Occupational determinants of chlorpyrifos adducts to plasma cholinesterase in chlorpyrifos exposed agricultural workers in Washington State

Aaron Riutta

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Committee:

Christopher Simpson

Harvey Checkoway

Jonathan Hofmann

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Chapter 1: Background

Physical/Chemical Properties of Chlorpyrifos:

Chlorpyrifos is a broad-spectrum chlorinated organophosphorus (OP) insecticide, acaricide, and miticide utilized around the world to control a variety of pests. It is primarily a non-systemic contact poison that interferes with the activity of acetylcholinesterase (AChE), an enzyme essential for nervous system function for humans and insects alike. Chlorpyrifos is the common name for 0,0-diethyl-0-(3,5,6-trichloro-2-pyridinyl)-phosphorothioate. This chemical is a colorless to white crystalline solid that possesses a mild mercaptan-like (thiol) odor. With a low solubility of 1.12 mg/L of water at 24^{0}C (Racke, 1993) and being hydrophobic, chlorpyrifos is generally mixed with oily liquids instead of water before application (ATSDR, 1997). TABLE 1 below summarizes the physical and chemical characteristics of chlorpyrifos (Eaton, 2008).

TABLE 1: Chlorpyrifos—Physical/chemical properties

CAS no.	2921-88-2
Chemical formula	$C_9H_{11}Cl_3NO_3PS$
Molecular weight	350.57
Melting point	41–42^{0}C
Boiling point	Decomposes at ~160^{0}C
Density at 43.5^{0}C	1.398 g/cm^3
Water solubility at 20^{0}C	0.7 mg/L
Water solubility at 25^{0}C	2.0 mg/L
Organic solvent solubility	79% w/w in isooctane
	43% w/w in methanol readily in others
Partition coefficients	Log *K*ow4.82
	Log *K*oc3.73
Vapor pressure at 20^{0}C and 25^{0}C	1.87 × 10^{-5} mm Hg
Henry's law constant at 25^{0}C	1.23 × 10^{-5} atm-m^3/mol
Conversion factors at 25^{0}C	1ppm = 14.3 mg/ m^3
	1 mg/ m^3 = 0.07 ppm

FIGURE 1 below represents the chemical structure of chlorpyrifos. Like other OPs, chlorpyrifos is considered a derivative of phosphoric acid (H_3PO_4) or phosphonic acid (H_3PO_3) in which all hydrogen atoms are replaced by organic moieties and one or more (1 in the case of chlorpyrifos) oxygen atoms are replaced by a sulfur and/or nitrogen atom (sulfur in the case of chlorpyrifos) (Krieger et al., 2010). OPs also possess "L" or "Leaving" groups so named because they are the groups that are displaced when the OP phosphorylates acetylcholinesterase. The L group is the most reactive and variable substituent of the OP and is also most susceptible to hydrolysis (Krieger et al., 2010). In the case of chlorpyrifos the L group is trichloro pyridinol.

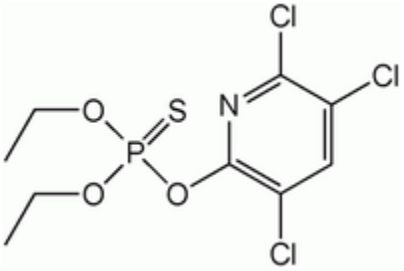

FIGURE 1: Chemical Structure of Chlorpyrifos

Environmental Fate and Transport:

Chlorpyrifos enters the environment primarily through direct application, but can also be found as a result of volatilization, from spills (leaching and/or runoff), improper disposal of chlorpyrifos contaminated waste and bio transfer by plants. Because of its tendency to adhere tightly to soil particles (mainly due to a strong affinity for soil colloids), it generally stays in the area where it has been applied, minimizing the likelihood of being

included in runoff and contaminating nearby waterways and groundwater sources (USGS, 1997). If it does mobilize and end up in surface waters, it is generally thought to remain on or near the surface where it tends to evaporate quickly (ATSDR, 1997). Chlorpyrifos has a vapor pressure of ~1.9 $X10^{-5}$mm Hg at 25^0C (Racke, 1993), which is considered "semi-volatile". In this form, chlorpyrifos can travel great distances if it is not degraded.

Chlorpyrifos has been shown to naturally degrade under sunlight (UV radiation), bacteria and certain chemical processes (ATSDR, 1997). In soils, it has been reported to have a half-life of several months whereas on plants it has a half-life more on the order of days to weeks (CDC, 2009). Overall chlorpyrifos has been shown to have a half-life 60-120 days under normal conditions (Krieger et al., 2010). Chlorpyrifos generally degrades to form TCP (3, 5, 6-trichloropyridinol), the principle metabolite of chlorpyrifos and then degrades further into organochlorine compounds and carbon dioxide (Krieger et al., 2010). Photo-oxidation and reaction with ozone (Junwang, 2010) have also been shown to convert chlorpyrifos into its oxon form, which is described in greater detail below but is considered to be a much more toxic form. When disposal of chlorpyrifos is necessary, the United Nations Environment Program recommends disposal of chlorpyrifos via incineration, adsorption and landfilling (IRPTC, 1989).

A major concern of scientists is the effects that OPs like chlorpyrifos have on non-target species. Although much of the focus of scientific research has been on human health effects (which will be discussed in depth later in this paper), the effects of OPs on other species are also of great scientific interest. The overall threat that chlorpyrifos poses to all organisms in an environment is nearly impossible to determine based on current scientific understanding. Inter-species susceptibility has been shown in different studies to be

extremely variable. In rats, for example, it has been shown that chlorpyrifos can act as an immunosuppressant (Krieger et al., 2010), whereas in four species of fish indigenous to Australia, chlorpyrifos had little to no detectable immunotoxicity (Harford et al., 2005). Conversely, organisms of the class aves (birds) have been shown to be particularly susceptible to OP pesticides (Kreiger et al., 2010). Ultimately chlorpyrifos has been shown, albeit to varying degrees, to be toxic to not only humans but many other organisms in a variety of ecosystems. As such is widely considered to be ecologically detrimental, especially if it is not controlled and monitored.

History of Use:

OPs like chlorpyrifos were originally developed to replace organochlorine pesticides. Organochlorine pesticides were banned in North America and Europe starting in the 1960s due to the fact that these pesticides broke down slowly in the environment and bioaccumulated to toxic levels in some species. OPs in contrast broke down much more rapidly in the environment than did organochlorines making them less persistent and presumably less of a health concern for humans and wildlife alike (Krieger et al., 2010). Chlorpyrifos was first registered by DowElanco in 1965 under the trade names Dursban as well as Lorsban, and was primarily used as a control for soil-borne insects on a variety of food crops. Chlorpyrifos has had a number of other trade names including: Brodan, Detmol UA, Dowco 179, Empire, Eradex, Paqeant, Piridane, Scout and Stipend. It has since been in heavy use, primarily in agricultural settings on crops ranging from, but not limited to: Cotton, corn, almonds, orange trees and apple trees. Up until 2001, Chlorpyrifos was considered by the manufacturer to be safe for widespread household use (U.S.EPA, 2006)

and was first registered for household use as a termiticide in the US in 1980 (Racke, 1993). Its role was later expanded to include the control of cockroaches, fleas and termites, and was the primary active ingredient in some pet flea and tick collars (ATSDR, 1997). In 1993 it was estimated that approximately 1.7 million pounds of chlorpyrifos was used in household settings annually (Cink, 1993).

As more research and case studies surfaced about the various human health effects associated with exposure to chlorpyrifos, the EPA banned its use in households in 2001, and the ban was fully implemented in 2002. Even pre and post construction, structural applications for the control of termites were phased out by 2005 (U.S.EPA, 2006). With the sharp decline in household usage of chlorpyrifos in the US (3% of pre restriction quantities), comparisons between a pre restriction time period of 1998-2001 and post restriction time period of 2002 through 2006 show a decrease in chlorpyrifos usage by approximately half (Eaton, 2008). This illustrates an important point, that although household application accounted for a sizable portion of chlorpyrifos use even in the face of its phasing out, chlorpyrifos was still being widely utilized in agricultural settings, with approximately 8.8 million pounds being used between 2002 and 2006 (Eaton, 2008). According to the EPA, the current use is mainly attributed to chlorpyrifos' current, registered uses including: food and feed crops, greenhouses, golf course turf, non-structural wood treatments, ant bait stations and as an adult mosquitocide.

Human Exposure, Metabolism and Health Effects:

Human routes of exposure to chlorpyrifos have been shown to differ depending on whether an individual or group works with the OP pesticide (agricultural workers like applicators and industry workers that formulate the chemical), or are in the general population. For the general population, the oral route dominates as the primary exposure pathway as the ingestion of contaminated food or soil is not uncommon (particularly for children with high hand-to-mouth transfer rates) (CDC, 2009). Dermal and inhalation routes are also prevalent in the general population (CDC, 2009). According to the Fourth National Report on Human Exposure to Environmental Chemicals by the CDC, the 1994 randomly selected US citizens surveyed from 1999 – 2000 had an overall geometric mean of a chlorpyrifos metabolite, (urinary 3,5,6-Trichloro-2-pyridinol, of 1.58 (95%CI = 1.35 – 1.85) micrograms/gram of creatinine. The next survey years of 2001 – 2002 saw an increase of the metabolite of chlorpyrifos in 2,508 citizens surveys to 1.73 (1.49 – 2.01) micrograms/gram of creatinine. Based on the years surveyed, it could be suggested that most of this exposure could have been attributed to house-hold use of pesticides, which would have still be phasing out of use having only just been banned a year earlier. It is important to note that while these data indicate that a significant proportion of the population had detectable levels of the metabolite of chlorpyrifos, suggesting exposure to the pesticide, the CDC study did not explore potential associations between chlorpyrifos exposure and any particular health outcome.

In contrast, occupational exposures are usually more attributed to inhalation and dermal routes (CDC, 2009). Numerous studies have looked into occupational exposures to chlorpyrifos ranging from looking at OP manufacturing (Albers, 2009, Burns 2005), to the

handling and application of pesticides in agricultural use (Hofmann 2009). As chlorpyrifos is still so widely used in agriculture, understanding its negative impact on humans and the risk factors associated with increased exposure is exceedingly important, especially since a dose-response curve has not been established for health effects associated with low dose exposure to the OP pesticide.

Once exposure occurs, organophosphorothioates, like chlorpyrifos, are converted into the oxon form which is considered more toxic (greater AChE inhibitor) than the original chlorpyrifos compound (CDC, 2009). After the formation of the oxon species, further metabolic processes result in hydrolysis which in turn leads to the formation of TCPy, dialkyl phosphate and other metabolites (CDC, 2009). Ultimately, chlorpyrifos has a half-life in the human body of around 27 hours, and it is primarily expelled from the body in the urine (Nolen et al., 1984). The main metabolic pathways for chlorpyrifos in humans are two catalytic reactions involving the enzymes: CYP and PON1. CYP (cytochrome P450) works to catalyze the oxidation of chlorpyrifos (formation of the oxon). PON1 (or serum paraoxonase/arylesterase) is coded by the *PON1* gene and is an enzyme that hydrolyzes some of the OP pesticides to varying degrees resulting in a metabolite that does not inhibit AChE. Diazinon oxon and chlorpyrifos oxon are effectively detoxified by PON1 whereas oxons of parathions are more effectively detoxified by CYP (Krieger et al., 2010). Other pesticides that have been commonly used in agriculture are not metabolized by PON1. Interestingly, not all organisms have the *PON1* gene and even if they can (as in the case of humans) they express it to varying degrees (Krieger et al., 2010), and may express different alloforms. These are determined by the Q192R polymorphism.

Cholinesterases (ChE) are a family of enzymes that serve as a catalyst for the hydrolysis of acetylcholine into choline and acetic acid. Acetylcholine plays a crucial role in nervous system function. ChE can be primarily classified into two types; acetylcholinesterase and butyrylcholinesterase (BuChE). The difference between the two types are that AChE is found primarily in the nerve synapses and red blood cells and hydrolyses acetylcholine more efficiently, whereas BuChE is formed primarily in the liver, is present in blood plasma, and hydrolyses butyrylcholine more quickly (Krieger et al., 2010). Chlorpyrifos, like other OP pesticides, acts through inhibition of neuronal cholinesterase enzyme activity (Ecobichon, 2001). When exposure to high levels of chlorpyrifos occurs, this inhibition produces signs indicative of excess acetylcholine and the resulting overstimulation of the peripheral and central nervous systems that use it as a neurotransmitter (Krieger et al., 2010). The inhibition of cholinesterase enzymes is known to result in an overabundance of acetylcholine at the nerve terminals and has been linked to acute effects like: nausea, vomiting, weakness, paralysis, seizures and cholinergic effects (CDC, 2009). Observations from studies on chlorpyrifos and other OP pesticides have found that OP exposure also may be linked with certain neurological effects, developmental disorders and autoimmune disorders (Lotti, 2001, Aldridge, 2005 and Betancourt, 2006). Long lasting/chronic impairments in neurobehavioral performance (reduced verbal attention, memory, visual attention, flexibility in thinking) have also been reported as a result of chlorpyrifos exposure (Krieger et al., 2010).

Key to this research specifically, is the fact that chlorpyrifos will bond with BuChE enzymes to form adducts. The BuChE-chlorpyrifos adducts form when the active site serine at the 198 position in BuChE binds to the central phosphorus atom of the chlorpyrifos-

oxon, displacing the trichloropyridinol (TCPy) leaving group. This leaves a diethyl phosphate adduct attached to BuChE. The diethyl phosphate adduct can spontaneously hydrolyze, regenerating active BuChE. Alternatively, the adduct can lose one of its two ethoxy substituents in a process known as 'aging', generating a monoethyl phosphate adduct that is resistant to further hydrolysis. The monoethyl phosphate leaves the BuChE permanently inactivated. Similar reactions occur with dimethoxy substituted OPs (eg. azinphosmethyl), creating methyl and dimethyl-phosphate adducts. The different adducts can be distinguished based on HPLC (high performance liquid chromatography) retention time and mass:charge ratio, which will be discussed in the methods section when we discuss the analytical process for measurement of adduct concentration.

In Washington State, chlorpyrifos is one of the most common/heavily used OP pesticides in use for agriculture, particularly around apple, pear and cherry orchards. As a result, chlorpyrifos has been the subject of a number of studies in the region, aimed at assessing exposures and/or contamination of agricultural workers (Hofmann, 2009, Ecobinchon, 2001, L&I, 2011 and Rauh, 2011). The health concerns surrounding exposure to chlorpyrifos have spurred groups such as the National Resource Defense Council (NRDC) and the Pesticide Action Network of North America (PANNA) to petition that it have its registrations cancelled altogether, although this has not been successful to date (PANNA, 2007).

Rules and Regulations:

Pesticide use is primarily regulated under two laws: the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) and the Food Quality Protection Act (FQPA). The

FQPA regulates allowable levels of pesticides in foods and FIFRA mandates that all pesticides distributed or sold in the United States be registered by the U.S. EPA (U.S.EPA, 2006). This registration must include scientifically based data, specifically showing that the pesticide will not cause unreasonable risk to human health or the environment when used as directed by product labeling. The problem is that recent studies have shown that agricultural workers are being exposed to high enough levels of chlorpyrifos to potentially result in adverse health effects despite adherence to printed safeguards (Hofmann, 2009). Although there is no established OSHA PEL for chlorpyrifos, NIOSH has established a REL TWA of 0.2 mg/m^3. This guidance was intended to protect the worker from the acute effects of chlorpyrifos, but does not take into account the potential for chronic effects and also assumes inhalation as the major source of exposure largely ignoring the dermal pathway. The CDC points out that human health effects arising from chlorpyrifos at low environmental exposures are unknown (CDC, 2009). It has been hypothesized that chronic exposure to OP insecticides at levels previously considered to be non-toxic may have an adverse effect on the human peripheral nervous system and result in neuropathy (Aldridge, 2005). Recently, research done using in vitro and in vivo animal studies suggest that effects on neurotransmission, neuronal morphogenesis and behavioral changes may occur at doses that are below where ChE inhibition can be detected (Aldridge, 2005, Betancourt, 2006, Howard, 2005, Riccer, 2006 and Slotkin, 2006). Laboratory animal studies have shown that even short term exposures at concentrations previously categorized as "non-toxic" can have a detrimental effect on a developing nervous system and can negatively influence emotional and cognitive functions, social responses and sex related behavioral patterns (Krieger et al., 2010). Other studies have also shown impaired neurodevelopment

of human children, and that a decrease in pesticide exposure decreases the degree of impairment (Krieger et al., 2010). In a longitudinal birth cohort study by Rauh et al. undertaken in 1997 - 2004, they noted a full scale intelligence quotient decrease of 1.4% and a working memory decrease of 2.8% per 4.61 picograms/gram increase in plasma concentration of chlorpyrifos metabolites (Rauh, 2011).

In 2004, Washington State's Department of Labor and Industries (L&I) implemented a program aimed at monitoring pesticide exposures amongst agricultural workers who handle or are potentially exposed to OP pesticides. Administered by the Division of Occupational Safety and Health (DOSH), the program requires agricultural employers to provide medical monitoring for workers who handle toxicity Category I or II organophosphorus or N-methyl-carbamate cholinesterase-inhibiting pesticides. Blood samples are collected from workers at baseline each year (i.e. prior to the start of each spray season) and tested for AChE and BuChE activity. Workers who handle cholinesterase-inhibiting pesticides for 30 or more hours in any consecutive 30-day period provide a follow-up (post-exposure, aka ”periodic”) blood sample that is also tested for AChE and BuChE activity. The post exposure and pre exposure activity measurements are compared, and percentage depression is calculated. DOSH assumes depression of AChE and BuChE activity is caused by exposure to pesticides, and set a level of >20% inhibition to as indicative of too much exposure.

In TABLE 2 below, the previous five years of data has been condensed and displayed together by year to show the temporal trends in DOSH’s Cholinesterase Monitoring Program. DOSH policy was updated in 2011 to include an enforcement approach in addition to its traditional consulting approach. This was put in place to give it some legal

"teeth" so as to be able to more effectively require employers to meet compliance standards, particularly if they have been warned in the past (L&I, 2011).

TABLE 2: Participation and Monitoring Outcomes in the Washington State Cholinesterase Monitoring Program since 2007*

	2007	2008	2009	2010	2011
# Growing operations**	226	218	217	315	388
# Handlers submitting baseline tests	1857	2013	2056	1989	2017
# Handlers declining testing	167	192	229	Data not collected	Data not collected
# Working baselines	120	71	29	51	43
# Handlers with >1 periodic test	386	314	249	257	186
# Periodic tests	532	495	286	316	202
# Handlers with ChE depression to work evaluation level	49 (12.6%)	21 (6.7%)	15 (6.1%)	8 (3.1%)	6 (3.2%)
# Handlers with ChE depression to exposure removal level	18 (4.6%)	1 (0.1%)	7 (2.8%)	0	0
Total # handlers with AL ChE depression	67 (17.3%)	22 (7.0%)	22 (8.8%)	8 (3.1%)	6 (3.2%)***
# Handlers with reported pesticide illness	0	0	0	0	0

* Participation and outcomes during the years that chemical analysis was conducted through the Washington State Public Health Laboratory (2004-06) is included in prior reports. http://www.lni.wa.gov/Safety/Topics/AtoZ/Cholinesterase/default.asp

**A growing operation is defined as a specific site or orchard. An employer may have multiple growing operations.

*** Four (4) Serum and two (2) RBC cholinesterase depressions.

[A] Work Evaluation Level refers to workers with >20% cholinesterase activity depression

[B] Removal Level refers to workers with RBC depression of ≥30% or serum depression ≥40%

[C] AL (Action Level) refers to workers with either work evaluation or removal levels

Washington State has developed legislation that is designed to protect the agricultural workers and can be found under WAC 296-307, called the "Safety Standards for Agriculture". This code not only includes proper tool use and rules related to other safety concerns, but specifically talks about pesticides like chlorpyrifos, and the requirements in place that surround their (pesticides) usage. Of particular interest are Parts H – J-1 that covers PPE (personal protective equipment) and includes the pesticide worker protection standard, requirements for pesticide recordkeeping and the cholinesterase monitoring rule. The aforementioned parts of the Washington State regulation reference related codes of federal regulation (CFRs) which provide a national framework and were designed to protect everyone (not just specifically workers).

Current regulations rely on the test that measures ChE % depression. There are a number of issues with this test that make looking for alternatives to it a worthwhile venture. For example, measurement of ChE & depression is not very sensitive (LLOD ~20% as discussed below). It is also not very specific to a given pesticide as a number of pesticides, like diazonon and parathion, and even non-pesticide exposures, can depress activity of ChE enzymes.

Hypothesis & Specific Aims:

Our primary hypothesis was that measurement of chlorpyrifos adducts to BuChE provide a reliable indicator of occupational exposure to chlorpyrifos. To test this hypothesis we addressed the following specific aims:

Specific Aim 1: Using data obtained from pesticide handlers and applicators from Central Washington, we assessed the relationship between chlorpyrifos-BuChE adduct fraction and depression of BuChE enzyme activity (which is the current/established "gold standard").

Our second hypothesis is that formation of chlorpyrifos adducts to BuChE will be modified by *PON1* status. To test this hypothesis we addressed the following specific aim:

Specific Aim 2: We assessed the relationship between indicators of *PON1*. The indicators of *PON1* we looked were; arylesterase, paraoxonase, diazoxonase, C-108T genotype, Q192R genotype and Q192R phenotype.

Our third hypothesis is that measurement of chlorpyrifos adducts to BuChE can be used to identify risk factors for chlorpyrifos exposure in agricultural workers. To test this hypothesis we addressed the following specific aim:

Specific Aim 3: We identified potential occupational practices that significantly affect chlorpyrifos-BuChE adduct fraction.

Chapter 2: Methods

Analytical Methods:

With an ever increasing interest both on the part of the civilians (workers and employers) and the government, the need to clearly identify factors associated with excess occupational exposure to chlorpyrifos is critical. The method utilized as the "gold standard" to infer exposure is ChE percent depression. BuChE depression is sometimes preferred over AChE depression because, although both are widely considered to be good surrogate markers for early biologic effects related to OP/carbamate (CB) pesticide exposure (USEPA, 2000), BuChE inhibition is considered to be a more sensitive marker of exposure. This is because OP pesticides like chlorpyrifos, diazinon and malathion inhibit BuChE to a greater degree than they do AChE (Fenske et al 1997, Lotti 2001). Aim 1, in effect, tests how the measurement of adduct concentration/fraction compares to the "gold standard" currently used (i.e. BuChE% depression).

Blood samples were collected from each participant and the following variables were determined: *PON1* status/Q192R phenotype, *PON1* genotype, AChE activity, BuChE activity, chlorpyrifos-BuChE adduct concentration as described briefly above. *PON1* Q192R phenotype was assigned based on the ratio of diazoxonase to paraoxonase activity. Determination of *PON1* status involved the measurement of arylesterase activity; and determination of the Q192R phenotype as described above. In addition, the University of Washington Center for Ecogenetics and Environmental Health Functional Genomics Laboratory (Seattle, WA) assessed two *PON1* single nucleotide polymorphisms (SNPs): Q192R (rs662, amino acid change) and C-108T (rs705379 in the 5' promoter region that

affects PON1 levels). The *PON1* genotyping assays utilized the ABI TaqMan detection system based genotyping assays. DNA extraction was completed for each blood sample using the QIAGEN Qiamp DNA Blood mini kit (QIAGEN, Valencia, CA). Each assay was performed in triplicate according to protocols established by the Furlong laboratory using a plate reader.

Study Participants and Dataset:

This project focuses on the data from a cross-sectional study of 92 central Washington State agricultural workers that were enrolled in the much larger Washington State's Department of Labor & Industry's Division of Occupational Safety and Health (DOSH) administered Agriculture Cholinesterase Monitoring Program. This study's focus is on workers that were potentially exposed to chlorpyrifos during the OP/CB spray seasons (April-July) and provided both a baseline (pre-spray) and follow-up (post-exposure) sample spanning four years (2007-2010). The Central Washington Occupational Medicine (CWOM) Clinic (Yakima, WA) collected blood samples on our behalf, in parallel with the samples they were collecting for the State's pesticide monitoring program. CWOM notified the UW when follow-up testing was to occur on workers. The subjects were recruited at the clinic during their follow-up testing. In all, 232 serum samples were taken over this four year period, including 128 from subjects for whom we attempted to assess adduct fraction. The majority of these samples, classified as "follow-up", were collected after workers handled OP or carbamate pesticides for 30 hours in a 30-day period. A smaller set of samples, classified as "depressionary follow-up", were collected after workers tested positive for ChE depression during a periodic sampling event earlier in the season. The

remaining few samples were classified as “working baseline,” indicating the worker did not provide a baseline sample prior to handling pesticides. Samples were stored frozen (-80^{0}C) before use.

A total of 128 samples were selected to be analyzed for OP adducts. A pilot batch of eight samples was analyzed first to evaluate the assay. Of these eight, four had no ChE depression while the other four had depressions greater than 20% relative to the individuals’ baseline samples. Two of the four depressed samples were depressionary follow-up samples. Following analysis of the pilot batch, 100 samples were selected randomly from the remaining set. However, prior to selection, all working baseline samples and the remaining depressionary follow-up samples were removed from the collection of possible samples. Following selection of the 100 random samples, all remaining samples with at least 20% depression and all remaining depressionary follow-up samples were selected. These 128 samples will serve as the source of data for this thesis.Since the information on subject demographics is based on the computer survey results, the demographics only represent the survey subpopulation (N=92) and not necessarily the overall number of samples collected (N=128). This discrepancy is attributed to the fact that the dataset included many duplicates by person over the four years of data collection. Characteristics associated with these 92 samples are described in Table 4.

Upon agreeing to be a part of this study, participants were given a computer-based survey (see appendix A for the survey format) on tablet computers to collect the desired information. Of the 128 samples, 120 (94%) of those samples had associated surveys. The 6% loss of sample pool is may be due to the fact that some of the samples were depressionary follow-up samples, taken from workers who had already filled out the

survey.

Due to the majority of workers being Hispanic, the survey was administered in either English or Spanish depending on the language preferred by the given participant. The survey itself was designed by the University of Washington's Pacific Northwest Agricultural Safety and Health Center (PNASH) and has been administered since 2006 (Hofmann, 2009). These survey questions were displayed on the tablet computer screen along with an audio-recording in addition to icons or photos used to represent possible answers for the questions.

The survey, in its entirety, consisted of 64 questions included with their labels and possible answers. The following information was collected via the survey for each participant: Crops treated, pesticides used, pesticide handling activities performed, spray equipment used, duration and frequency of pesticide handling, PPE used and the condition and storage of said PPE, decontamination practices, acute exposure events, pesticide safety training, symptoms potentially related to OP/CB exposure, non-occupational risk factors for BuChE inhibition and subject demographics. Questions related to potential sources of exposure were limited to 30 days prior to the interview and follow-up ChE test. This is because it has been suggested by M Lotti, (2001) that this 30-day period would be the most etiologically relevant in terms of risk of BuChE inhibition, as the BuChE activity levels recover over time.

Statistical Analysis:

For this project we used StatCorp LP's STATA11 statistical software.

The analysis will be broken into three parts that will mirror our specific aims:

Aim 1: Our first specific aim will be addressed by first calculating the Spearman's rank correlation coefficient to determine how well chlorpyrifos-BuChE adduct fraction and percentage depression of BuChE enzyme activity relate to one another. Also of interest is the sensitivity and specificity of the adduct fraction test compared the BuChE percent depression.

Aim 2: For the second aim, we use a linear regression model to assess the potential relationship between chlorpyrifos-BuChE adduct fraction and *PON1* activity (namely arylesterase), and also looked at this relationship when stratified by Q192R phenotype.

Aim 3: For our third aim we sought to determine what characteristics of the participants and their work practices were associated with the presence of chlorpyrifos adduct.To address this, we utilized a Chi Square or Fisher's Exact tests (depending on the expected n values), followed by logistic regression on those variables that are found to be statistically significantly associated (i.e. p-value < 0.05).

Determination of OP Adducts to BuChE:

The crux of this research is our ability to determine chlorpyrifos adduct fraction derived from individual worker's blood samples. Blood sample volumes in this study ranged from 0.6 – 1.8 ml with a mean of 1.3 ml. For the determination of the concentrations of the chlorpyrifos-BuChE adduct, the samples were analyzed using HPLC/MS/MS, with the mass spectrometer operated in the Multiple Reaction Monitoring (MRM) mode. Whole blood samples were thawed and volumes were measured. Sample

volume permitting, 1.8 ml was applied to a 2-ml procainamide affinity gel column. When less than 1.8 ml was available, the entire sample was used and the actual volume was recorded. The procainamide gel and blood sample mixture and allowed to set for 30 minutes for binding of BuChE. Following incubation, the gel was washed with 20mM phosphate buffer containing 100mM sodium chloride to remove unwanted proteins and other blood components and then the BuChE was eluted with the same phosphate buffer containing 1000mM sodium chloride. The eluate containing BuChE was concentrated by centrifugal filtration and washed twice with 50mM ammonium bicarbonate to remove the phosphate buffer and salt. The final concentrated BuChE sample was acidified with formic acid, amended with pepsin and incubated for two hours at 37ºC. Following pepsin digestion, samples were spiked with internal standards (13C-labelled nonapeptide [FGES*AGAAS] and 13C/15N-labelled nonapeptide containing a phosphoserine residue). Peptides of interest were separated from pepsin and larger peptides by another centrifugal filtration step.

Extracts and calibration standards were analyzed by HPLC-MS/MS. Transitions were monitored to identify the unadducted BuChE active site peptide and six adducts of that peptide including methylphosphate (MMOP), dimethylphosphate (DMOP), ethylphosphate (MEOP), diethylphosphate (DEOP), phosphoserine and dehydroalanine. Quantification was conducted with the internal standards mentioned previously and external calibration standards of the unmodified nonapeptide and the nonapeptide containing a phosphoserine residue.

Quality Control Procedures:

Samples were typically prepared in batches of eight or sixteen. Included with each batch were a method blank and one or two positive control samples. The positive control samples were prepared at the onset of the project by pooling blood from four unexposed individuals and then treating different portions with chlorpyrifos oxon or methylparaoxon. Some of the treated blood was allowed to age at 37ºC before being recombined, mixed, separated into 1.8 ml aliquots and stored at -80ºC. Positive control samples contained the four main adducts (DMOP, MMOP, DEOP, MEOP) as well as unadducted nonapeptide and provided a tool to monitor intra and inter-batch precision as well as consistency of the HPLC-MS/MS instrument. Additional quality control procedures for monitoring LC-MS/MS performance included analysis of solvent blanks and repeated analysis of selected samples and calibration standards.

After LC/MS/MS analysis, the resulting peaks were reviewed for quality and assigned a peak code according to the criteria described in TABLE 3 below.

TABLE 3: Peak Code Determination Criteria for the Adduct Concentration

Peak Code	Criteria
1	No peak above noise level detected at any MRM transition
2	Quant MRM above noise level; both qualifiers at noise level
3	Quant and one qualifier ≥2 times the noise; other qualifier at noise level
4	All three transitions ≥2 times the noise
5	All three transitions substantially greater than the noise level

The LOQ was calculated only for the monoethyl adduct because no other adducts were detected. The nonapeptide was abundant in every sample so it was not necessary to calculate the LOQ for the nonapeptide in the blood-extract matrix. Three MRM transitions

were collected for each sample in order to characterize the adduct concentrations; one was used for quantification and the other two qualifiers, were used for confirmation. Thirty-one of the 128 samples were assigned with a peak code of 1 (i.e. these samples were considered non-detects) and thus were used to calculate the LOQ. The noise level was quantified for these 31 samples by integrating the background noise peak at the expected retention time of the monoethyl adduct. The noise peak areas were divided by the corresponding phosphoserine nonapeptide internal standard and the ratio was converted to a concentration using the calibration curve associated with that sample batch. Calibration was by linear regression with a 1/concentration weighting factor. The calculated concentration in the extract was converted to blood concentration by dividing by the actual blood volume used for each individual sample. Mean and standard deviation values were calculated for both the extract and blood concentrations.

The LOQs for the extract and blood concentrations were calculated as the mean plus two times the standard deviation of the "concentration" of the noise peaks. The extract and blood LOQs were converted to an "adduct fraction" LOQ by dividing the LOQs by the sum of the mean nonapeptide concentration plus the LOQ/square root of two. For this dataset the LOQ for the monoethyl adduct was calculated as 0.7101 pg/µl blood and the monethyl adduct as a fraction of the total adducted plus the non-adducted nonapeptide is 0.06. In this study we will use the commonly accepted method of inferring values less than or equal to the LOQ as LOQ divided by the square root of two. When this is done, our values for the adduct concentration are 0.5021 pg/µl blood and the adduct fraction value is 0.042 (4%) in blood.

Chapter 3: Results and Discussion

Variables of Interest:

In addition to the demographic data, we were interested in factors that have been found in previous studies to be correlated either positively or negatively to BuChE depression. The primary variables of interest for specific aim 1 are BuChE % depression and chlorpyrifos adduct fraction. Aim 2 was interested in looking at indicators of *PON1* and the relationship of those indicators and chlorpyrifos-BuChE adduct fraction. As such, the variables of interest here were arylesterase activity and *PON1* Q192R phenotype. We were also interested in looking briefly at paraoxonase activity as it related to adduct fraction. The variables of interest for specific aim 3 in this study were based on the literature (Hofmann, 2009) and are as follows: chlorpyrifos adduct fraction, smoking status, use of full faced respirators, use of chemical resistant boots, storage of PPE at work, mixing and loading of pesticides and whether a worker cleaned the spraying equipment. In addition to the above variables, there are other variables that will also be examined based on Hofmann's (2009) findings. Unlike the variables above, the following variables were not shown to be statistically significant in their correlation with BuChE% depression, but had comparatively low p-values (<0.20). These variables are as follows: Cleaning pesticide containers, gloves worn under nitriles, use of chemical resistant apron, and number of activities without decontamination (2 and 3+). The selection of the 0.20 p-value cutoff was selected to provide a greater range of variables that might be more likely to be correlated with adduct formation in blood.

TABLE 4: Subject Demographics and Work Characteristics

Characteristic	Subcategory	N=92 n (%)
Race	Hispanic	91 (99)
	African-American	1 (1)
	Other	0 (0)
Sex	Male	92 (100)
	Female	0 (0)
Crops*	Apples	78 (91.8)
	Cherries	13 (15.3)
	Grapes	7 (8.2)
	Pears	29 (34.1)
	Peaches	4 (4.7)
	Apricots	2 (2.4)
	Plums	1 (1.2)
	Other	0 (0)
	Multiple	35 (41.2)
	Unsure	0 (0)
Cigarette use	Non Smoker	58 (69.0)
	Less than every day	16 (19.0)
	Every Day	10 (11.9)
Alcohol use	Never	28 (33.3)
	<1 day/week	22 (26.2)
	1 day/week	23 (27.4)
	2-3 days/week	7 (8.3)
	4-6 days/week	1 (1.2)
	Every Day	3 (3.6)

* These characteristics are NOT mutually exclusive
** This field represents those who responded that they received training but did not know if the instructor was trained/certified

The adduct levels are reported as a fraction (i.e. ratio of adducted cholinesterase over total cholinesterase (non-adducted plus adducted) as opposed to being left as a concentration. The reasoning behind the fraction being preferred over the absolute concentration is because BuChE levels vary widely between individuals depending on their age and health status (Lotti, 2001). Adduct fraction is an analogous measurement to the percent change metric of the state legislation regarding cholinesterase inhibition. Thus policy makers can easily interpret our findings and put our results into the proper context.

Although there is a concern of other OP pesticides interfering with the BuChE% depression signal, this concern is not shared when looking at the chlorpyrifos adduct fractions of the workers.

Results from our analyses are described below. In our sample set, the adduct fraction data were not normally distributed, whereas % BuChE inhibition was. Therefore, a simple linear regression or linear mixed effects regression using adduct as the outcome was not pursued. For Aims 1 and 2 we focused on the indicators commonly associated with *PON1*. For our analysis in Aim 3, we converted adducts and BuChE % depression data points from continuous into categorical data. This was done because we were more interested in looking at comparisons and relationships from the standpoint of pass/fail (direction) more so than looking for a dose-response relationship (magnitude) of sorts. For the purposes of our study we were more interested in determining if people who wore a sweatshirt under their PPE while spraying were more likely to yield detectable adduct levels than we were at finding out how much adduct formation occurred as a result of wearing the sweatshirt.

TABLE 5: Summary of adduct and BuChE data

Parameter	**N = 127 n (%)**	**Mean**	**Median**	**Range**
Adduct fraction	56 (44)	0.1486	0.0423	0.038 – 0.726
% BuChE depression	20 (16)	-0.0863	-0.0699	-0.166 - 0.491

Aim 1:

The goal of aim 1 is to compare the new adduct analysis to the currently utilized BuChE % depression test. The hope would be that the adduct test might be able to provide a more sensitive option in order to better detect ChE depression in agricultural workers.

With a more sensitive test, workers will be protected to a greater extent because the method will identify those workers that have been exposed to chlorpyrifos that might have been missed by the BuChE test.

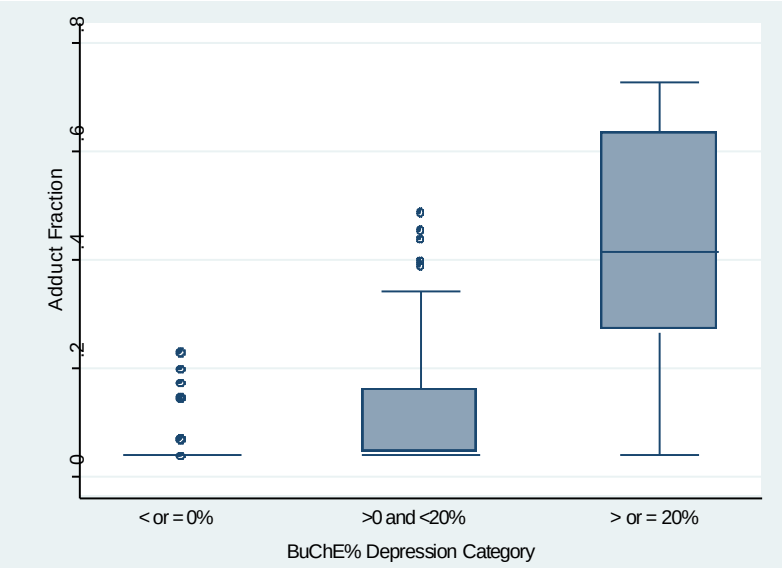

FIGURE 2a: Box Plot of BuChE % Depression (Categorical) v. Adduct Fraction (Continuous)

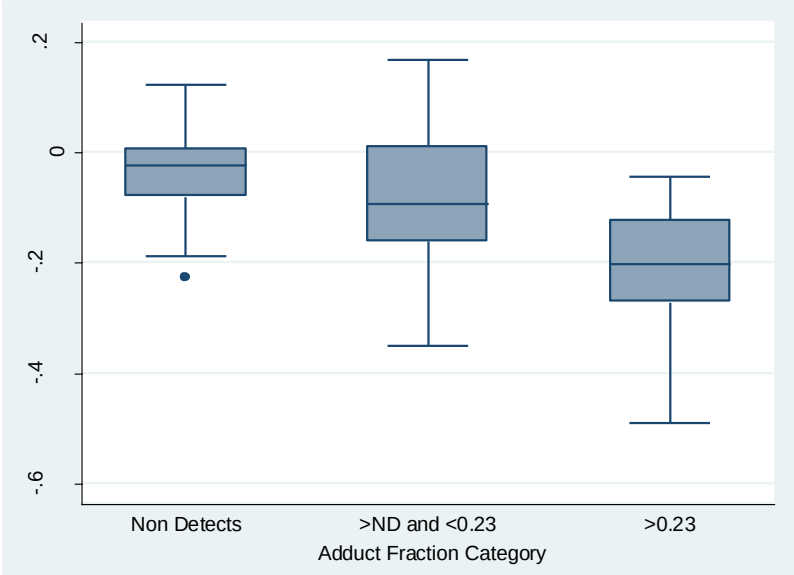

FIGURE 2b: Box plot of change in BuChE (i.e. BuChE depression) v. adduct fraction category

The above graphs are botha box and whisker graphs that show how adduct fraction compares to BuChE% depression. FIGURE 2a shows measurements of adduct fraction level (Y-axis) stratified by BuChE % depression category (X-axis), with 0 being those people that had less than or equal to 0% depression and are therefore definitely not experiencing BuChE depression, subjects falling under the 1 category are the people that are have BuChE depression of greater than 0% but less than 20% ("maybe" depressed), and category 3 represents the subjects that would be considered to have plasma cholinesterase depression based on the current Washington State Cholinesterase Monitoring Program threshold of greater than or equal to 20% (Smith, 2011). FIGURE 2b shows the BuChE depression (continuous) by adduct fraction category and reflects what was seen in FIGURE 2a, that the higher the levels of adduct you detect, the more BuChE Depression you will observe in our dataset. Below is a table summarizing the box plot above, and also presents the mean and standard deviation of adduct fraction for each of the three categories of BuChE% depression.

TABLE 6: Adduct fraction by BuChE % Depression Category

BuChE Category	**1***	**2****	**3*****
N	30	76	20
1%	0.04	0.04	0.04
25%	0.04	0.04	0.27
Median	0.04	0.04	0.41
75%	0.04	0.17	0.64
99%	0.23	0.49	0.73
Mean	0.07	0.11	0.41
SD	0.05	0.12	0.22

*This category represents samples that were less than or equal to 0% depression
**This category represents samples that were greater than 0% but less than 20%
***This category represents samples that were greater than or equal to 20%

We also evaluated the degree of association between the two assays. Since, the BuChE% depression is the current method used for regulatory purposes, this will serve as our reference variable, with adduct fraction as the test variable. We used a Spearman's correlation test because the adduct data is not normally distributed. For this test we obtained a Spearman's rho of -0.6014 indicating a degree of correlation between the two assays ($p < 0.0001$). The direction of the association is negative because the BuChE % inhibition was expressed as a negative value.

In addition, we determined the sensitivity and specificity of the adduct fraction method when compared to the BuChE% depression. Consistent with the Washington State Cholinesterase monitoring program, a decrease in BuChE activity of 20% or more was used as the threshold to define a positive outcome from the BuChE activity assay. The adduct fraction was coded in a binary fashion – "detected" or "not detected". The results of this analysis can be seen below.

The sensitivity for this analysis is very high at 95% which indicates that only 5% of the samples (N=1) that were classified as positive for BuChE% depression were negative for OP-BuChE adducts (failed to be identified by the adduct assay). The specificity (66%), on the other hand was not as high, meaning that of the people that were classified as negative with respect to BuChE% depression, 34% were (incorrectly according to the gold standard) found to be possess measurable levels of the OP- adduct. On the surface this might seem to be a less than ideal value, but this is actually a good result when it is considered that the adduct fraction test is much more sensitive in its ability to detect a previous exposure. With a lower limit of detection of just over 4% (0.042) as discussed above in the methods section, the adduct assay is much more sensitive than the currently

utilized ChE/BuChE test, which sports an LLOD of 20%. With this in mind the 66% specificity makes sense as the adduct test would pick up many potential exposures that the BuChE test would not.

The last test that we performed as a part of aim 1 was to create a receiver operator character (ROC) curve, and estimate the area under it. By including both the sensitivity and specificity simultaneously, inclusion of an ROC curve is important because it will provide for a good measure of overall performance when compared to the BuChE % depression test (0.5 or lower indicates an uninformative test) (Searles Nielsen, 2008). The ROC curve can be seen below in FIGURE 3. The area under the curve was 0.91 meaning that the results of our assay yield results that are more than chance alone ($p<0.001$).

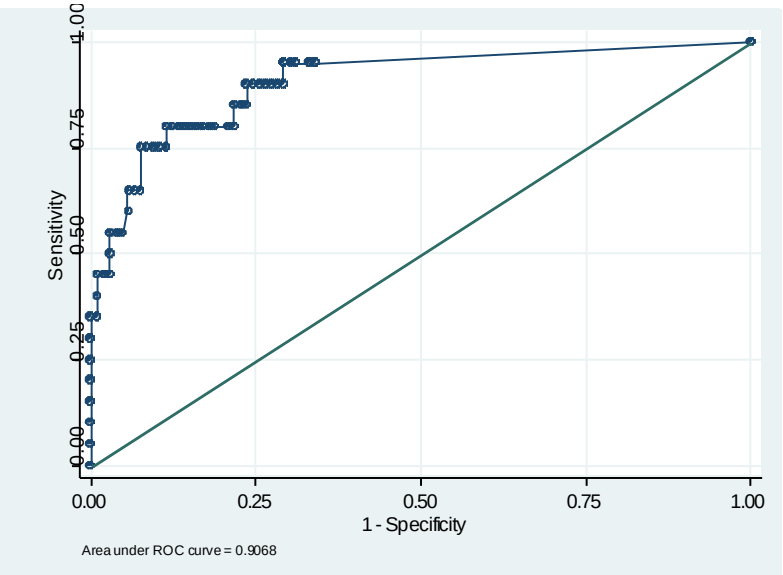

FIGURE 3: ROC for BuChE% Depression (Categorical-dichotomous) v. Adduct Fraction (Continuous)

The 20% BuChE % depression cut off used in the state program is based on a formal analysis of variance in Washington state data in 2004 (SACCM, 2004). There is some debate on whether this value is appropriate. With this question in mind we performed two additional

sensitivity analyses at lower cut off values for BuChE% depression using our dataset. TABLE 7 below compares the findings for both sensitivity and specificity and also shows our calculated ROC area under the curve for each cut off including the currently used 20%. We can see that as you increase the cut off, you get a trade off with much better sensitivity (net gain of 23%) for lower specificity (net loss of 8%). The ROC curve also yields favorable results for the current cut off as discussed above.

TABLE 7: Comparison of Sensitivity, Specificity and ROC Curve Analyses for three different BuChE % Depression Cut Offs.

BuChE Cut off	Sensitivity	Specificity	ROC Area*
10%	72	74	0.7910
15%	86	68	0.8353
20%	95	66	0.9068

*The ROC curve was based on the continuous adduct fraction data, which was not the case for the sensitivity/specificity values that were based on the dichotomous adduct variable (detect v. non-detect)

Aim 2:

The goal of Aim 2 was to test the relationship between adduct fraction (continuous) and various indicators of *PON1*. The indicators of *PON1* we investigated were arylesterase activity, paraoxonase activity, diazoxonase activity, C-108T genotype, Q192R genotype and Q192R phenotype. Ultimately, only the continuous arylesterase variable turned out to be strongly associated with adduct ($p<0.001$). It is worth noting that all of the categorical indicators of PON1 and the categorical/tertile variables were not found to be statistically significantly associated with adduct fraction with the lowest p-value being found with arylesterase at $p = 0.224$. That said, we were still interested in seeing what effect PON1 Q192R phenotype might have on adduct fraction, and on the relationship between arylesterase and adduct fraction. Arylesterase has two associated graphs (FIGURES 4 and 5) comparing the tertiles of arylesterase activity (surrogate for PON1 levels) to adduct

fraction without, and with stratification by *PON1* Q192R phenotype respectively. Traditionally, arylesterase activity is considered to be the best indicator of *PON1* levels, and previous studies have shown an association between low levels of *PON1* enzyme and susceptibility to BuChE enzyme inhibition due to chlorpyrifos exposure (Hofmann, 2009), and so this project will begin there.

FIGURE 4 below shows the general relationship between arylesterase activities (divided into tertiles) and adduct fraction. According to FIGURE 4, there is a general positive trend of increasing adduct fraction as the arylesterase activity increases (as seen in the arylesterase tertiles). We note that the relationship is opposite to what we might have expected, with higher levels of arylesterase being associated with high adduct fraction being observed. One thought on this interesting finding is that the workers with low levels of *PON1* (low arylesterase activity) might be "self-selecting" into jobs or tasks that result in lower levels of exposure as they might be experiencing more overt symptoms associated with CPF exposure.

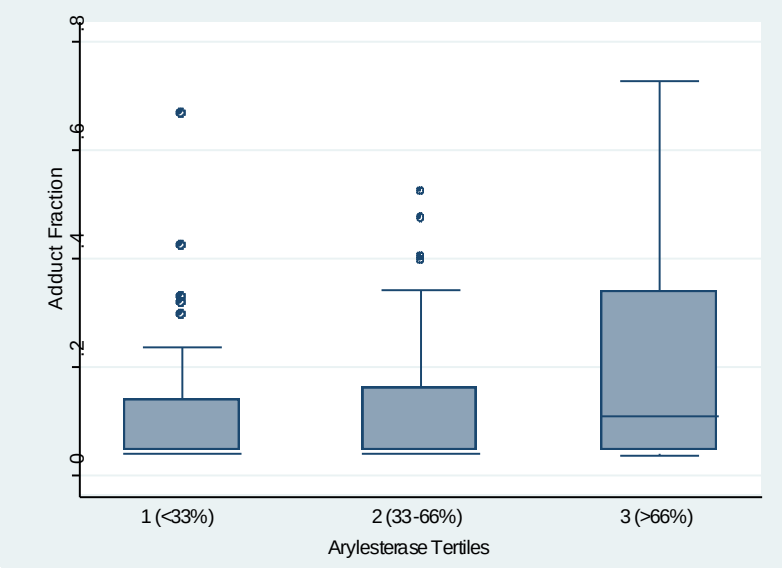

FIGURE 4: Box plot showing the relationship between Adduct (Continuous) and Tertiles of Arylesterase activity.

1 refers to the bottom tertile of AREase activity (U/mL), 2 the middle and 3 the upper.

FIGURE 5 below, takes the same relationship between AREase and adduct fraction after stratification by PON1 Q192R phenotype. The critical thing to emphasize in FIGURE 5 is the effect of PON1 Q192R phenotype. In particular, subjects with the RR phenotype (high catalytic efficiency form of PON1) have lower adduct fractions than subjects with QR or QQ phenotypes. This can also be seen in TABLE 8.

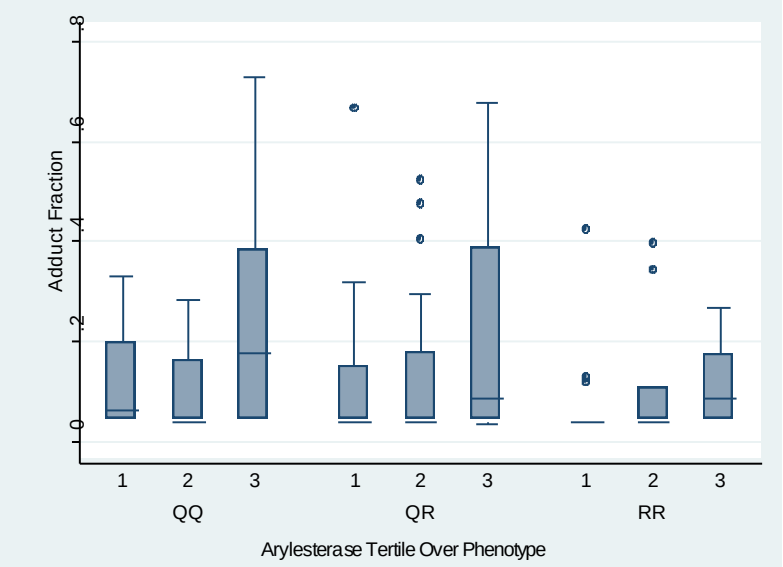

FIGURE 5: Box Plot Showing the Relationship Between Adduct (Continuous) and Arylesterase Tertiles Stratified by *PON1* Q192R Phenotype.
1 refers to the bottom tertile of arylesterase concentration (U/mL), 2 the middle and 3 the upper tertile.

TABLE 8: CPF-BuChE adduct fraction by Arylesterase Activity (U/mL), stratified by Q192R Phenotype

	Arylesterase Activity		
Q192R Phenotype	**Low (<113)**	**Moderate (113 – 160)**	**High (>160)**
QQ	n = 12	n = 11	n = 17
Adduct frn (mean+sd)	0.126+0.108	0.091+0.081	0.244+0.246
QR	n = 16	n = 21	n = 20
Adduct frn (mean+sd)	0.136+0.164	0.135+0.158	0.210+0.220
RR	n = 14	n = 11	n = 5
Adduct frn (mean+sd)	0.081+0.103	0.113+0.130	0.124+0.098

We were also interested in seeing if there was an interaction between arylesterase activity, adduct fraction and Q192R phenotype. This was tested by performing a likelihood ratio test. The LRT p-value was not significant suggesting there was no interaction (p = 0.466).

Of the three enzyme activities measured (arylesterase, diazoxonase, paraoxonase), paraoxonase was also associated with adduct fraction (p = 0.173). In this case, high paraoxonase activity was associated with a decrease in adduct fraction, as would be expected. This relationship can be seen in FIGURE 6.

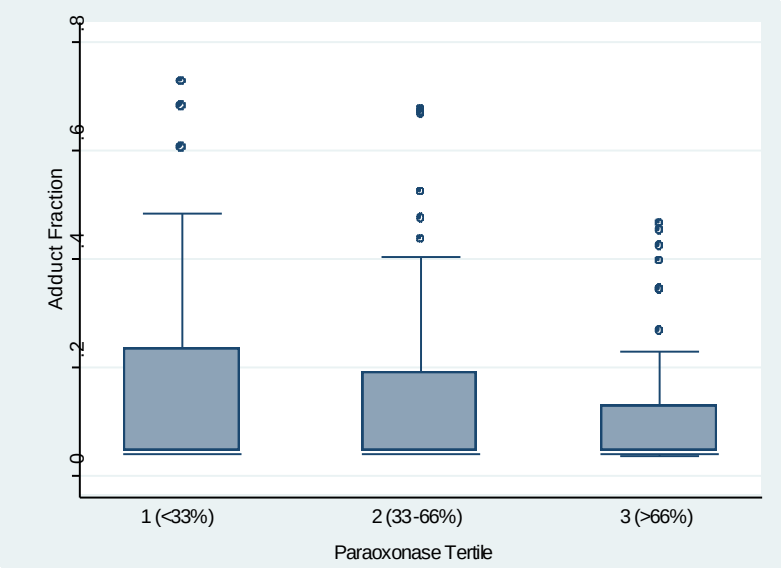

FIGURE 6: Box Plot showing the Relationship Between Adduct Fraction (Continuous) and Tertiles of Paraoxonase Activity.
1 refers to the bottom tertile of paraoxonase activity (U/mL), 2 the middle and 3 the upper.

Aim 3:

Now that we have established that the adduct assay is a viable test with excellent sensitivity, reasonable specificity and a lower LLOD than the currently utilized BuChE%

depression test, the next step is to see what risk factors are associated with adduct being measured.

TABLE 9: Survey Results From Randomly Selected WA Agricultural Workers Compared to the Dichotomous Adduct Fraction Variable

Variable	Sub Variable	All N = 99 n (%)	Adduct present N = 37 n (%)	Adduct absent N = 62 n (%)	p-value
Year	2007	28(28)	9(24)	19(31)	0.469
	2008	28(28)	13(35)	15(24)	
	2009	21(21)	9(24)	12(19)	
	2010	22(22)	6(16)	16(26)	
Age (years)	<30	32(32)	15(41)	17(27)	0.246
	30-39	43(43)	16(43)	27(44)	
	40+	24(24)	6(16)	18(29)	
Survey		92(93)	37(100)	55(89)	0.034

Variable	Sub Variable	All N = 92 n (%)	Adduct Yes N = 37 n (%)	Adduct No N = 55 n (%)	p-value
Application	Tractor	80(87)	35(95)	45(82)	0.074
	Weed Sprayer	7(8)	3(8)	4(7)	0.882
	Tower Sprayer	6(7)	3(8)	3(5)	0.612
	Backpack Sprayer	1(1)	0(0)	1(2)	1.000
	Other	4(4)	1(3)	3(5)	0.646
	Did Not Apply	3(3)	0(0)	3(5)	0.271
Handling Activities					
	Mix or load	72(78)	31(84)	41(75)	0.292
	Repair Equipment	28(30)	14(38)	14(25)	0.206
	Enter Storage	36(39)	17(46)	19(35)	0.272
	Enter Field	12(13)	3(8)	9(16)	0.249
	None	5(5)	1(3)	4(7)	0.343
	Control Traffic	12(13)	3(8)	9(16)	0.249
	Supervisor	4(4)	0(0)	4(7)	0.146
Cleaning Activities					
	Spray Equipment	57(62)	23(62)	34(62)	0.973
Person	Protective Equipment	68(74)	27(73)	41(75)	0.866
	Storage	19(21)	9(24)	10(18)	0.475
	After Spray	5(5)	1(3)	4(7)	0.343
Pesticide	Containers	43(47)	18(49)	25(45)	0.763
	None	4(4)	2(5)	2(4)	1.000
Pesticide Used					
	Chlorpyrifos	46(50)	15(41)	31(56)	0.137
	Azinphos	22(24)	9(24)	13(24)	0.940
	Carbaryl	34(37)	14(38)	20(36)	0.886

	Phosmet	6(7)	3(8)	3(5)	0.613
	Diazinon	0(0)	0(0)	0(0)	
	Methidathion	3(3)	1(3)	2(4)	1.000
	Methamidophos	2(2)	0(0)	2(4)	0.514

TABLE 9 Cont.

Variable	Sub Variable	All N = 92 n (%)	Adduct Yes N = 37 n (%)	Adduct No N = 55 n (%)	p-value
Dichlorvos		1(1)	1(3)	0(0)	0.402
	Dimethoate	3(3)	2(5)	1(2)	0.562
	Other	8(9)	2(5)	6(11)	0.358
	Unsure	8(9)	3(8)	5(9)	0.870
Last Exposure					
	Today	7(8)	4(11)	3(5)	0.451
	Yesterday	11(12)	4(11)	7(13)	
	2-7 Days Ago	37(40)	16(43)	21(38)	
	8-14 Days	12(13)	5(14)	7(13)	
	15-30 Days	14(15)	7(19)	7(13)	
	More than 30 Days	9(10)	1(3)	8(15)	
Hours of Exposure (Hours)					
	Less than 10	16(17)	7(19)	9(16)	0.955
	10-19	16(17)	7(19)	9(16)	
	20-29	9(10)	3(8)	6(11)	
	30-39	23(25)	11(30)	12(22)	
	40-49	8(9)	4(11)	4(7)	
	50+	10(11)	5(14)	5(9)	
Crops					
	Apples	84(91)	36(97)	48(87)	0.314
	Cherries	17(18)	5(14)	12(22)	0.258
	Grapes	6(7)	1(3)	5(9)	0.200
	Pears	32(35)	16(43)	16(29)	0.227
	Peaches	2(2)	1(3)	1(2)	1.000
	Apricots	1(1)	0(0)	1(2)	1.000
	Plums	1(1)	0(0)	1(2)	1.000
	Other	0(0)	0(0)	0(0)	
	No Pesticides Used	0(0)	0(0)	0(0)	
Tractor Enclosed Cab		2(2)	2(5)	0(0)	0.181
Unclog Spray Nozzles		73(79)	33(89)	40(73)	0.263
Unclean Spray Equipment Used		48(52)	21(57)	27(49)	0.849
Glove Use Among Those Who Conducted Mechanical Repairs					
	No	2(2)	1(3)	1(2)	1.000
	Latex Gloves	8(9)	3(8)	5(9)	0.403
	Chem Resistant Gloves	19(21)	10(27)	9(16)	0.686
	Cloth Gloves	1(1)	0(0)	1(2)	1.000
	Leather Gloves	4(4)	3(8)	1(2)	0.596
	Used Other Gloves	0(0)	0(0)	0(0)	
Performs Repairs Inside Pest. Tank		1(1)	1(3)	0(0)	1.000
Pesticide Contamination on Skin, Mouth or Eyes					
		40(43)	20(54)	20(36)	0.092
Handles Wet Equipment After Spray		19(21)	9(24)	10(18)	0.475
Time Between Spray and Shower					

	<1 Hour	20(22)	7(19)	13(24)	0.519
	1-2 Hours	37(40)	18(49)	19(35)	
	2-4 Hours	22(24)	8(22)	14(25)	
	5+ Hours	10(11)	2(5)	8(15)	
Not Until Next Morning		2(2)	1(3)	1(2)	

TABLE 9 Cont.

Variable	Sub Variable	All N = 92 n (%)	Adduct Yes N = 37 n (%)	Adduct No N = 55 n (%)	p-value
Handled Pesticides at Other Sites		2(2)	2(5)	0(0)	0.159
Fit-Tested in Last 12 Months		72(78)	31(84)	41(75)	0.894
Frequency of Seal Checks					
	Never	3(3)	3(8)	0(0)	0.060
	Sometimes	19(21)	9(24)	10(18)	
	Always	57(62)	20(54)	37(67)	
Reparatory Protection					
	Dust Mask	2(2)	1(3)	1(2)	0.928
	Half-Faced	79(86)	32(86)	47(85)	
	Full faced or PAPR	11(12)	4(11)	7(13)	
Changed Cartridges					
	Hard to Breath	4(4)	2(5)	2(4)	0.746
	Smelled Pesticides	14(15)	7(19)	7(13)	
	1/ Day	37(40)	14(38)	23(42)	
	1/ Week	16(17)	8(22)	8(15)	
	1/ Month	6(7)	1(3)	5(9)	
	1/ Year	2(2)	1(3)	1(2)	
	Unsure	0(0)	0(0)	0(0)	
Eye Protection					
	Safety Glasses	65(71)	30(81)	35(64)	0.135
	Goggles	11(12)	2(5)	9(16)	
	Face Shield	5(5)	1(3)	4(7)	
	Other	0(0)	0(0)	0(0)	
	Did Not Use	0(0)	0(0)	0(0)	
Head Covering Used					
	Rain Hat	14(15)	8(22)	6(11)	0.177
	Chem Resistant Hood	56(61)	18(49)	38(69)	0.049
	Other	0(0)	0(0)	0(0)	
	Didn't Wear	1(1)	0(0)	1(2)	1.000
	Baseball Cap Only	11(12)	7(19)	4(7)	0.101
Gloves too Thick					
	No	38(41)	13(35)	25(45)	0.324
	Yes	54(59)	24(65)	30(55)	
Gloves Used					
Chem Resistant Gloves Only		55(60)	21(57)	34(62)	0.683
Disposable Under Chem Resist		18(20)	7(19)	11(20)	
Cloth Under Chem Resist		18(20)	9(24)	9(16)	
	Leather Only	1(1)	0(0)	1(2)	
	Cloth Only	0(0)	0(0)	0(0)	
	Other	0(0)	0(0)	0(0)	
	Didn't Wear	0(0)	0(0)	0(0)	
Footwear Used					

	Chem Resist Boots	92(100)	37(100)	55(100)	
TABLE 9 Cont.		**All**	**Adduct Yes**	**Adduct No**	
		N = 92	**N = 37**	**N = 55**	
Variable	**Sub Variable**	**n (%)**	**n (%)**	**n (%)**	**p-value**
Protective Clothing Used					
	Chem Resist Overalls	67(73)	28(76)	39(71)	0.614
	Chem Resist Jacket	78(85)	30(81)	48(87)	0.418
	Chem Resist Apron	9(10)	5(14)	4(7)	0.323
	Chem Resist coveralls	15(16)	7(19)	8(15)	0.578
	Other	0(0)	0(0)	0(0)	
	Didn't Wear	0(0)	0(0)	0(0)	
Condition of PPE					
	Poor-Fair	32(35)	14(38)	18(33)	0.249
	Good	43(47)	19(51)	24(44)	
	Excellent	17(18)	4(11)	13(24)	
Wear Sweatshirt Under PPE					
	Never	22(24)	4(11)	18(33)	0.036
	Sometimes	24(26)	10(27)	14(25)	
	Always	45(49)	23(62)	22(40)	
Wear Baseball Cap Under PPE					
	Never	29(32)	7(19)	22(40)	0.081
	Sometimes	11(12)	6(16)	5(9)	
	Always	51(55)	24(65)	27(49)	
Wear Bandana Under PPE					
	Never	60(65)	22(59)	38(69)	0.542
	Sometimes	20(22)	10(27)	10(18)	
	Always	11(12)	5(14)	6(11)	
Hand Washing Supplies					
	Water	72(78)	30(81)	42(76)	0.703
	Bar/Liquid Soap	76(83)	29(78)	47(85)	0.274
	Chem Hand Sanitizer	20(22)	8(22)	12(22)	0.946
	Hand Wipes	8(9)	3(8)	5(9)	0.849
	Shop Towels	56(61)	24(65)	32(58)	0.589
	Cloth Towel	3(3)	1(3)	2(4)	1.000
	Other	1(1)	0(0)	1(2)	1.000
	Don't Wash Hands	0(0)	0(0)	0(0)	
Unable to Wash Hands After Spray		7(8)	3(8)	4(7)	0.922
Activities During Break					
	Drink Beverage	55(60)	21(57)	34(62)	0.479
	Eat	31(34)	13(35)	18(33)	0.908
	Smoke	2(2)	0(0)	2(4)	0.510
	Cell Phone	24(26)	12(32)	12(22)	0.301
	Two-way Radio	1(1)	0(0)	1(2)	1.000
	Urinate	47(51)	23(62)	24(44)	0.115
	Use Portable Toilet	33(36)	12(32)	21(38)	0.486
	Never Stopped	8(9)	3(8)	5(9)	0.828
Washed Hands Before Beverage					
	Never	1(1)	0(0)	1(2)	0.026
	Sometimes	28(30)	15(41)	13(24)	
	Always	26(28)	6(16)	20(36)	

TABLE 9 Cont.		All N = 92	Adduct Yes N = 37	Adduct No N = 55	
Variable	**Sub Variable**	**n (%)**	**n (%)**	**n (%)**	**p-value**
Washed Hands Before Food					
	Never	0(0)	0(0)	0(0)	0.558
	Sometimes	3(3)	2(5)	1(2)	
	Always	28(30)	11(30)	17(31)	
Washed Hands Before Cell Phone					
	Never	8(9)	4(11)	4(7)	0.660
	Sometimes	12(13)	7(19)	5(9)	
	Always	4(4)	1(3)	3(5)	
Washed Hands Before Urination					
	Never	12(13)	7(19)	5(9)	0.066
	Sometimes	24(26)	14(38)	10(18)	
	Always	11(12)	2(5)	9(16)	
Washed Hands Before Toilet					
	Never	5(5)	3(8)	2(4)	0.069
	Sometimes	11(12)	6(16)	5(9)	
	Always	17(18)	3(8)	14(25)	
Wore Contaminated Clothes		27(29)	16(43)	11(20)	0.043
Years of Handling Pesticides					
	One or Less	21(23)	13(35)	8(15)	0.075
	2-3	24(26)	7(19)	17(31)	
	4-5	19(21)	10(27)	9(16)	
	6-10	20(22)	5(14)	15(27)	
	11+	6(7)	2(5)	4(7)	
Application License		26(28)	11(30)	15(27)	0.841
Pesticide Training		10(11)	3(8)	7(13)	0.456
Medical History Of Liver Disease		1(1)	1(3)	0(0)	0.407
Use of Tylenol					
	Never	43(47)	17(46)	26(47)	0.771
	Sometimes	47(51)	20(54)	27(49)	
	Frequently	0(0)	0(0)	0(0)	
Alcohol Use (Days/Week)					
	Never	33(36)	12(32)	21(38)	0.726
	<1	19(21)	7(19)	12(22)	
	1	23(25)	10(27)	13(24)	
	2-3	11(12)	6(16)	5(9)	
	4-6	1(1)	0(0)	1(2)	
	Every Day	3(3)	2(5)	1(2)	
Cigarette Use					
	Every Day	10(11)	5(14)	5(9)	0.551
	Some Days	16(17)	8(22)	8(15)	
	Not At All	64(70)	24(65)	40(73)	
Use of Pesticides at Home					
	Never	68(74)	28(76)	40(73)	0.963
	Sometimes	20(22)	8(22)	12(22)	
	Frequently	2(2)	1(3)	1(2)	

TABLE 9 Cont.		All N = 92	Adduct Yes N = 37	Adduct No N = 55	
Variable	**Sub Variable**	**n (%)**	**n (%)**	**n (%)**	**p-value**
Pesticide Related Illness		8(9)	3(8)	5(9)	0.947
Symptoms					
	Skin Irritation, Rash	7(8)	2(5)	5(9)	0.483
	Eye Irritation	21(23)	14(38)	7(13)	0.007
	Nose or Throat Irritation	11(12)	5(14)	6(11)	0.755
	Blurred Vision	5(5)	2(5)	3(5)	0.959
	Dizziness	3(3)	2(5)	1(2)	0.566
	Asthma	1(1)	0(0)	1(2)	1.000
	Headache	27(29)	14(38)	13(24)	0.175
	Nausea or Vomiting	1(1)	0(0)	1(2)	1.000
	Diarrhea	3(3)	0(0)	3(5)	0.266
	Other	1(1)	0(0)	1(2)	1.000
	Trouble Breathing	4(4)	1(3)	3(5)	0.641
	No Symptoms	35(38)	10(27)	25(45)	0.054
Health Status					
	Poor-Fair	30(33)	13(35)	17(31)	0.832
	Good	48(52)	20(54)	28(51)	
	Excellent	12(13)	4(11)	8(15)	
Country of Education					
	Mexico	81(88)	33(89)	48(87)	1.000
	USA	4(4)	2(5)	2(4)	
	Both Mexico and USA	4(4)	2(5)	2(4)	
	None	1(1)	0(0)	1(2)	
Level of Education (Mexico)					
	Part Primary	19(21)	5(14)	14(25)	0.499
	Completed Primary	31(34)	14(38)	17(31)	
	Completed Middle	23(25)	10(27)	13(24)	
Completed High School and Above		12(13)	6(16)	6(11)	
Level of Education (USA)					
	<6th Grade	3(3)	2(5)	1(2)	0.486
	6th Grade	1(1)	1(3)	0(0)	
	High School	4(4)	1(3)	3(5)	
	College	0(0)	0(0)	0(0)	
Literate in English					
	Not At All	50(54)	19(51)	31(56)	0.357
	Not Very Well	27(29)	14(38)	13(24)	
	Fairly Well	13(14)	4(11)	9(16)	
	Very Well	0(0)	0(0)	0(0)	
Home Location					
	In Town	56(61)	20(54)	36(65)	0.182
	Country Away From Orchard	7(8)	3(8)	4(7)	0.922
	Country Near Orchard	10(11)	4(11)	6(11)	0.940
	Within Orchard	12(13)	9(24)	3(5)	0.010
	Other	4(4)	1(3)	3(5)	0.641

TABLE 9 above summarizes the results from the chi squared tests run on the subset of the overall population that were selected randomly in order to tease out variables that are statistically significant from those that are not. The following variables showed statistically significant associations with higher prevalence of chlorpyrifos adduct (associated p-values in brackets): Those workers that took the survey (0.034), those who wore previously contaminated clothing while spraying (0.043), workers who wore sweatshirts under their PPE (0.036), workers who reported eye irritation (0.007), and those who lived and slept in an orchard (0.010). The following variables showed statistically significant associations with reduced prevalence of chlorpyrifos adduct (associated p-values in brackets): those that wore chemical resistant hoods (0.049), those who washed their hands before drinking a beverage after spraying (0.026).

The next step was to use logistic regressions to determine the strength of association with adduct fraction prevalence for the variables identified via the chi squared test. The goal of this was to come up with an Odds ratio (OR) and corresponding p-value to further characterize the relationship. TABLE 10 below shows the key variables identified in TABLE 8 with associated ORs and p-values. The table itself is divided into two parts. The top section shows variables that were found to be statistically significantly associated with adduct fraction presence when the data was restricted to the random samples only. The bottom section shows the variables, that when the data was not restricted to only random, were found by the Chi Square or Fisher's Exact tests to be significantly associated and the corresponding OR and p-values associated with the logistic regression analysis. In it, we can see that for the variables; use of chemical resistant hoods and washing before drinking, we note a protective relationship with workers who do these activities having lower odds of

chlorpyrifos exposure. The opposite is true for the remaining variables (those with ORs >1.00), with the eye irritation and home location of "orchard" being the worst (ORs = 4.00 and 5.36 respectively). Regarding the survey variable, it is plausible that the workers are more likely to agree to take the survey if they believe they are being exposed than they would if they weren't getting exposed and just wanted to go home or didn't care. This was somewhat supported by the results of our analysis when taking the survey was tested against adduct fraction (OR=6.02, p-value = 0.098).

TABLE 10: Logistic Regression of Risk Variables for Random Population and Adduct Fraction For Random and All Samples Respectively

	Random		**All**	
Variable (Random)	**OR**	**p-value**	**OR**	**p-value**
Chem. Resistant Hood	0.33	0.021	0.34	0.011
Wash Before Beverage	0.37	0.017	0.31	0.017
Sweatshirt Under PPE	2.02	0.014	1.74	0.020
Wore Contaminated Clothes	2.62	0.045	2.64	0.024
Eye Irritation	4.00	0.009	3.25	0.008
Home in Orchard	5.36	0.018	9.67	0.001
	Random		**All**	
Variable (All)	**OR**	**p-value**	**OR**	**p-value**
Wash Before Portable Toilet	0.33	0.041	0.26	0.011
Wash Before Urinating	0.44	0.067	0.48	0.085
Home in Town	0.56	0.184	0.33	0.005
Performs Regular Seal Checks	0.65	0.066	0.61	0.017
Skin, Eye, Mouth Exposure	2.06	0.095	2.30	0.027

In addition to just focusing on the samples that were selected randomly, we also ran the chi square tests for each of the variables in TABLE 9 on the larger data set of 128 samples and identified the following list of variables as being significantly associated with adduct fraction and their p-values: Worker was exposed to pesticide through their skin, eyes or mouth (0.026), handled pesticides at other work sites (0.026), performed regular

seal checks (0.043), wore sweatshirts under PPE (0.050), washed their hands before drinking a beverage during their break (0.014), washed their hands before urination during their break (0.024), washed their hands before using a portable toilet during their break(0.017), wore contaminated clothes (0.022), reported eye irritation symptoms (0.007) and home location of both in town (0.005) and again a very strong association of those workers that lived within the orchard (<0.001). This test was not considered to be the main analysis because of the concern that the hand-selection of the 20 samples hand-picked for their suspected exposure levels and the 8 pilot samples might introduce bias into the sample group.

Of those variables, workers that performed regular seal checks, washed their hands during their breaks and home location in town have a lower prevalence of chlorpyrifos adducts (ORs = 0.61, 0.48 for washing before urination and 0.26 for washing before toilet and 0.33 respectively), whereas the remaining variables are risk factors associated with a higher prevalence of chlorpyrifos adducts.

Study Limitations:

This study focused on the entire data pool of N = 128 samples described above for aims 1 and 2. Aim 3 largely focused on the subset of data that have both the survey and the adduct measurements and had been randomly selected (N = 92). One issue with the sub dataset containing the survey data is that it relies on self-reporting which can a present a plethora of issues related to data interpretation (Johnson et al, 2005). Although self-reporting has been noted to be an issue, the logistics involved with independently verifying the responses for each individual is impossible so we will recognize this as a source of

unavoidable uncertainty in our data, fully recognizing that this reporting bias might not be random.

Another source of uncertainty is our use of the presence of a common imputation equation, which simply divides the LOQ by the square root of 2 when we had non-detects for adduct. Although this imputation is a source of uncertainty, with the adduct fraction assay being considerably more precise than is the current BuChE % depression test we were able to get many more detects for the adduct fraction test than for the BuChE % depression test. More sophisticated approaches to imputing data below the LOD may have increased our statistical power, but were considered unlikely to materially alter the findings we reported. Another limitation is that we had multiple examples of where a person was sampled multiple times over the 4 years. For our analysis we treated them as individual samples rather than try to account for the subject effect in our models.

Arguably one of the largest limitations is the absence of objectively measured exposure data for the individuals involved in the study. Although BuChE % depression is used as an indicator of OP exposure, it does not provide information on specifically how or even when an individual was exposed. No instruments were used to determine chlorpyrifos concentrations in air nor were swipe samples taken of the environments or equipment etc. We would recommend that future studies consider undertaking some exposure measurements in conjunction with the survey and blood samples to provide a better picture of how measured exposure equates to both the adduct/BuChE% depression and the surveys.

Conclusions:

Based on the results above, we conclude that the CPF-BuChE adduct fraction assay (with a LLOD of ~4%) is more sensitive at identifying chlorpyrifos exposed workers than the current "gold" standard (BuChE % depression, LLOD ~20%). With this in mind it is critical that further testing be done on the validity of this type of testing with the ultimate goal of potentially providing a better, more accurate assay to measure the effects of chlorpyrifos exposure that is specific to the commonly used pesticides with the ultimate intent of providing better protection for the workers. This is not to say that the adduct assay is not without its limitations. One such limitation is the fact that it is specific to CPF. Although CPF is the most commonly used pesticide in WA State and incidentally for the workers who participated in this study, it is by no means the only pesticide used. Another issue that would be major from an implementation standpoint is the expense and time needed to perform this assay, even when compared with BuChE % depression. It is possible that as technology continues to evolve that this process might become easier and even cheaper in the future, but as it stands now, this is not the case.

Aim 2 showed us that PON1 Q192R phenotype did influence adduct fraction: subjects with the RR genotype (produces the isoform of PON1 enzyme with high catalytic efficiency towards chlorpyrifos oxon) in general had lower adduct levels that subjects with QR or QQ phenotypes. Somewhat counter intuitively, subjects with higher arylesterase activity (indicating they had high levels of the PON1 enzyme) tended to have higher adduct levels.

Aim 3 highlighted several variables that were found to be associated with measurable levels of adduct. Some variables were protective (associated with lower

prevalence of adduct). This category included variable associated with PPE use (e.g. wearing a chemical resistant hood), and variables associated with good hygiene practices (washing hands before drinking beverages). Others were found to be risk factors associated with higher prevalence of adduct. Variables in this category included wearing a personal sweatshirt under the workers' PPE, or wearing clothing contaminated with pesticide. Eye irritation was also associated with greater prevalence of OP adduct, suggesting that workers who experience symptoms of eye irritation have likely been exposed to pesticide., then they are being exposed to something, possibly the pesticide. One of the strongest risk factors associated with OP adducts was living within an orchard. Conversely, living in town was associated with lower prevalence of chlorpyrifos adducts. Perhaps it would be prudent to inform employers that those families living on their orchards also be considered for testing of pesticide exposure. If this is found to be a risk factor, then the workers families might be exposed unbeknownst to them.

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