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## Blood $\alpha$ -Synuclein in Agricultural Pesticide Handlers in Central Washington State

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### Abstract

Epidemiologic studies suggest that occupational exposure to pesticides might increase Parkinson disease risk. Some pesticides, such as the organophosphorus insecticide chlorpyrifos, appear to increase the expression of  $\alpha$ -synuclein, a protein critically involved in Parkinson disease.

Therefore, we assessed total blood cell  $\alpha$ -synuclein in 90 specimens from 63 agricultural pesticide handlers, mainly Hispanic men from central Washington State, who participated in the state's

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**Conflicts of Interest:** The authors have no potential conflicts of interest to report.

**Human Subjects:** The present work used blood samples from a biorepository created for research. The blood samples used in the present work were originally obtained through a study approved by the University of Washington Institutional Review Board (IRB application #29460 "Identifying Risk Factors for Cholinesterase Depression among Pesticide Handlers"). This IRB also approved the biorepository (IRB application #33592 "Organophosphate Exposure and Effects among Agricultural Pesticide Handlers"), and provided an exemption for the present specimen-based analysis (IRB application #42290 "Preclinical Markers of Parkinsonism and Organophosphorus Pesticide Exposure in Washington State Farmworkers") on 2/23/2012 because this activity does not meet the federal regulatory definition of "human subjects" research under 45 CFR 46.102(f) and does not require review by the IRB. All subjects provided written informed consent to participate in the original study and to have their blood samples archived in the biorepository for future research.

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cholinesterase monitoring program in 2007–2010. Additionally, in age-adjusted linear regression models for repeated measures, we assessed whether  $\alpha$ -synuclein levels were associated with butyrylcholinesterase-chlorpyrifos adducts or cholinesterase inhibition measured in peripheral blood, or with self-reported pesticide exposure or paraoxonase (*PONI*) genotype. There was no evidence by any of those indicators that exposure to chlorpyrifos was associated with greater blood  $\alpha$ -synuclein. We observed somewhat greater  $\alpha$ -synuclein with the *PONI* -108T (lower paraoxonase enzyme) allele, and with 10 hours of exposure to cholinesterase inhibiting insecticides in the preceding 30 days, but neither of these associations followed a clear dose-response pattern. These results suggest that selected genetic and environmental factors may affect  $\alpha$ -synuclein blood levels. However, longitudinal studies with larger numbers of pesticide handlers will be required to confirm and elucidate the possible associations observed in this exploratory cross-sectional study.

### MeSH Keywords

$\alpha$ -Synuclein; Aryldialkylphosphatase; Chlorpyrifos; Parkinson Disease; Pesticides

## 1. Introduction

Parkinson disease (PD) is a progressive, neurodegenerative movement disorder that affects up to 2% of the population older than age 60 (Elbaz et al. 2002; Wright Willis et al. 2010). The signs of PD, which include slow movement, tremor, rigidity and postural impairment, result from the loss of dopaminergic neurons in the substantia nigra region in the midbrain. Both genetic and environmental factors are believed to contribute to the development of sporadic PD (Wirdefeldt et al. 2011b). Pesticides are among the environmental factors suspected to increase PD risk (Wirdefeldt et al. 2011a). A recent meta-analysis (Allen and Levy 2013) suggests a 34% increased risk of PD in relation to occupational exposure to either insecticides or herbicides. Notably, some pesticides appear to increase  $\alpha$ -synuclein ( $\alpha$ -syn) in cell line models of dopaminergic neurons (Chorfa et al. 2013; Slotkin and Seidler 2011).  $\alpha$ -Syn is a protein that is critically involved in PD. It aggregates in the brain as the major component of Lewy bodies, a pathological hallmark of PD (Spillantini et al. 1997). Genetic mutations in *SNCA*, the gene coding for  $\alpha$ -syn, are strongly associated with both familial and sporadic forms of the disease, notably including those that increase  $\alpha$ -syn (Maraganore et al. 2006; Mata et al. 2010; Singleton et al. 2003). Lower methylation of the *SNCA* promoter, as has been observed among PD patients' brains, is also associated with increased  $\alpha$ -syn (Jowaed et al. 2010; Matsumoto et al. 2010).

Of the pesticides thus far associated with greater  $\alpha$ -syn or *SNCA* expression in neurons, the one most commonly employed in agriculture (USDA 2013) is the organophosphorus insecticide chlorpyrifos (Slotkin and Seidler 2011). Therefore, we investigated the relation between  $\alpha$ -syn and occupational chlorpyrifos exposure. We hypothesized that, among agricultural workers exposed to chlorpyrifos,  $\alpha$ -syn would be increased among those with highest exposures. We tested this hypothesis in a population of actively working agricultural pesticide handlers in an apple-growing region of central Washington State in 2007–2010. We measured  $\alpha$ -syn in peripheral blood cells, where the protein is observed at readily

detectable levels (e.g. 24,800 ng/mL, approximately one thousand times greater than levels in plasma) (Barbour et al. 2008). The extent to which blood and brain  $\alpha$ -syn are correlated remains to be established directly. However, the *SNCA* Rep1 259 length allele, very well-established as protective with regard to PD (Maraganore et al. 2006), is associated with lower *SNCA* expression or  $\alpha$ -syn protein levels in humans in both the midbrain (Linnertz et al. 2009) and peripheral blood mononuclear cells (Fuchs et al. 2008) relative to longer alleles. When accounting for total protein content,  $\alpha$ -syn levels in these cells are similar to the level in red blood cells (Barbour et al. 2008), where >98% of blood  $\alpha$ -syn resides (Barbour et al. 2008; Shi et al. 2010). In addition, an *SNCA* gene triplication that increases risk of PD is estimated to double levels of  $\alpha$ -syn in the blood (Miller et al. 2004).

## 2. Materials and Methods

### 2.1. Source and selection of specimens

We obtained lithium heparin-treated packed whole blood cells from a biorepository at the University of Washington Department of Environmental and Occupational Health Sciences (Seattle, WA). The biorepository contains blood samples collected during an earlier study of occupational and genetic determinants of serum cholinesterase inhibition in adult agricultural workers (Hofmann et al. 2009; Hofmann et al. 2010b). This study population is comprised mainly of Hispanic male pesticide handlers who had been recently exposed to cholinesterase inhibiting (organophosphorus and/or carbamate) insecticides for >30 hours in a 30 day period while working in tree fruit orchards in the Yakima Valley of central Washington State. Whole blood was originally collected from these workers by venipuncture into heparinized vacutainer tubes. It was then spun at 2,500 rpm to separate plasma and cells and then stored in separate cryovials in a refrigerator for up to three days before transfer to a  $-80^{\circ}\text{C}$  freezer in the biorepository.

The present work is based on a sample of 128 specimens originally selected from the biorepository to assess the feasibility of using chlorpyrifos-butrylcholinesterase (BuChE) adducts as a measure of chlorpyrifos exposure and cholinesterase inhibition (Riutta et al. 2012). This sample, drawn from specimens obtained in the years 2007–2010, included 100 selected from the repository at random. The other 28 were selected because they were from workers who had experienced cholinesterase depression in the previous two weeks (depressionary follow-up samples) or were in an earlier pilot study. Sufficient (>0.5 mL packed blood cells) remained to attempt the  $\alpha$ -syn assay for 90 (70%) of the specimens, and  $\alpha$ -syn was measured in all 90. Of those, 70 had been selected at random. Workers in the original study could have participated in more than one year, and could have provided multiple specimens in the same year if they experienced high levels of exposure or cholinesterase depression. Of the 90 specimens, 63 were from unique subjects, and 27 were repeat specimens collected from 18 subjects. Two-thirds of the pairs were collected in different years (18 pairs), five pairs 36–56 days apart, and four pairs one week apart.

The original studies and biorepository were approved by the University of Washington Institutional Review Board, which also provided an exemption for the present specimen-based analysis. All subjects provided written informed consent to participate in the original study and to have their blood samples archived in the biorepository for future research.

## 2.2 Assessment of blood $\alpha$ -syn

Whole blood cells were assayed for total  $\alpha$ -syn ( $\mu\text{g/mL}$ ) in random order on two plates with positive and negative controls following an established protocol (Hong et al. 2010; Shi et al. 2010). Briefly, red blood cells were lysed and the diluted supernatant was incubated with  $\alpha$ -syn capture and detection antibodies, respectively rabbit anti- $\alpha$ -synuclein antibody (ASY-1, which largely recognizes the C-terminal of the protein) (Jensen et al. 2000) and biotinylated anti-human  $\alpha$ -synuclein antibody (R&D systems, Minneapolis, MN, USA). We quantitated  $\alpha$ -syn using a LiquiChip Luminex 200TM instrument (Qiagen, Valencia, CA, USA). The intra-assay coefficient of variation was 0.5%, and the inter-assay coefficient of variation was 3%.

## 2.3 Assessment of chlorpyrifos exposure and other factors

We included both laboratory- and interview-based indicators of exposure to chlorpyrifos and related factors. Our primary exposure variable of interest was chlorpyrifos-BuChE adducts (Riutta et al. 2012). This is the percent BuChE with a monoethyl phosphate adduct (generated when chlorpyrifos attaches to BuChE and ages irreversibly) assessed by high-performance liquid chromatography tandem mass spectrometry using 1.8 mL of whole blood. The limit of detection is at approximately 4% adducts. Also available were data on the percent change (relative to the subject's pre-spray level) of red blood cell acetylcholinesterase (AChE) and serum BuChE activity from the State of Washington's cholinesterase monitoring program (Wilson et al. 2009). In addition, we used Taqman detection system based assays to assess two functional *PON1* single nucleotide polymorphisms, Q192R and C-108T. The *PON1* gene codes for the enzyme paraoxonase, which detoxifies the activated intermediate (oxon) of chlorpyrifos. Chlorpyrifos-oxonase activity, which is influenced by the C-108T and Q192R polymorphisms, markedly affects susceptibility to chlorpyrifos (Costa et al. 2013). Paraoxonase also detoxifies the oxon of diazinon, which also was applied in apple orchards during the study years in Washington, though less widely (6–9% of bearing acres vs. 39–64% for chlorpyrifos).

In addition to these laboratory-based measures, data from interviews with the agricultural workers who provided the specimens were available for 82 (91%) of the specimens. These data had been collected via a computer-based questionnaire that the workers completed in person in Spanish or English (Hofmann et al. 2010a). The questionnaire assessed ethnicity, sex, tobacco smoking and work history, including total years handling agricultural pesticides, and the duration and type of cholinesterase inhibiting insecticides handled within the 30 days preceding collection of the associated blood sample. The questionnaire specifically inquired about 11 cholinesterase inhibiting insecticides. We report results for those reportedly handled by >5% of the participants within 30 days of blood collection: chlorpyrifos, azinphos-methyl and carbaryl. Study participants did not report using diazinon.

## 2.4 Statistical analysis

We used Stata version 11.1 (StataCorp, College Station, TX) for all statistical analysis. We conducted linear regression models suitable for repeated measures, specifically generalized linear models, with  $\alpha$ -syn ( $\mu\text{g/mL}$ , continuous) as the outcome variable. We report  $\beta$  coefficients and 95% confidence intervals (CIs), characterizing the difference in  $\alpha$ -syn levels

across strata of each of the variables of interest, while adjusting for age (continuous) because of its association with  $\alpha$ -syn (Barbour et al. 2008; Shi et al. 2010). Among workers who completed the questionnaire, all but one was a Hispanic man, so we were unable to adjust for race, ethnicity and gender. We did not adjust for tobacco smoking or experimental plate because these changed the observed coefficients by <10%. We repeated all analyses while excluding the 20 specimens not selected at random, or excluding the specimens collected in 2007. We conducted the latter sensitivity analyses because there were technical issues in the cholinesterase measurements for that year (WSDOSH 2007), and preliminary analyses indicated marked differences in  $\alpha$ -syn in specimens from 2007 vs. other years. In a more exploratory separate analysis, we compared  $\alpha$ -syn values between paired blood specimens to assess the extent to which blood  $\alpha$ -syn changes over time within an individual.

### 3. Results

#### 3.1 Characteristics of participants

Among participants who provided interview data, all but one was a Hispanic man (Table 1). Age when providing the first specimen ranged from 20 to 60 years (median 33 years). All participants had been handling pesticides for 15 years, with a majority (71%) handling pesticides for 5 years. The median blood  $\alpha$ -syn was 79.0  $\mu\text{g}/\text{mL}$  (range 33.6 to 126.5  $\mu\text{g}/\text{mL}$ ; mean 77.5  $\mu\text{g}/\text{mL}$ ; standard deviation 18.9  $\mu\text{g}/\text{mL}$ ; not shown in tables).

#### 3.2 Factors associated with blood $\alpha$ -syn

There was no indication that blood  $\alpha$ -syn increased with increasing BuChE-chlorpyrifos adducts or with BuChE inhibition (Table 2). AChE inhibition was associated with greater blood  $\alpha$ -syn ( $p_{\text{trend}} = 0.05$  overall and excluding year 2007), but this was due to a single worker with >20% AChE inhibition and blood  $\alpha$ -syn at the 99<sup>th</sup> percentile. Self-reported exposure to chlorpyrifos or other commonly-reported cholinesterase inhibiting insecticides in the preceding 30 days also were not associated with blood  $\alpha$ -syn. However, workers who reported they did not know what pesticides they used in the preceding 30 days had markedly greater blood  $\alpha$ -syn compared to other workers ( $p=0.006$ ; Table 2). This association was not attenuated by adjustment for level of experience, as there was no association between blood  $\alpha$ -syn and total years handling pesticides after adjusting for age.

We observed no dose-response associations between blood  $\alpha$ -syn and days since handling these pesticides, total number of hours handling them, or number of days handling them for >8 hours (Table 2). At the same time a possible threshold effect was suggested as blood  $\alpha$ -syn was generally greater among workers who had handled pesticides for 10 hours, compared to workers who had handled pesticides for <10 hours in the preceding 30 days. Blood  $\alpha$ -syn was also greater in specimens collected in 2007 than in later years, and among carriers of the *PON1* -108T allele (Table 2). There was no association between *PON1* Q192R genotype and blood  $\alpha$ -syn, with or without adjustment for the C-108T polymorphism.

In a fuller multivariable model (Table 3), the above associations remained (all  $p < 0.02$ ). These associations were insensitive to the exclusion of any individual specimen, and results

were also similar when we focused on the randomly selected specimens (data not shown in tables).

Because the association between  $\alpha$ -syn and *PONI* C-108T remained in the fuller model, we explored whether this association was modified by any indicator of chlorpyrifos exposure. The association between  $\alpha$ -syn and *PONI* C-108T did not clearly differ by recent chlorpyrifos exposure (self-reported exposure or chlorpyrifos-BuChE adducts, all interaction p-values >0.23, data not shown in tables). There also was no indication that the association was stronger in workers with the greatest number of years of exposure.

### 3.3 Blood $\alpha$ -syn in longitudinal specimens

When we explored the extent to which blood  $\alpha$ -syn levels might change over time, we observed that in some instances there were marked fluctuations from year to year. These ranged from a 63% decline to an 80% increase from one year to the next in 18 paired samples, but on average there was little change. The largest decline was for a worker whose first specimen was from 2007, but a drop after 2007 was not universally observed. There also were marked fluctuations in blood  $\alpha$ -syn within the same year: Among the specimen pairs taken in the same year because the worker reached the 30 hours of exposure in 30 days threshold a second time, three of four workers showed marked increases from the end of the first to the end of the second exposure period (73–115% increases in blood  $\alpha$ -syn). Blood  $\alpha$ -syn did not clearly go up or down for the three additional workers (four pairs) whose same-year blood specimens were taken only one week apart.

## 4. Discussion

To our knowledge this is the first study to assess  $\alpha$ -syn, the hallmark protein of PD, in a cohort of workers occupationally exposed to pesticides. Our primary aim was to examine whether exposure to chlorpyrifos, as measured by BuChE-chlorpyrifos adducts, was positively associated with  $\alpha$ -syn in blood cells. We found no evidence of this, nor of an association between blood  $\alpha$ -syn and self-reported chlorpyrifos exposure. Similarly, there was no association between blood  $\alpha$ -syn and BuChE inhibition, which is notable because chlorpyrifos more efficiently inhibits BuChE compared to AChE (Eaton et al. 2008). There also was no association between blood  $\alpha$ -syn and the other two cholinesterase-inhibiting insecticides that we were able to consider, azinphos-methyl and carbaryl. Although our findings were thus generally negative for these types of pesticides, we did observe increased blood  $\alpha$ -syn levels among those with the T allele at position -108 in the *PONI* promoter region, the allele associated with lower levels of paraoxonase enzyme expression due to disruption of a transcription factor binding site (Deakin et al. 2003). This association may reflect the enzyme's more generic anti-oxidant potential (Shih et al. 1998), rather than its ability to hydrolyze the oxons of chlorpyrifos and diazinon, given our negative results for chlorpyrifos and apparently limited exposure to diazinon. The absence of an association between  $\alpha$ -syn and *PONI* Q192R genotype, which is thought to influence the catalytic efficiency of chlorpyrifos oxon hydrolysis (Costa et al. 2013), underscores this possibility.

Our secondary analyses identified other potential indicators of pesticide exposure associated with greater  $\alpha$ -syn in blood cells: Handling unknown pesticides in the preceding 30 days,

handling pesticides for 10 hours in the preceding 30 days, and providing a blood sample in 2007 (vs. 2008–2010). Given the number of comparisons made, and our modest sample size, some or all of these secondary findings may be due to chance, particularly since dose-response associations were not clearly present. In particular, we cannot account for the markedly greater blood  $\alpha$ -syn in 2007 compared to later years. Despite compelling evidence that rotenone, paraquat and maneb may relate to increased  $\alpha$ -syn and risk of PD (Bové and Perier 2012; Chorfa et al. 2013; Pan-Montojo et al. 2010; Pezzoli and Cereda 2013; Tanner et al. 2011), there do not appear to have been year-to-year changes in the use of these or similar compounds that might explain the year to year changes in  $\alpha$ -syn that we observed (USDA 2013). Paraquat and mancozeb (manganese-containing fungicide like maneb) were each applied to 11–16% of acres producing apples in Washington State during the study years, while neither rotenone nor maneb were individually listed in this survey in any relevant survey years (2007, 2009 or 2011; no survey data for 2008 or 2010). Because none of these four pesticides were included on our questionnaire, which focused on cholinesterase inhibitors, we were unable to directly evaluate their potential effect on blood  $\alpha$ -syn. This is a limitation, as is our inability to consider some combined measure of total pesticide exposure, or perhaps a more biologically relevant measure (e.g. total oxidative stress potential). However, there were marked changes in use of other pesticides between 2007 as compared to all later survey years (2009 and 2011). These include reductions in the proportion of apple acres treated with chlorpyrifos and azinphos-methyl, and commensurate increases for some insect pheromones and the neonicotinoid insecticides imadacloprid and thiacloprid (USDA 2013). Changes were not limited to insecticides, however. For example, the portion treated with any herbicide dropped from 61% in 2007 to 40–43% in 2009 and 2011, and the use of sulfur as a fungicide increased from 30% in 2007 to 47–48% in 2009 and 2011 despite slight decreases in the total portion of acres treated with fungicides.

The finding that blood  $\alpha$ -syn was greater in workers who handled pesticide(s) that they could not specify could indicate that blood  $\alpha$ -syn might be affected by any number of pesticides, even those not registered for use on apples in 2007–2010. However, we speculate that a lack of knowledge about what pesticide was recently applied also may indicate less rigorous use of personal protective equipment. Although we were unable to examine the association between  $\alpha$ -syn and the use of personal protective equipment, we were able to consider data on the pesticide handlers' years of experience, and that was unrelated to  $\alpha$ -syn.

Our exploratory analysis of blood  $\alpha$ -syn changes over time suggested that blood  $\alpha$ -syn can fluctuate markedly within an individual. Notably, three of four workers experienced substantial increases during the course of the spray season. To our knowledge, longitudinal changes in blood  $\alpha$ -syn have not been previously examined. Therefore, this observation must be interpreted with caution given the very small number of paired samples available for this analysis. Moreover, all of our findings, including this one, remain somewhat difficult to interpret with regard to PD because as noted above, it is unknown the extent to which blood  $\alpha$ -syn indicates  $\alpha$ -syn levels in the brain or PD risk.

## 5. Conclusions

Some pesticide exposures or susceptibility factors may affect  $\alpha$ -syn levels in blood cells, but we observed little evidence that chlorpyrifos or other cholinesterase inhibitors increase blood  $\alpha$ -syn in this contemporary sample of workers in the U.S. with little cholinesterase depression. Longitudinal studies in a larger sample of more highly exposed workers in which pre- and post-spray  $\alpha$ -syn levels are measured in blood will be needed to confirm our largely negative findings, and explore the few positive findings suggested. Ideally, future epidemiologic studies should be designed to also test the hypothesis that other pesticides, such as rotenone, maneb, mancozeb and paraquat, or combinations of pesticides, increase  $\alpha$ -syn concentrations in blood and to the extent possible within the brain as well. These investigations may help identify what occupational factors among pesticide handlers might increase PD risk, which also could have implications for risk among the general public with potential exposure to agricultural pesticides.

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## Abbreviations

<b>AChE</b>	acetylcholinesterase
<b><math>\alpha</math>-syn</b>	$\alpha$ -synuclein
<b>BuChE</b>	butyrylcholinesterase
<b>PON1</b>	paraoxonase
<b>PD</b>	Parkinson disease

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### Highlights

- We assess  $\alpha$ -synuclein in blood from active agricultural pesticide handlers
- Blood  $\alpha$ -synuclein is not associated with butyrylcholinesterase-chlorpyrifos adducts
- Blood  $\alpha$ -synuclein is not associated with other indicators of chlorpyrifos exposure
- Blood  $\alpha$ -synuclein is associated with paraoxonase genotype

**Table 1**

Characteristics of agricultural pesticide handlers providing blood specimens, central Washington State, 2007–2010

	<b>All workers N=63 n (%)</b>	<b>Interviewed workers N=57 n (%)</b>
Male	--	57 (100)
Hispanic	--	56 (98)
Educated in Mexico	--	53 (96) <sup>a</sup>
Age first provided a specimen, years		
Range	20–60	20–60
Median	33	33
Mean (standard deviation)	34.5 (9.1)	33.9 (8.8)
Years handling pesticides <sup>a</sup>		
1	--	15 (27)
2–3	--	13 (24)
4–5	--	11 (20)
6–10	--	13 (24)
11–15	--	3 (5)
Year first provided a specimen		
2007	11 (17)	8 (14)
2008	26 (41)	25 (44)
2009	16 (25)	14 (25)
2010	10 (16)	10 (18)

<sup>a</sup>Percent excludes 2 without data on smoking, education and years handling pesticides

**Table 2** Blood α-synuclein and potential indicators of exposure to cholinesterase inhibiting insecticides among agricultural pesticide handlers, central Washington State, 2007–2010

	All Years			Excluding Year 2007		
	N=90 n	Mean α-synuclein (µg/mL)	Age-adjusted relative mean in µg/mL (95% CI)	N=79 n	Mean α-synuclein (µg/mL)	Age-adjusted relative mean in µg/mL (95% CI)
<b>% Butyrylcholinesterase-chlorpyrifos adducts<sup>a,b</sup></b>						
>20	22	75.8	-3.9 (-13.8, 5.9)	20	74.7	-2.4 (-12.5, 7.8)
>5 to <20	14	76.7	-2.1 (-13.3, 9.2)	13	72.9	-4.4 (-15.8, 7.0)
5	53	78.8	Reference	45	77.2	Reference
<b>% Butyrylcholinesterase inhibition</b>						
>20	14	78.1	0.3 (-11.9, 12.6)	12	76.7	1.6 (-11.2, 14.4)
>5 to <20	40	77.9	1.0 (-7.6, 9.6)	35	75.5	0.01 (-8.9, 8.9)
5	36	76.9	Reference	32	75.4	Reference
<b>% Acetylcholinesterase inhibition<sup>b</sup></b>						
>20	2	99.8		1	- <sup>c</sup>	
>5 to <20	4	76.3	7.2 (-8.5, 22.9)	3	70.0	8.6 (-9.8, 27.0)
5	83	77.1	Reference	74	75.2	Reference
<b>Blood collected because of cholinesterase inhibition in the previous recent blood sample</b>						
Yes	8	85.9	8.8 (-5.0, 22.7)	8	85.9	11.7 (-1.6, 25.1)
No	82	76.7	Reference	71	74.5	Reference
<b>Used chlorpyrifos<sup>d</sup></b>						
Yes	51	76.8	-2.0 (-10.3, 6.2)	48	75.3	-2.5 (-11.0, 6.0)
No	31	78.4	Reference	27	77.7	Reference
<b>Used azinphos-methyl<sup>d</sup></b>						
Yes	13	71.1	-8.2 (-19.0, 2.7)	11	68.9	-9.1 (-20.6, 2.4)
No	69	78.6	Reference	64	77.4	Reference

	All Years			Excluding Year 2007		
	N=90 n	Mean $\alpha$ -synuclein ( $\mu\text{g/mL}$ )	Age-adjusted relative mean in $\mu\text{g/mL}$ (95% CI)	N=79 n	Mean $\alpha$ -synuclein ( $\mu\text{g/mL}$ )	Age-adjusted relative mean in $\mu\text{g/mL}$ (95% CI)
<b>Used carbaryl<sup>d</sup></b>						
Yes	22	79.6	3.1 (-5.8, 12.1)	21	80.2	5.7 (-3.3, 14.6)
No	60	76.6	Reference	54	74.6	Reference
<b>Used other organophosphorus or carbamate insecticides<sup>d,e</sup></b>						
Yes	15	71.8	-6.9 (-17.1, 3.3)	15	71.8	-5.5 (-15.6, 4.6)
No	67	78.6	Reference	60	77.3	Reference
<b>Used unknown pesticide<sup>d</sup></b>						
Yes	7	93.1	19.8 (5.7, 33.9)	6	94.0	21.8 (6.9, 36.6)
No	75	75.9	Reference	69	74.6	Reference
<b>Time since last handled insecticides<sup>f</sup></b>						
1 day	18	76.3	-0.1 (-11.4, 11.1)	16	72.5	-1.7 (-13.4, 10.0)
>1 day and 1 week	30	77.1	0.3 (-9.4, 10.0)	29	77.4	2.8 (-7.1, 12.8)
>1 week	32	78.4	Reference	28	77.0	Reference
<b>Hours handled insecticides<sup>d</sup></b>						
40	11	72.6	2.6 (-11.3, 16.4)	11	72.6	5.5 (-7.8, 18.9)
30-39	20	79.5	9.5 (-2.4, 21.5)	20	79.5	12.5 (0.8, 24.1)
20-29	10	82.9	12.2 (-2.2, 26.6)	9	84.7	17.2 (2.9, 31.6)
10-19	16	76.9	7.2 (-5.3, 19.8)	14	71.8	5.0 (-7.6, 17.5)
<10	14	69.4	Reference	12	66.8	Reference
<b>Days applying insecticides for &gt;8 hours<sup>d,f</sup></b>						
5	8	78.7	6.2 (-12.1, 24.4)	7	80.4	9.6 (-9.3, 28.5)
3-4	37	77.7	4.5 (-9.7, 18.6)	36	77.3	5.5 (-9.0, 20.0)
1-2	29	77.7	3.9 (-10.6, 18.4)	25	74.4	1.6 (-13.4, 16.6)
0	8	73.5	Reference	7	72.5	Reference
<b>Years handling pesticides<sup>g</sup></b>						
11-15 years	7	72.8	-11.3 (-32.1, 9.6)	7	72.8	-9.5 (-30.6, 11.6)
6-10 years	22	76.1	-3.7 (-16.9, 9.4)	20	75.6	-4.0 (-17.8, 9.8)
4-5 years	15	80.5	2.2 (-11.1, 15.6)	13	76.2	-1.9 (-15.7, 11.9)

	All Years			Excluding Year 2007		
	N=90 n	Mean $\alpha$ -synuclein ( $\mu\text{g/mL}$ )	Age-adjusted relative mean in $\mu\text{g/mL}$ (95% CI)	N=79 n	Mean $\alpha$ -synuclein ( $\mu\text{g/mL}$ )	Age-adjusted relative mean in $\mu\text{g/mL}$ (95% CI)
2-3 years	19	78.3	-0.2 (-13.0, 12.5)	17	76.5	-1.9 (-15.2, 11.3)
1 year	17	76.2	Reference	16	76.8	Reference
<b>PONI C-108T genotype</b>						
TT	22	80.9	7.5 (-2.5, 17.5)	18	77.8	5.7 (-4.8, 16.2)
CT	32	79.7	6.6 (-2.5, 15.6)	28	78.4	6.3 (-3.0, 15.5)
CC	36	73.5	Reference	33	72.1	Reference
<b>PONI Q192R genotype</b>						
QQ	28	75.6	-2.3 (-13.0, 8.4)	25	72.2	-3.2 (-14.4, 7.9)
QR	40	78.5	0.8 (-9.3, 10.8)	36	78.2	2.7 (-7.8, 13.2)
RR	22	78.2	Reference	18	75.4	Reference
<b>Year of blood collection</b>						
2010	22	75.3	-15.9 (-28.8, -2.9)			
2009	23	83.0	-8.3 (-21.2, 4.6)			
2008	34	70.9	-20.2 (-32.4, -8.0)			
2007	11	91.1	Reference	0	--	--

<sup>a</sup>In whole blood, limit of detection at approximately 4% adducts

<sup>b</sup>Excludes 1 subject for whom assay data were not available

<sup>c</sup> $\alpha$ -syn at 95<sup>th</sup> percentile; exact value not shown to protect confidentiality (N=1)

<sup>d</sup>Within previous 30 days, assessed by in-person questionnaire (Hofmann 2010b), excludes up to 19 without questionnaire data

<sup>e</sup>Phosmet (N=4), methidathion (N=1), methamidophos (N=2), dichlorvos (N=1), dimethoate (N=1), other (N=8) excluding diazinon and malathion, which were included on the questionnaire but not reportedly used

<sup>f</sup>Also adjusted for blood sample type (routine vs. cholinesterase depression follow up)

<sup>g</sup>Excludes 10 without data on number of years handling pesticides

**Table 3**Predictors of blood  $\alpha$ -synuclein among agricultural pesticide handlers, central Washington State, 2007–2010

	Mutually-adjusted relative mean $\alpha$ -synuclein in $\mu\text{g/mL}$ (95% CI) <sup>a</sup> N=90	p-value
Used unknown (vs. specified) pesticides <sup>b</sup>	19.4 (5.1, 33.7)	0.008
Handled insecticides 10 (vs. <10) hours <sup>b</sup>	14.7 (4.3, 25.2)	0.006
Year 2007 (vs. 2008–2010)	18.7 (7.0, 30.4)	0.002
<i>PONI</i> C-108T (per T allele) <sup>c</sup>	5.7 (0.8, 10.6)	0.02
Pesticide handler age (per year)	0.3 (−0.1, 0.6)	0.13

<sup>a</sup> Also adjusted for no interview data on hours and/or type of pesticides in preceding 30 days, to allow the inclusion of all specimens in the mutually-adjusted model

<sup>b</sup> In preceding 30 days

<sup>c</sup> Modeled linearly (0, 1 or 2 T alleles) as indicated as appropriate according to likelihood ratio test; estimate shown is per allele (i.e. CT vs. CC, and TT vs. CT); accordingly the estimate for TT vs. CC is 11.5 (95% CI 1.7, 21.2)