 can even affect the toxicity of compounds that are not substrates (Jansen et al. 2009). Genetic variation in CYPs has been shown to impact oxon formation *in vitro* (Dai et al. 2001; Tang et al. 2001; Crane et al. 2012) and we have shown here that it impacts ChE inhibition in a population occupationally exposed to AZ. The impacts of CYP variants will be dependent on the amount of enzyme expressed which can be affected by the amount of induction or inhibition by diet and prescription usage (Tamasi et al. 2003; Jana and Paliwal 2007). In order for estimations of risk to be accurate, they need to incorporate metabolic and genetic information in addition to the RPFs.

We believe the knowledge gained from the studies in the preceding chapters adds evidence to the need to incorporate such data into the cumulative risk assessment model for OP pesticides and to move away from a simple model of dose additivity. The data presented here can also serve as an initial dataset to begin this integration.

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Appendix 1 – *In Vitro* Metabolism of OP Pesticide by CYP450s

Pesticide	Experimental Model	Assay	[OP] Tested	Metabolizing enzyme(s) examined	Desulfuration Reaction - Produce oxon product	Dearylation Reaction - Produce DAPs and leaving group	Reference	Comments
azinphos-methyl	Microsomes (Supersomes) expressing single CYPs	Formation of oxon metabolite	0.05-25 µM	CYP1A1, CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP3A4	Intrinsic clearance (CL) values: 2C19 > 2B6 > 1A2 > 1A1 > 2C9 > 2C8 > 3A4		Buratti et al. (2002) Environ Tox Pharm 11:181-90	% Contribution: 1A2> 2C19 > 2C9 > 3A4> 2B6> 2C8
	Human liver microsomes	Correlation with isoform activities	10 µM	CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3A4, CYP2E1, CYP4A11	1A2, 3A4, 2B6		Buratti et al. (2003) Tox Appl Pharm 186:143–54	
	Human liver microsomes	Isoform specific inhibitors' effect on AChE inhibition	28.5 µM	CYP1A1, CYP1A2, CYP2C9, CYP2D6, CYP2E1, CYP3A4	2D6 > 3A4 = 2E1		Sams et al. (2000) Tox Lett 116:217–21	
	Reconstituted CYP3A4	Formation of oxon and TCP	100 µM	CYP3A4*1 (WT), F189S (CYP3A4*17), L293P (CYP3A4*18), M445T (CYP3A4*3), P467S (CYP3A4*19)	Relative to CYP3A4 WT: F189S - lower activity; L293P - higher activity	Relative to CYP3A4 WT: F189S - lower activity; L293P - higher activity	Dai et al. (2001) JPET 299(3):825–31	All forms of CYP3A4 produced more oxon than TCP

chlorpyrifos Cont.	Human Lymphoblast Cell Culture	Formation of oxon and TCP	100 µM	CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP3A4	**2B6** > 1A2 = 2C9 = 2C19 = 3A4	**2C19** > **2C9** = 3A4 > 1A2 = 2B6	Tang et al. (2001) DMD 29(9):1201–04	Desulfuration/ Dearylation ratio: 2B6 > 1A2 > 3A4 > 2C9 > 2C19
	E.coli expressing CYP2C19 variants	Formation of oxon and TCP	100 µM	CYP2C19*1B, CYP2C19*8, CYP2C19*6, CYP2C19*5	CYP2C19*1B	**2C19*1B** > 2C19*8 > 2C19*6 = 2C19*5		
	Human liver microsomes	Correlation with CYP450 content in individually-derived microsomes	100 µM	CYP2B6, CYP2C19, CYP2D6, CYP3A4	correlated with 2B6 and 3A4 content	highest in individual with high 2C19 but low 3A4 content		
		Pool of 10 male or female samples	2-100 µM	no specific enzymes tested	Higher activity in females than males*	Higher activity in females than males*		*Based both on protein and P450 content
	Microsomes expressing single CYPs	Formation of oxon and TCP	100 µM	CYP1A1, CYP1A2, CYP2A6, CYP3A4, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6	**2B6** > **1A2** = 3A4 > 2C19 = **1A1** = 2C8 = 2C9 = 2D6 = 2A6	**2C19** > 3A4 > 2B6 = 2C8 = 2C9 = 2D6 = 1A2 = 1A1 = 2A6	Vitozzi et al. (2001) Environ Internat 26:125-9	
	Microsomes expressing single CYPs	Formation of oxon metabolite	0.005-2.5 µM	CYP2B6, CYP1A1, CYP1A2, CYP2C19, CYP3A4	Intrinsic clearance (ICL) values: 2B6 > 1A1 > 1A2 > 2C19 > 3A4		Buratti et al. (2002) Environ Tox Pharm 11:181-90	% Contribution: 1A2 > 3A4 > 2B6 > 2C19
		Formation of oxon and TCP	25 µM	CYP2C19, CYP3A4, CYP2B6, CYP1A2	1A2, 3A4, **2B6**, 2C18	1A2, 3A4, 2B6, **2C19**		
			100 µM	CYP2C19, CYP3A4, CYP2B6, CYP1A3	1A2, 3A4, **2B6**, 2C18	**3A4**, 2B6, **2C19**		
	Human liver microsomes	Correlation with isoform activities	5 µM	CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19,CYP2D6, CYP3A4, CYP2E1, CYP4A11	1A2, 3A4, 2B6		Buratti et al. (2003) Tox Appl Pharm 186:143–54	
		Immunoinhibition	5 µM	CYP1A2, CYP2A6, CYP2B6, CYP2C, CYP3A4, CYP2E1	1A2, 3A4, 2B6			Highest Inhibition: 2B6 + 1A2

chlorpyrifos Cont.	Human liver microsomes	Isoform specific activity	12.5 µM	CYP2C19, CYP2B6, CYP1A2, CYP2C9, CYP2D6, CYP2A6, CYP3A4	**2B6** > **1A2** > 2C19 > **2A6** = **2C9** = **3A4** > **2D6**	**2C19**	Sams et al. (2004) Xenobiotica 34(10):861–73	
			100 µM	CYP2C19, CYP2B6, CYP1A2, CYP2C9, CYP2D6, CYP2A6, CYP3A4	**2B6** > **1A2** > 2C19 > 2A6 = 2C9 = 3A4 > 2D6	**2C19** > 2B6 = **2C9** = **2D6** > 1A2		
		Isoform specific inhibition	25 µM	CYP3A4, CYP1A2, CYP2C9, CYP2D6	3A4 = **1A2** > 2C9 > 2D6	**3A4** > 2C9 = 1A2, 2D6		
	Recombinant enzymes	Formation of oxon	0-2.5 µM	CYP3A4, CYP3A5, CYP3A7	3A4 > 3A5 > 3A7		Buratti et al. (2006) Tox Lett 167:245–55	
	Recombinant enzymes	Formation of oxon and TCP metabolites	100 µM	CYP3A4, CYP3A5, CYP1A2, CYP2B6, CYP2C8, CYP2E1, CYP2D6*1, CYP2C19, CYP2C9*1	2D6*1 > **2B6** = **3A5** > **3A4** > 2C19 > 2C8 = **1A2**	**2C19** > **2D6*1** > 3A5 > 3A4 > 2B6 > 2C8 > 1A2	Mutch & Williams (2006) Toxicology 224:22–32	Ratio of oxon/TCP formation: 1A2 > 2B6 > 2C8 > 3A5 = 3A4 > 2D6*1 > 2C19
	Human liver microsomes	Correlation with P450 activity	100 µM	CYP3A4/5, CYP2C8, CYP2C19, CYP1A1/2, CYP2B6, CYP2E1, CYP2D6		3A4/5, 2C8, 2C19, 1A1/2		only TCP formed, no oxon
	Recombinant CYP450s	Formation of oxon and TCP	0-60 µM	CYP1A2, CYP2B6, CYP2C19, CYP3A4, CYP3A5, CYP3A7	Intrinsic Clearance (ICL): **2B6** > **1A2** > 2C19 > 3A4 > 3A5 > 3A7	Intrinsic Clearance (ICL): **2C19** > 1A2 > 2B6 > 3A4 > 3A5	Foxenberg et al. (2007) DMD 35(2):189–93	
	Human liver microsomes	Correlation of TCP and oxon formation with P450 activity	20 µM	CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4/5, CYP4A9/11	3A4/5 > 2B6 > 2C8 > 2C19	2B6 > 2C8 > 2C9 = 3A4/6	Croom et al. (2010) Toxicology 276:184–191	
			100 µM	CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4/5, CYP4A9/12	3A4/5 > 2C19 > 2B6 > 2C8	3A4/5 > 2B6 > 2C8 > 2C19 > 2A6		
		Total normalized rates of oxon and TCP formation		CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP3A4	3A4 > **2B6** > 1A2 = 2C19 = 2C9	3A4 > **2C19** > 2C9 = 2B6 = 1A2		

chlorpyrifos Cont.	Recombinant CYP450s	Formation of oxon and TCP		CYP2B6, CYP2C8, CYP2C9*1, CYP2C9*2, CYP2C9*3, CYP2C19, CYP3A4, CYP3A5, CYP3A7				Desulfuration/ Dearylation ratio: CYP2B6 > CYP2C9*1, CYP3A4, CYP2C19
diazinon	Human liver microsomes	Isoform specific inhibitors' effect on AChE inhibition	32.9 µM	CYP1A1, CYP1A2, CYP2C9, CYP2D6, CYP2E1, CYP3A4	2D6, 3A4		Sams et al. (2000) Tox Lett 116:217–21	
	recombinant CYP450s (Supersomes)	Formation of oxon and PYR	500 µm	CYP1A2, CYP2A6, CYP2B6, CYP2C9*1, CYP2C19, CYP2D6, CYP3A4	**2C19** > **2C9*1** > **1A2** > **2D6** > **3A4** > 2B6 > 2A6	3A4 > **2B6** > 2D6 > 1A2 > **2A6** = 2C9 > 2C19	Kappers et al. (2001) Tox Appl Pharm 177:68–76	Ratio of oxon/PYR formation: 2C19 > 2C9*1 > 1A2 > 3A4 > 2D6 > 2A6 > 2B6
		Inhibition of CYP450 reactions by diazinon	2.5-25 µm	CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4				No reaction products: 2C19 > 1A2 > 2C9 > 2D6
	Human Liver microsomes	Correlation with isoform activities	50 µM	CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4/5, CYP4A11	2C19, 1A2*			*oxon and PYR formation combined
			500 µM	CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4/5, CYP4A11	1A2, 2C19, 2D6*, 2C9*			*oxon and PYR formation combined
		Isoform specific inhibitors	50 µM	CYP1A2, CYP2C9, CYP2D6, CYP2C19, CYP3A4	3A4			
			500 µm	CYP1A2, CYP2C9, CYP2D6, CYP2C19, CYP3A4	3A4, 1A2, 2C9, 2C19(?)			
	Microsomes expressing single CYPs	Formation of oxons and aryl alcohols	100 µM	CYP3A4, CYP2C19, CYP1A2, CYP2B6, CYP1A1, CYP2C8, 2CYPC9, CYP2D6, CYP2A6	3A4 = 2C19 > 1A2 > 2B6 = 2C8 = 2C9 = 2D6 > 1A1 > 2A6	**3A4** > 2C19 > 1A2 > 2B6 = 2C8 = 2C9 = 2D6 > 1A1 > 2A6	Vitozzi et al. (2001) Environ Internat 26:125-9	

diazinon Cont.	Microsomes expressing single CYPs	Formation of oxon metabolite	0.5-25 µM	CYP1A2, CYP2C19, CYP2E1, CYP3A4, CYP2B6	Intrinsic clearance (ICL) values: 1A2 > 2C19 > 2E1 > 3A4 > 2B6		Buratti et al. (2002) Environ Tox Pharm 11:181-90	% Contribution: 1A2 > 3A4 > 2E1 > 2C19 > 2B6
		Formation of oxon and IHMP	25 µM	CYP2C19, CYP3A4, CYP2B6, CYP1A2	1A2 = 3A4 = 2B6 = 2C19	**2C19** > 1A2 = 3A4 = 2B6		
			100 µM	CYP2C19, CYP3A4, CYP2B6, CYP1A3	1A2 = 3A4 = 2C19 > 2B6	1A2 = 3A4 = 2C19 = **2B6**		
	Human liver microsomes	Correlation with P450 activity	250 µM	CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19,CYP2D6, CYP3A4, CYP2E1, CYP4A11	3A4, 2B6		Buratti et al. (2003) Tox Appl Pharm 186:143–54	
		Immunoinhibition	250 µM	CYP1A2, CYP2A6, CYP2B6, CYP2C, CYP3A4, CYP2E1	1A2, 2B6, 3A4			Highest inhibition: 2B6 + 1A2 + 3A4
	Human liver microsomes	Isoform specific activity	12.5 µM	CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3A4	2C19 > 1A2 > 2B6 > 3A4 > **2D6**	**2C19** > 1A2 > **2B6** > 3A4	Sams et al. (2004) Xenobiotica 34(10):861–73	
			100 µM	CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3A4	1A2 > 2C19 > 2D6 > 2B6 = 3A4	**2C19** > 1A2 > **2B6** > 3A4 > 2D6 > 2C9		
	Recombinant enzymes	Formation of oxon and IHMP	500 µM	CYP3A4, CYP3A5, CYP1A2. CYP2B6, CYP2C8, CYP2E1, CYP2D6*1, CYP2C19, CYP2C9*1	2D6*1 > 3A5 > 2C19 > 3A4 > 1A2 > 2B6 > 2C8	**3A5** > **2D6*1** > **2C19** > **3A4** > 1A2 = 2B6 = 2C8	Mutch & Williams (2006) Toxicology 224:22–32	Ratio of oxon:IHMP - 1A2 > 2B6 = 2C8 > 2D6*1 > 3A4 > 2C19 > 3A5
	Human liver microsomes	Correlation with P450 activity	50 µM	CYP3A4/5, CYP2C8, CYP2C19, CYP1A1/2, CYP2B6, CYP2E1, CYP2D6	3A4/5	3A4/5, 2C8, 2C19		
			500 µm	CYP3A4/5, CYP2C8, CYP2C19, CYP1A1/2, CYP2B6, CYP2E1, CYP2D6	3A4/5	3A4/5, 2C19		

fenthion	Recombinant enzymes	Formation of oxon	0-200 µM	CYP3A4, CYP3A5, CYP3A7	3A4	Burrati et al. (2006) Tox Lett 167:245–55	sulfoxide formation: CYP3A4>CYP3A5>CYP3A7
	Human liver microsomes	Immunoinhibition	5 µM	CYP3A4, CYP1A2, CYP2C9, CYP2B6, CYP2C19	2B6	Leoni et al. (2008) Tox Appl Pharm 233:343–52	
			100 µM	CYP3A4, CYP1A2, CYP2C9, CYP2B6, CYP2C19	2B6		
		isoform specific inhibitors	5 µm	CYP3A4, CYP1A2, CYP2C9, CYP2B6 + CYP2C19	1A2, 2B6 + 2C19		
			100 µM	CYP3A4, CYP1A2, CYP2C9, CYP2B6 + CYP2C19	1A2, 2C9, 2B6 + 2C19		
	Recombinant enzymes	Formation of oxon	"Low"	CYP1A2, CYP2C9, CYP2B6, CYP2C19, CYP3A4, CYP1A1	Intrinsic clearance (ICL) values: 2B6 > 1A2 > 2C19 > 2C9 > 3A4		% Contribution: 1A2 > 2B6 > 2C19 = 2C9 > 3A4
			"High"	CYP1A2, CYP2C9, CYP2B6, CYP2C19, CYP3A4, CYP1A1	Intrinsic clearance (ICL) values: 2B6 > 1A2 > 2C19 > 2C9 > 3A4		% Contribution: 2B6 > 1A2 > 3A4 > 2C9 > 2C19
malathion	Human liver microsomes	correlation with isoform activities	50 µM	CYP1A2, CYP2D6, CYP3A4, CYP2C9, CYP2B6, CYP2C19, CYP2E1, CYP4A11, CYP2A6	1A2	Buratti et al. (2005) DMD 33(3):295-302	
			400 µM	CYP1A2, CYP2D6, CYP3A4, CYP2C9, CYP2B6, CYP2C19, CYP2E1, CYP4A11, CYP2A6	3A4, 2B6		
		Immunoinhibition	50 µM	CYP3A4, CYP1A2, CYP2B6, CYP2A6, CYP2E1, CYP2D6, CYP1A1	1A2, 2B6*		*only in 1 of 3 microsomes tested
			400 µM	CYP3A4, CYP1A2, CYP2B6, CYP2A6, CYP2E1, CYP2D6, CYP1A1	3A4, 2B6		

malathion Cont.	Recombinant human P450s	Formation of oxon	0.01-50 µm	CYP1A1, CYP1A2, CYP2C19, CYP3A4, CYP2B6, CYP2C9, CYP2C8	Intrinsic clearance (ICL) values: 2C19 > 1A2 > 2B6 > 1A1 > 3A4 > 2C9 > 2C8			% Contribution: 1A2 > 2C19 > 3A4 > 2B6 = 2C9 > 2C8
	Recombinant enzymes	Formation of oxon	0-50 µM	CYP3A4, CYP3A5, CYP3A7	3A4 > 3A7 > 3A5		Buratti et al. (2006) Tox Lett 167:245–55	
parathion	Human liver microsomes	Isoform specific inhibitors on parathion metabolism*	250 µM	CYP3A4, CYP2E1, CYP1A2, CYP2C9/10, CYP2D6, CYP2A6, CYP2B6	3A4		Butler & Murray (1997) JPET 280(2):966–73	*sum of oxon and PNP formation
		Inhibition of CYP450 metabolism of testosterone	0-50 µM	CYP2C9, CYP3A4	2C9, 3A4			
	lymphoblastoid microsomes w/expressed CYP450 cDNA	production of oxon and PNP	250 µM	CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4	**2B6** > **2D6** = 3A4 > **1A2** > **2E1** > 2C19 > 2C9 > 2C8 > 1A1	3A4 > 2B6 > 1A2 > 2D6 > 2A6 > 1A1		
	Human liver microsomes	Correlation with isoform activities	200 µM	CYP3A4/5, CYP2B6, CYP1A1/2, CYP2E1	3A4/5		Mutch et al. (1999) Tox Lett 107:177–87	
		CYP class specific inhibitors	20 µM	CYP1A, CYP2B, CYP2C, CYP3A	3A > 1A = 2B = 2C			
			200 µM	CYP1A, CYP2B, CYP2C, CYP3A	3A > 1A = 2B = 2C			
		Inhibition of A-esterases with EDTA (5 mM)	1000 µm (saturating)	PON1?		72% of control PNP production		
	CYP450 expressing cell lines	Formation of oxon and PNP	200 µM	CYP3A4, CYP3A5, CYP1A1, CYP2B6, CYP2E1, CYP2C8 CYP2C9-Arg144, CYP3A4, CYP3A5	3A4 > 3A5 > 2B6 = 1A1 > 2C8	**3A4**		* only isoform to produce both oxon and PNP
	Human liver microsomes	Isoform specific inhibitors' vs. AChE inhibition	17.1 µM	CYP1A1, CYP1A2, CYP2C9, CYP2D6, CYP2E1, CYP3A4	2D6, 3A4		Sams et al. (2000) Tox Lett 116:217–21	

parathion Cont.	Microsomes expressing single CYPs	Formation of oxon	0.05-10 µM	CYP1A1, CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP3A4	Intrinsic clearance (ICL) values: 1A2 > 2B6 > 2C19 > 1A1 > 3A4 > 2C8 > 2C9		Buratti et al. (2002) Environ Tox Pharm 11:181-90	% contribution: 1A2 > 3A4 = 2C19 > 2B6 > 2C8 = 2C9
	recombinant CYP450s (Supersomes)	Formation of oxon and PNP	5 - 400 µm	CYP3A4, CYP3A5, CYP1A2, CYP2B6, CYP2C8, CYP2E1, CYP2C19, CYP2D6, CYP2C9*1	Vmax/Km: 1A2 > 2C19 > 3A5 > 2D6 > 3A4 > 2E1 > 2C8 > 2B6	Vmax/Km: 1A2 > 3A4 > 2B6 > 3A5 > 2C19 = 2C8 > 2E1	Mutch et al. (2003) Arch Toxicol 77: 313–20	At 5 µM, 3A4, 3A5, 1A2, & 2C19 most able to form oxon while 3A4, 3A5, & 1A2 produced most PNP
	Human liver microsomes	Correlation with P450 activity	200 µM	CYP2C8, CYP2C19, CYP3A4/5	2C8, 3A4/5			
		Correlation with P450 protein expression	200 µM	CYP2C8, CYP2C19, CYP3A4/6	2C8, 3A4 + 3A5			
		Correlation with P450 activity	5 µM		1A1/2			
		Correlation with P450 protein expression	5 µM		2C8			
	Human liver microsomes	Correlation with P450 activity	10 µM	CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19,CYP2D6, CYP3A4, CYP2E1, CYP4A11	1A2, 2B6		Buratti et al. (2003) Tox Appl Pharm 186:143–54	
			250 µM	CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19,CYP2D6, CYP3A4, CYP2E1, CYP4A11	1A2, 2B6, 3A4			
		Immunoinhibition	10 µM	CYP1A2, CYP2A6, CYP2B6, CYP2C, CYP2E1, CYP3A4	1A2, 2B6, 3A4			Highest inhibition: 1A2
			250 µM	CYP1A2, CYP2A6, CYP2B6, CYP2C, CYP2E1, CYP3A4	1A2, 2B6, 3A4			Highest inhibition: 2A6 + 1A2 + 2B6
	Recombinant enzymes	Formation of oxon	0-50 µM	CYP3A4, CYP3A5, CYP3A7	3A4 > 3A7 > 3A5		Buratti et al. (2006) Tox Lett 167: 245–55	

parathion Cont.	Recombinant enzymes	Formation of oxon and PNP	200 µM	CYP3A4, CYP3A5, CYP1A2. CYP2B6, CYP2C8, CYP2E1, CYP2D6*1, CYP2C19, CYP2C9*1	3A5 > 3A4 > **2D6*1** > 1A2 > 2C19 > 2E1 > 2C8 > 2B6	**3A4** > **1A2** = **3A5** > **2B6** > 2C19 > 2C8 = 2E1	Mutch & Williams (2006) Toxicology 224:22–32	Ratio oxon/PNP: CYP2D6*1 > 2E1 > 2C19 > 2C8 > 3A5 > 1A2 > 3A4 > 2B6
	Human liver microsomes	Correlation with P450 activity	5 µm	CYP3A4/5, CYP2C8, CYP2C19, CYP1A1/2, CYP2B6, CYP2E1, CYP2D6	2C8, 1A1/2, 2D6	1A1/2		
			20 µm	CYP3A4/5, CYP2C8, CYP2C19, CYP1A1/2, CYP2B6, CYP2E1, CYP2D6	2C8, 3A4/5, 2D6			No correlation with PNP formation observed
			50 µm	CYP3A4/5, CYP2C8, CYP2C19, CYP1A1/2, CYP2B6, CYP2E1, CYP2D6	3A4/5	3A4/5, 2C8, 2C19		
			200 µM	CYP3A4/5, CYP2C8, CYP2C19, CYP1A1/2, CYP2B6, CYP2E1, CYP2D6	2C8, 3A4/5			No correlation with PNP formation observed
			500 µM	CYP3A4/5, CYP2C8, CYP2C19, CYP1A1/2, CYP2B6, CYP2E1, CYP2D6	3A4/5	3A4/5, 2C19		
	Recombinant CYP450s	Formation of oxon and PNP	0-70 (?) µM	CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP3A4, CYP3A5, CYP3A7	Intrinsic clearance (ICL) values: 2C19 > 2B6 > 1A2 > 3A4 > 2C9 > 3A5	Intrinsic clearance (ICL) values: 2C19 > 1A2 > 2B6 > 3A4 > 2C9 > 3A7 > 3A5	Foxenberg et al. (2007) DMD 35(2):189–93	

(Bolded values indicate higher production of one metabolite over the other)

Vita

EDUCATION

- Doctor of Philosophy Candidate in Toxicology, Risk Emphasis Degree, Department of Environmental and Occupational Health Sciences (DEOHS), School of Public Health, University of Washington, Seattle, Washington. 2006-2012 (expected graduation in June). 3.80 cumulative GPA. Advisor: Prof. Elaine M. Faustman.
  Dissertation Title: "Integrating Biomarkers of Organophosphate Pesticides in an Agriculturally Exposed Population."

- Bachelor's of Science in Biochemistry, Minor in Chemistry; Bachelor's of Science in Zoology and Ecology/Evolution/Conservation Biology (Double Degree), University of Washington, Seattle. 2000-2004; 3.62 cumulative GPA, while working part time.

HONORS/AFFILIATIONS

Nominee, Student Representative for Risk Assessment Specialty Section (RASS), Society of Toxicology; February 2012
Society of Toxicology Graduate Student Travel Support Award; December 2011
2nd Place Student Poster Presentation (Tie), Pacific Northwest Association of Toxicologists Annual Meeting, North Bonneville, WA; October 2011.
DEOHS Outstanding Student Award for Doctoral Student - School of Public Health Awards Ceremony, 2011
NIEHS Environmental Pathology/Toxicology (EP/T) Training Fellow *October '08 - Present*
Society of Toxicology Graduate Student Member *'09-Present*
Beta Beta Beta Biological Honor Society, *March '03 – December '04*
Dean's List – 11 of 15 quarters during course of undergraduate studies
Annual Dean's List – '00-'01 and '03-'04 academic years

PUBLICATIONS

Robinson JF, **Z Guerrette**, Yu X, Hong S, Faustman EM. A systems-based approach to investigate dose- and time-dependent methylmercury-induced gene expression response in C57BL/6 mouse embryos undergoing neurulation. Birth Defects Research Part B: Developmental and Reproductive Toxicology 2010 89(3):188-200.

PRESENTATIONS

Curran, CA, TJ Black, BJ Granger, **ZN Guerrette**, KL Ireland, EA Marchesini, RC Schneider, CE Spardlin, MR Sternberg, JM Grassley, JL Cabarrus, WW Major III, and CE Grue, "Brain Cholinesterase Inhibition in Juvenile Rainbow Trout Exposed to Carbaryl - Benchmark Concentrations for Active Ingredient vs. Formulated Products," Pacific North West-SETAC 13th Annual Meeting; Port Townsend, WA; April 16th 2004. Presented by ZN Guerrette

POSTER PRESENTATIONS

Guerrette, ZN, EG Moreira, WC Griffith, DB Barr, GD Coronado, B Thompson, EM Vigoren, X Yu, RJ Richter, CE Furlong, EM. Faustman. "Association Between PON1 Status and Blood Cholinesterase Activities in Farmworkers." SOT 51th Annual Meeting, San Francisco, CA; March 2012.

McDonald, KM, WC Griffith, **ZN Guerrette**, EM Vigoren, M Vredevoogd, B Thompson, EM Faustman. "Characterizing Stability of Organophosphates in Home Environmental Dust." SOT 51th Annual Meeting, San Francisco, CA; March 2012.

Lin, Y, **ZN Guerrette**, T Aliwarga, T Senn, D Whittington, M Vredevoogd, EM Vigoren, EM Faustman. "Creating Virtual Biobanking Using a Global Metabolimics Approach." SOT 51th Annual Meeting, San Francisco, CA; March 2012.

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TEACHING EXPERIENCE

Teaching Assistant for undergraduate/graduate introductory toxicology class *March 08 - June 08*

- Guided students though material covering the toxic nature of natural and man-made compounds.
- Produced and presented a lecture on developmental and reproductive toxicology.
- Constructed exams using new and previously generated questions.

Teaching Assistant for a graduate course in risk assessment *September 07 - December 07*
September 08 - December 08

- Assisted students in their study of the risk assessment and management process.
- Provided a demo for Crystal Ball modeling software.
- Produced and presented a lecture on the application of toxicogenomics in risk assessment.
- Constructed exams using new and previously generated questions.

Lead Tutor for Howard Hughes Biology Fellows, *September 04 - June 05*

- Produced and organized study materials for student use during review sessions.
- Led students in discussion of material presented in the introductory biology courses.
- Assisted students with questions concerning laboratory and lecture material.