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Longitudinal Assessment of Chlorpyrifos Exposure and Self-Reported Neurological Symptoms in Adolescent Pesticide Applicators

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ABSTRACT

Objectives: Occupational exposure of organophosphorus pesticides (OPs) such as chlorpyrifos (CPF) in adolescents is of particular concern because the potential vulnerability of the developing neurological system. The objectives of the study were to examine how neurological symptoms reported over the CPF application season vary across time, whether these effects are reversible post application and if there are any associations between CPF biomarkers and neurological symptoms in an adolescent study population.

Methods: Egyptian adolescent CPF applicators (n=57) and non-applicators (n=38) were recruited for a longitudinal study. Self-reported data for 25 neurological symptoms were collected at 32 time points over the 7-month period before, during and after CPF-application. Urine and blood samples were collected for CPF-specific biomarkers urinetrichloro-2-pyridinol (TCPy), and blood cholinesterase.

Results: We observed increased reporting of neurological symptoms among both applicators and non-applicators after several weeks of repeated CPF application. Applicators demonstrated a greater percentage of neurological symptoms relative to baseline than the non-applicators after accounting for potential covariates. Similar models revealed that cumulative TCPy was positively and significantly associated with the average percentage of symptoms, but only among the applicators. Associations of the change butyrylcholinesterase (BChE) from pre to post application season with several subclasses of symptoms were also found significant or marginally significant.

Conclusions: These observations reinforce the growing concern regarding the neurotoxic health effects of CPF in adolescents and the importance of exposure prevention during the application season.

STRENGTHS AND LIMITATIONS OF THE STUDY:

- This is the first longitudinal study showing the association between specific organophosphorus pesticide exposure and reporting of neurological symptoms in adolescent applicators.
- Symptoms in applicators are compared with symptoms in non-applicator thus showing the effect of environmental CPF exposure in general population.
- The study is also novel in its approach to include prospective measures of biomarkers of CPF exposure and effect and to examine their associations with neurological symptoms.
- The non-specific nature of many of the symptoms is a limitation of the current study.
- Small sample size is another limitation study that may have influenced the significance levels of exposure-outcome relationships.
- Results of the study may be generalizable only to agricultural communities with similar sociodemographic characteristics.

What this paper adds

- It is not fully understood how neurological symptoms vary across time in adolescents exposed to specific organophosphorus pesticide.
- Applicators are more likely to report increased symptoms compared to non-applicators.
- Repeated occupational exposure to CPF increases the reporting of acute neurological symptoms during the CPF application season and the symptoms may persist for months after the cessation of exposure in both applicators and non-applicators.
- Cumulative biomarker of CPF exposure also demonstrates association with neurological symptoms in applicators.
- Reduction of CPF exposure among the adolescent applicators should be a public health priority since neurological symptoms remained elevated even after the cessation of CPF application.

INTRODUCTION

High prevalence of agricultural use of organophosphorus pesticides (OPs) has been recognized as a major global public health challenge for agriculture-based communities due to their associations with neurological outcomes. Immediate or short-term neurological signs and symptoms ranging from less severe (headache, dizziness, nausea etc.) to more severe (muscle weakness, bronchospasm, change in heart rate etc.) were all reported after occupational OP exposure [1]. These short-term symptoms were reported as early as 48 hours after acute exposure [2]. Although high levels of occupational OP exposure can be associated with symptoms persisting for several years [3], repeated moderate to low exposures can also produce chronic neurological symptoms and deficits in neurobehavioral performance [4]. Converging

evidence regarding the associations between OP exposures and neurological symptoms is based on adult occupational studies conducted in a wide range of study settings. These include comparisons between exposed and non-exposed farmworkers in the US [5], South Africa [6], Nicaragua [7 8], Kenya [9], Sri Lanka [10]and Egypt [11]. These studies have used self-reported questionnaire data containing non-specific neurological symptoms. Additional evidence for the effect of pesticides on somatic and mood symptoms are also found in the literature [2 12].

Although less commonly studied, OP exposures were also found to be associated with neurological symptoms in children and adolescents. In developing countries children and adolescents are engaged in OP application and this presents a major public health concern[13]. Even in the US, adolescents can be involved in mixing and applying pesticides[14 15]. Because of their smaller body size, the biological doses of pesticides (for children and adolescents may be substantially higher than adults [16], making them more vulnerable to neurological effects. Animal and human studies have also suggested that paraoxonase (PON 1)—an organophosphate detoxifying enzyme—is less active in younger populations making them more vulnerable to OP toxicity[17 18]. An Egyptian cross-sectional study found adolescent pesticide applicators reporting more neurological symptoms and neuromuscular problems than controls [19 20]. Association between environmental OP exposure and neurological symptoms was also demonstrated in children living in an Indian agricultural community [21].

Biomarkers have been used to characterize OP exposure in epidemiological and occupational studies. Urinary trichloro-2-pyridinol (TCPy), a relatively specific CPF metabolite of exposure, which is eliminated in the urine with a half-life of 27 hr following exposure [22]. Due to the ease and non-invasiveness of collection of urine samples, TCPy is widely recognized as a useful biomarker of exposure, particularly in children and adolescents [23 24]. The classic mode of OP toxicity is manifested by the inhibition of cholinesterase. Both blood acetylcholinesterase (AChE) and butyrylcholinesterase

(BChE) are biomarkers of effect with BChE being more sensitive to inhibition by OP pesticides[25]. A small number of adult studies found associations between inhibition of cholinergic activities with self-reported symptoms [9 10]; however, this relationship has rarely been examined in adolescent studies.

Understanding the relationship between OP exposure and the change in neurological symptoms across time (temporal change) is important because application-related exposure follows a seasonal pattern in most areas. Moreover, specific OP exposure is important to track the changes in symptom reporting over time. Two longitudinal studies with agricultural workers demonstrated that short-term neurological signs and symptoms were associated with initial acute episodes of exposure, which eventually advanced into long-term sequelae [7 12]. However, these studies did not characterize exposure and did not identify any specific OP that was being applied.

To investigate whether occupational exposure to CPF is associated with self-reported neurological symptoms, we compared adolescent applicators exposed to CPF with adolescent non-applicators working and residing in Egypt through a prospective study. Typically, CPF is the primary insecticide used by pesticide applicators in Egyptian cotton fields, including adolescent applicators, and offered us a unique exposure environment with well characterized occupational exposure. The possibility of potential confounding effects of other neurotoxic pesticides was minimal because of limited use of other pesticides in the study area. We attempted to answer the critical questions of how repeated exposures to OP determines reporting of neurological symptoms, how neurological symptoms vary across time during the exposure season, if these effects could reverse at the cessation of exposure and whether there are any associations between OP biomarkers and neurological symptoms in the adolescent study population. A questionnaire was administered pre-, mid- and post-CPF application season to examine changes in self-reported symptoms across time.

METHODS

Study area and population

A prospective study was conducted in Menoufia Governorate, Egypt from April 2010 to January 2011. Two of the nine districts of Menoufia, Al-Shohada and Berket El-Sabea were chosen randomly to conduct the study (Supplementary Figure 1). In Egypt, adolescents are hired seasonally to apply pesticides to cotton fields and the schedule of pesticide applications to the cotton crop is regulated by the Ministry of Agriculture. In the year of 2011, approximately 2100 liters of OPs were applied on approximately 5700 acres of cotton fields (personal communication with the Ministry of Agriculture). Chlorpyrifos is the primary OP applied in the districts of Menoufia governorate from mid-June to early August. Although there are slight variations in the timing of CPF application between the two districts (Supplementary Figure 2) the application patterns are consistent across field stations.. The typical workday was from 8am-12pm and from 3pm-7pm, six days per week. Because there is no regulation in Egypt for mandatory use of personal protective equipment (PPE), dermal exposure and inhalation were both considered as the potential route of exposure in this population [19]. Recently, [26] reported that dermal exposure and subsequent absorption through the skin accounted for 94-96% of the total dose of chlorpyrifos in Egyptian pesticide applicators.

Recruitment and data collection

Fifty-eight male adolescents aged 12-21, hired seasonally by the Ministry of Agriculture to spray pesticides in the cotton fields were recruited from two field stations in the Menoufia governate (i.e. Al-Shohada and Berket El-Sabea, field station 1 and field station 2, respectively). Forty adolescent non-applicators were recruited through convenience sampling (word of mouth, direct communication with utilizing contacts through the staff from the local Ministry of Agriculture) from the same districts as the applicators for the cotton crop. These adolescents never worked in the field as pesticide applicators. We excluded one adolescent from the final analysis due to inconsistency in participating in study activities and two other subjects for questionable sample integrity, resulting in a final sample size of 95 (57

applicators and 38 non-applicators). Written informed consent was obtained from all participants and their legal guardian (for those under 18). All the subjects were monetarily compensated for their time during the questionnaire survey, medical examination and biological samples (~$5 per visit). The study was approved by the OHSU IRB in June 2009, and by the Medical Ethics committee of the Faculty of Medicine, Menoufia University in July 2009.

Data collection occurred at the primary field station for each district. Pesticides applicators and supervisors meet in the field stations, which also provides storage area for the pesticides and the equipment used for application.

Outcome assessment

We developed a multiple time-point, 25-item, short-term neurological symptom questionnaire on the basis of the widely used Q16 questionnaire [27] and a modified version of the Q16 used in a previous study with licensed pesticide applicators [28]. The 25 symptoms were grouped into six domains: behavioral, autonomic, cognitive, sensory, motor and non-specific temporary disability (Table 1). There were five frequency choices (0-4) for each symptom ranging from "never" (coded as 0) to "everyday of the week" (coded as 4). Since more than 90% of the responses were between 0-2 (1=once a week and 2=once in every 2-3 days) we recoded each of the symptom response to "0" or "never" and "1" or "at least once a week or more." Beginning on June 2 of 2010 through January 2011 participants reported symptoms occurring in the past week through this symptom questionnaire administered 32 separate times, at least once per week) and spanning all relevant application periods in the season (pre-application, during application, and post-application). The number of positive responses was totaled for each person to yield a score ranging from 0–25; division by 25 produced the proportion of symptoms endorsed and this proportion was averaged across the 32 collection points to produce a season-level mean proportion of self-reported symptoms. Participants also completed a questionnaire at baseline addressing their socio-demographic status, household and occupational use of pesticides such as number of days of pesticide

application or mixing, medical history, safety practices and lifestyle activities including smoking status, hours of sleep at night, number of drinks containing caffeine.

Table 1. Domains of neurological symptoms

Domains	Symptoms
Behavioral Symptoms	1. Tense or anxious[#] 2. Excessively angry or irritable*[#] 3. Depressed or withdrawn*[#]
Autonomic Symptoms	4. Nausea[#] 5. Heavy sweating*[#] 6. Loss of appetite[#] 7. Fast heart rate*[#] 8. Excessive salivation
Cognitive Symptoms	9. Difficulty concentrating*[#] 10. Being absentminded and memory problem*[#]
Sensory Symptoms	11. Difficulty seeing at night[#] 12. Blurred or double vision[#] 13. Numbness in hands and feet[#] 14. Sense of smell or taste change[#] 15. Ringing in ears
Motor Symptoms	16. Difficulty with balance[#] 17. Weakness in arms and legs[#] 18. Involuntary movement of arms and legs[#] 19. Shaking in hands* 20. Difficulty speaking[#]
Temporary Disability (non-specific symptoms)	21. Dizziness[#] 22. Headache*[#] 23. Momentary loss of consciousness[#] 24. Fatigue*[#] 25. Insomnia[#]

*Symptoms used in Q-16 [27]

#Symptoms used in Agricultural Health Study [28]

Urine collection and analysis

Urine was collected in wide mouth plastic cups at eight time points between April 2010 and January 2011. We collected spot urine samples at the field station at the beginning of the work shift. The cups were subsequently transferred to the laboratory at Menoufia University in a cooler with wet ice. At the laboratory, 4 ml aliquots of urine were transferred into labeled 5 ml cryovials within hours of sampling and stored at –20 ºC. The banked urine samples were express mailed on dry ice to University of Buffalo laboratory for analysis of pesticide metabolites; duplicate samples were retained in the –20 ºC freezer at Menoufia. Urine samples in the field station at Berket El-Sabea district were collected one day after the collection date of the field station at Al-Shohada.

The method of urinary TCPy measurement (a primary metabolite of chlorpyrifos) has been described elsewhere [23]. Briefly, negative-ion chemical ionization gas chromatography–mass spectrometry was used that utilized 13C‑15N‑3,5,6‑TCPy as an internal standard. Jaffe reaction was used for colorimetric analysis of creatinine [29]. The within-run imprecision of this assay is very low (< 2% coefficient of variation and an intra-class correlation coefficient of 0.997). The quality control (QC) samples consisted of lab samples that were first analyzed for TCPy levels; these levels were non-detectable. Twenty aliquots were then spiked with 50ng of TCPy/mL of urine; these were then extracted and analyzed as per protocol. The recovery rates ranged from 92% - 98% with the average being 94.8%, SD = 0.931 and the CV% = 1.965, minimum detection level was 0.0501 ng. QC replicates had 94.75% recovery. Finally, cumulative urinary TCPy for each participant was determined by calculating the area under the curve for the plotted values for eight time intervals.

Blood collection and ChE analysis

To establish the baseline ChE activity, pre-application blood draws occurred on April 11 and June 2, 2010, prior to the start of the official government-regulated CPF application season. As with urine collection, blood draws in the field station at Berket El-Sabea were performed one day later. Changes in

both AChE and BChE levels from baseline to the end of CPF-application season (blood collected on September 4, 2010) were estimated. Blood samples were collected by venipuncture into 10mL lavender top (EDTA) vacutainer tubes and immediately placed on wet ice and transported to Menoufia University, where they were analyzed in duplicate for AChE and BChE activity using an EQM Test-Mate kit (EQM Research Inc., Cincinnati, OH, USA) as described previously [23].

Statistical analysis

We used SPSS version 18.0 and STATA (version 11; Stata Corporation, College Station, TX) for the statistical analysis. Sociodemographic variables were summarized and described using means and standard deviations for continuous responses and percentages for discrete outcomes; simple comparisons between applicators and non-applicators were completed using t-tests (continuous measures) or chi-square tests (discrete outcomes). Concentrations of TCPy, AChE and BChE exhibited pronounced right skewness and more than a 3-fold separation between the minimum and maximum observed values; consequently, these responses were log transformed prior to analysis to improve symmetry. Both AChE and BChE were expressed as a log-transformed ratio of post-application activity relative to pre-application activity prior to investigation of associations with average percentage of self-reported symptoms. All p-values are two-sided with significance judged relative to a 0.05 level.

Spearman correlation coefficients were used to estimate associations between urine and blood biomarkers and symptom scores. Self-reported neurological symptom counts were collected at 32 irregularly spaced dates over an eight-month period spanning from early June 2010 through early January 2011. These sample periods were collapsed into 10 separate non-overlapping intervals lasting between one and four weeks in length (Supplementary Figure 2). Symptom data from the first three dates (i.e first fifteen days of the study from June 2 to June 16), when no CPF was applied, was collectively taken to represent the baseline time interval (or time interval 1) against which symptom reporting from the other

nine remaining time intervals was evaluated. In five of these nine time intervals (between June 19 and July 21), application of CPF was reported in both field stations. Generalized estimating equations (GEE) [30] were used to model the proportion of neurological symptoms reported in each time interval while controlling for number of days worked (within five days of the symptom reporting date), home use of pesticides, age, education and income levels. The one fitted model was used to estimate changes over time, relative to the first time interval (June 2–June 16), for applicators and non-applicators as well as examine whether changes relative to baseline differed between the two groups (via group-by-time interaction).

RESULTS

Sociodemographic Characteristics

Ninety-two of the participants (97%) were between 12 and 18 years old with the remaining three between 19 and 21. The two groups, non-applicators and applicators, did not differ significantly in terms of age, educational status, family income, number of people in house, years of pesticide use at home, and insecticides and rodenticides use at home (Table 2). Compared to non-applicators, a significantly higher number of applicators lived close to the field (within 25 meters), had carpet in their homes and applied herbicides at home. Applicators had significantly lower BMI than non-applicators. On average, applicators had been working in the field for a little over 3 years.

Table 2: Sociodemogrphic characteristics for participants at baseline

Variables		Non-applicators (n=38) Mean (SD)	Applicators (n=57) Mean (SD)
Age		16.6 (2.4)	16.2 (1.6)
Education		9.8 (1.8)	9.9 (1.8)
Height (cm) *(34 non-applicators vs 30 app)*		166.3 (12.0)	163.4 (10.0)
Weight (kg) *(34 non-applicators vs 30 app)**		62.0 (15.4)	54.2 (8.6)
BMI (kg/m^2) (34 non-applicators vs 30 app)*		22.1 (3.7)	20.2 (2.2)
Number of people in house		5.6 (1.1)	6.0 (1.8)
Home pesticide use (years)* (19 Non-applicators & 44 App)*		1.6 (1.9)	2.5 (1.9)
Occupational application of pesticides (yrs)		-	3.1 (1.5)
Days/week of pesticide application		-	4.8 (1.3)
Hours/day of pesticide application		-	5.2 (0.7)
		% (n)	**% (n)**
Family Monthly Income (Low)		78.9 (30)	71.9 (41)
Applied pesticides in home in last 5 yrs (yes)*		47.4 (18)	78.9 (45)
Computer use (once a week or more)*		65.8 (25)	45.6 (26)
Carpet in house (yes)*		27.0 (10)	54.4 (31)
Live close to agricultural field (yes)*		23.7 (9)	50.9 (29)
Types of pesticides applied at home (24 vs 49)			
	Herbicides*	13.0 (3)	44.9 (21)
	Insecticides	83.3 (20)	93.9 (46)
	Rodenticides	16.7 (4)	14.3 (7)

*p<0.05 for group difference

Change in symptoms over time

We considered day 0-14 as baseline time interval (1st time interval) when no application of CPF was reported. Applicators began increased reporting of neurological symptoms at the beginning of the chlorpyrifos application season (at the 2nd time interval between days 17-21 of the study). The percentage of neurological symptoms increased during the application season and reached the peak at the 6th time interval representing days 45-48, the time when the chlorpyrifos application period ended. The highest peak of symptom reporting was observed at the 8th time interval representing days 63-77 (Table 3). Similar to the applicators, the non-applicators also demonstrated the highest increase in the proportion of neurological symptoms during the 8th time interval although the magnitude of the change was smaller (14 percentage point increase of symptoms relative to baseline interval). The change of neurological symptoms relative to baseline declined over the next two time intervals (9th and 10th) in both groups. For applicators, the percentage of reported symptoms at each of the nine subsequent time intervals was always higher than the percentage observed at baseline; non-applicators by contrast had a pattern of percentage of reported symptoms that both increased and decreased relative to baseline time interval over the course of the study (Figure 1).

When applicators and non-applicators are compared with respect to change in percentage of symptoms (relative to baseline), it was always the case that the change (percentage point change relative to baseline) for applicators was greater than the corresponding change for non-applicators even after adjusting for the covariates (Table 3).

Table 3. Estimated change (95% CI) from baseline in the percentage points of neurological symptoms reported at each of nine successive collected time points, shown separately for non-applicators and applicators.

	Time Intervals	Non-Applicators Adjusted Models*		Applicators Adjusted Models*		Change in difference between applicators and non-applicators relative to difference in baseline	
1	Days from Baseline (Day 0-14) June 2-June 14, 2010	b (% of Symptoms) (95% CI)	p-value	b (% of Symptoms) (95% CI)	p-value	b (% of Symptoms) (95% CI)	p-value
2	17-21 Jun 19-Jun 23	-2.74 (-4.61,-0.86)	0.004	4.08 (0.18,7.97)	**0.040**	6.81 (2.47, 11.15)	**0.002**
3	24-28 Jun 26-Jun 30	-2.68 (-5.18,0.17)	0.004	12.57 (7.75,17.38)	**<0.001**	15.25 (9.80, 20.69)	**<0.001**
4	31-35 Jul 3-Jul 7	4.45 (-0.80,9.71)	0.10	14.06 (9.21,18.90)	**<0.001**	9.60 (2.44, 16.77)	**0.009**
5	38-42 Jul 10-Jul 14	-2.28 (-4.75.0.17)	0.07	22.83 (19.25,26.40)	**<0.001**	25.11 (20.79, 29.43)	**<0.001**
6	45-48 Jul 17-Jul 21	0.46 (-2.37,3.30)	0.75	28.80 (24.27,33.35)	**<0.001**	28.35 (22.98, 33.72)	**<0.001**
7	52-59 Jul 24-Jul 31	4.95 (1.63,8.28)	**0.003**	24.01 (19.92,28.09)	**<0.001**	19.05 (13.80, 24.30)	**<0.001**
8	63-77 Aug 4-Aug 18	14.49 (11.45,17.55)	**<0.001**	30.10 (26.53,33.66)	**<0.001**	15.60 (10.95, 20.26)	**<0.001**
9	80-94 Aug 21-Sep 4	12.08 (8.72,15.44)	**<0.001**	29.17 (25.22,33.13)	**<0.001**	17.09 (11.93, 22.25)	**<0.001**
10	105-217 Sep 22-Jan 5[#]	9.22 (6.12,12.32)	**<0.001**	18.45 (14.30,22.59)	**<0.001**	9.22 (4.12,14.33)	**<0.001**

*Estimates have been adjusted for number of days worked applying pesticides, home use of pesticides, age, education and income level. CPF application time intervals are shaded in grey.

Associations of neurological symptoms with biomarkers

TCPy was detected in 100% of the samples. Summary statistics for TCPy, AChE and BChE of the study samples have been already reported by [31], Mean creatinine concentration of the urine samples was reported to be 1696 µg/ml with maximum of 4199 and a minimum of 164 µg/ml. In brief, the applicators had much higher mean and estimated median peak TCPy concentration than the non-applicators (mean: 719 vs 44.9 µg/g creatinine; estimated median 137 vs 19.7 µg/g creatinine). In our study sample, BChE was found to be more sensitive to CPF exposure than AChE, with median activity reduced by 37% from baseline in applicators and 13% in non-applicators during the CPF application period.

A scatter plot of cumulative TCPy (ug/g creatinine) against average percentage points of symptoms revealed distinct exposure-response gradients by pesticide application status (applicator vs non-applicator) (Supplementary Figure 3a). In addition, two other scatter plots of change in AChE activity and change in BChE activity from pre-application to post-application against percentage of symptoms also revealed effect measure modification by pesticide application status (Supplementary Figures 3b & 3c). Therefore, separate linear models for applicators and non-applicators were used to examine the associations of these three biomarkers with the outcome measures.

Log-transformed TCPy was positively associated with the average percentage of neurological symptoms in the regression models after adjusting for field stations, age, family income, home pesticide use and average number of hours worked in the field among applicators (b=2.68, p=0.007). However, non-applicators demonstrated positive but statistically non-significant associations between TCPy and symptoms. Among applicators, AChE and BChE activity was negatively and significantly associated with the average percentage of neurological symptoms in the unadjusted models. In the adjusted models these associations remained negative but became non-significant (Table 4).

Table 4. Summary of regression analysis for biomarkers of exposure & effect of chlorpyrifos predicting average percentage of neurological symptoms over the entire study stratified by applicator status

	Unadjusted Models			Adjusted Models*		
Explanatory variables	**B (se)**	**95% CI**	**p-value**	**B (se)**	**95% CI**	**p-value**
For Non Applicators						
Ln TCPy (mg/g Cr) (n=28)	0.29 (0.76)	-1.26,1.84	0.71	0.57 (0.79)	-1.06,2.20	0.47
Ln (Post AChE/Pre AChE) (n=21)	-1.25 (16.41)	-35.59,33.1	0.94	-6.57 (18.80)	-43.64,33.50	0.73
Ln (Post BChE/Pre BChE) (n=21)	2.23 (7.04)	-12.51,16.98	0.76	2.50 (7.63)	-13.77,18.77	0.75
For Applicators						
Ln TCPy (mg/g Cr) (n=42)	4.56 (0.63)	3.29,5.84	<0.001	2.68 (0.93)	0.78,4.57	0.007
Ln (Post AChE/Pre AChE) (n=28)	-24.21 (12.79)	-50.50,2.09	0.07	-11.60 (12.44)	-37.46,14.25	0.36
Ln (Post BChE/Pre BChE) (n=29)	-14.52 (4.61)	-23.97,-5.07	0.004	-7.33(5.93)	-19.63,4.97	0.23

*Regression models adjusted for field stations, age, family income, home pesticide use and average number of hours of work in the field over the entire application season (for applicators only)

When we examined biomarker-symptom relationship by subclasses of symptoms among the applicators we observed significant positive associations of log-transformed TCPy with behavioral, autonomic, cognitive, motor and sensory problems after accounting for sociodemographic and occupational covariates (Supplementary Table 1). The magnitudes of associations (adjusted betas) were greater for autonomic, cognitive and sensory symptoms than the two other subclasses. Although the log-transformed change in AChE activity was not associated with any of these subclasses, change in BChE activity demonstrated a significant association with average percentage of behavioral symptoms (p=0.04) and a marginally significant association with average percentage of cognitive symptoms (p=0.07) (Supplementary Table 1).

DISCUSSION

A self-reported symptom questionnaire has been globally recognized as the primary method to capture symptom data in exposed populations. The most common questionnaire utilized is the extended or modified versions of Q-16 [27], which has been used in many international studies including a study with Nicaraguans living close to cotton fields [8], Sri Lankan farmworkers [10] and Colorado agricultural communities [32]. However, time intervals between exposure and collection of symptom data in these studies varied from one month to twelve months [6 9 10 28 33-35]. Furthermore, the majority of studies have utilized cross-sectional design which lacks information about temporality.

To the best of our knowledge, this is the first longitudinal study on adolescents to look into the relationship between CPF and self-reported neurological symptoms. In this study, a gradual increase in neurological symptoms relative to the baseline time interval was observed among the applicators from the 2nd to the 8th time intervals (days 24-77 of the study during June 26 to August18, 2010) after accounting for the number of days worked during the week, home use of pesticides by the participant, age, education and family income levels. A significant 30 percentage point increase in the neurological symptoms relative to the baseline time interval was observed on the 8th time interval (days 63-77 of the study). This is perhaps due to a second short CPF application episode in the same season in the field station at Al-Shohada. Self-reported symptoms among applicators remained significantly elevated from the baseline time interval until day 217, approximately five months after the cessation of exposure showing evidence that despite discontinuation of CPF application, repeated exposure of this pesticide led to persistence of neurological health effects for several months. Compared with the applicators, the non-applicators showed relatively late reporting of neurological symptoms perhaps due to the low level environmental chlorpyrifos exposure. It is interesting to note that the non-applicators still reported approximately 9 percentage point more symptoms relative to baseline in the last time point (day 105-217). Residual CPF can survive in indoor environments for an extended period of time, can rapidly bind to soil and plants and

has a half-life of several months in soil [36 37]. We anticipate that because of these properties, CPF remained in the environment as a potential source of environmental exposure leading to increased symptom reporting among non-applicators.

The symptom reporting across time showed a recovery phase at the 10th time interval (day 105-207) when percentage of symptom reporting relative to baseline declined substantially from the previous time intervals (Table 3, Figure 1). Using the same sample, we recently demonstrated that both applicators and non-applicators experienced peak median BChE depression during the CPF application period but BChE returned to the baseline level by the end of the study (day 217/January 5, 2011) [31]. We anticipate that symptoms were following BChE activity pattern, i.e., as the BChE activity was returning back to the baseline level, recovery from the neurological symptoms was taking place.

Prior to this study, a cross-sectional study on Egyptian cotton field workers reported associations between OP exposure and neurological symptoms [19 20]. Similar to another Indian study on occupationally exposed adolescents [21], the previous Egyptian adolescent study [19 20] presented descriptive statistics to show the difference between exposed and unexposed adolescents in terms of the prevalence of various neurological symptoms without taking other sociodemographic confounders into account. Results of the present study were consistent with several longitudinal studies conducted in adult populations. In one study of occupationally and non-occupationally OP pesticide-exposed farmers and fishermen, delayed persistence of neurological symptoms were found during the two-year follow-up[7]. Results from a clinical examination of the same cohort found that there were deficits related to sensory function [38]. Another study, conducted over three years with Colorado farm workers, reported an association between OP exposure and symptoms of depression [12]. Consistency in the results across studies indicate that a Q-16 based self-reported questionnaire used in all of these studies is a reliable measure to estimate health effects resulting from OP (in this case chlorpyrifos) exposure.

Our study is also novel in its approach to include prospective measures of biomarkers. First, instead of using single-time point biomarker data (urinary TCPy) commonly used in cross-sectional studies, our study analyzed urinary TCPy levels at multiple time points. The collection of pre, during and post exposure samples resulting in a precise estimate of cumulative exposure from April 11 to January 5 [31]. This has enabled us to overcome a historical challenge in characterizing OP exposure and allows us to subsequently examine the association of cumulative exposure with neurological symptoms. An additional limitation often encountered by past studies was the absence of established baseline AChE and BChE levels. A recent adult study examining the variation of cholinesterase levels among OP pesticides and carbamate-exposed field-workers could not establish any baseline AChE/BChE due to the mobility of the migrant study population [39]. Another Egyptian adolescent study also reported greater reduction of acetylcholinesterase activity among the pesticide applicators compared to the controls [19 20]. By collecting blood samples prior to the start of the application season, baseline data were established, which allowed us to compute more precise measures of change in activities of AChE and BChE from pre-exposure to post-exposure periods.

Two previous studies of Kenyan and Palestinian farm workers, which measured cholinesterase levels before and after exposure, found associations between cholinesterase inhibition and respiratory, eye and neurological symptoms [9 40]. Potential occupational confounding factors (e.g residential application of pesticides and number of days worked in agriculture into account) that are associated with neurological symptoms [19 41]were not taken into account while examining exposure-outcome associations in these past studies . These potential confounding variables were included in our study questionnaires and later examined during statistical analysis.

We identified a comparison group (non-applicators) who were similar in demographic characteristics to our applicators. It is often true that control groups in occupational settings may not be truly unexposed[1]. In our study, close proximity to the agricultural field and application of pesticides at home were the two environmental factors offering some degree of OP exposure to the non-applicators as indicated by elevated urinary TCPy levels during the period of chlorpyrifos application to cotton fields [31]. To encounter this potential confounder, all statistical models were adjusted for these two variables in addition to other sociodemographic variables.

It is difficult to explain why we found no relationship between TCPy and neurological symptoms among the non-applicators when a delayed effect of environmental or passive CPF exposure on symptoms was evident among this subgroup in the corresponding GEE model (Table 3 & Figure 1). One possible explanation is that the range of cumulative exposure was much lower among the non-applicators (154 to 24,180 mg/g creatinine; median 2591 mg/g creatinine) compared to the applicators (232 to 28,260 mg/g creatinine; median 10318 mg/g creatinine). Small sample size and differences in cumulative exposure might have contributed to the non-significant association in the non-applicator subgroup. Some other undocumented environmental factors such as working during high temperature along with carrying a heavy backpack during CPF application might have positively confounded the association among the applicators.

The non-specific nature of many of the symptoms is a limitation of the current study. In addition, the biological significance of these self-reported symptoms is unknown. However, the goal of the study was not to establish that more symptoms lead to development of any neurological disease. Rather we attempted to examine how repeated or cumulative exposure to chlorpyrifos determined the pattern of neurological symptoms over the entire season. Five of the symptoms included in our questionnaire are considered non-specific, including, headache, dizziness, fatigue, loss of consciousness and insomnia. The

remaining 20 symptoms were classified into more specific neurological functions such as behavior, autonomic, sensory, cognitive or motor functions. When we excluded the five non-specific symptoms from the summary measure The estimated betas for the associations of exposure variables cumulative TCPy, change in AChE and BChE activities with average percentage of 20 neurological symptoms were 3.19 ($p<0.001$), -6.11 ($p=0.60$) and -9.49 ($p=0.05$) respectively after accounting for potential covariates.

Our study was conducted in an agricultural community in Egypt, which is relatively where families are primarily middle class to lower middle class. Results of our study may be generalizable only to agricultural communities with similar sociodemographic characteristics.

CONCLUSION

Our study is the first to demonstrate that repeated occupational CPF exposure is an important determinant of neurological symptoms in adolescent applicators and non-applicators across time, with symptoms peaking during the exposure season and partly recovering in months following exposure. The study also showed significant association between cumulative CPF exposure and symptoms, using cumulative urinary TCPy as a biomarker of exposure. Future studies are needed to assess the temporal and dose-dependent effects of repeated CPF exposure on neurological symptoms and neurobehavioral deficits in children, adolescents and adults to identify the most sensitive populations. Similar prospective studies with a larger population are also needed to assess the relationship between these endpoints and biomarkers of exposure, effect and susceptibility, ultimately identifying biomarkers, which may help protect sensitive population.

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Contributorship Statement

All authors have made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data in the study; Dr. Khalid Khan have taken the lead to draft the manuscript. Whereas other authors have revised the draft critically for important intellectual content; All of the authors have provided final approval of the version to be published. Dr. Diane Rohlman is the Principal Investigator of the study and has supervised each step of the manuscript development process. She has been listed as the Corresponding author.

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IRB Approval:

The study was approved by the OHSU IRB in June 2009 and by the Medical Ethics committee of the Faculty of Medicine, Menoufia University in July 2009.

Conflict of Interest Statement

None of the authors has any potential financial, personal or other conflict of interest, which could inappropriately influence this study.

Data Sharing Statement

Additional unpublished data from the study are available to Dr. Diane Rohlman (diane-rohlman@uiowa.edu), Dr. Ahmed Ismail (aa-ismail@hotmail.com) and Dr. James Olson (aa-ismail@hotmail.com) in Excel or SPSS datasets. They can be reached by email.

Abbreviations: CPF (chlorpyrifos); OP (organophosphorus); TCPy (3,5,6-trichloro-2-pyridinol); AChE (acetylcholinesterase); BChE (butyrylcholinesterase); CYP (cytochrome P450)

REFERENCES

1 Kamel F, Hoppin JA. Association of pesticide exposure with neurologic dysfunction and disease. *Environ Health Perspect* 2004;112:950-8.

2 Solomon C, Poole J, Palmer KT, Peveler R, Coggon D. Acute symptoms following work with pesticides. *Occup Med (Lond)* 2007;57:505-11.

3 Wesseling C, van Wendel de Joode B, Keifer M, London L, Mergler D, Stallones L. Symptoms of psychological distress and suicidal ideation among banana workers with a history of poisoning by organophosphate or n-methyl carbamate pesticides. *Occup Environ Med* 2010;67:778-84.

4 Alavanja MC, Hoppin JA, Kamel F. Health effects of chronic pesticide exposure: cancer and neurotoxicity. *Annu Rev Public Health* 2004;25:155-97.

5 Beseler C, Stallones L. Safety practices, neurological symptoms, and pesticide poisoning. *J Occup Environ Med* 2003;45:1079-86.

6 London L, Nell V, Thompson ML, Myers JE. Effects of long-term organophosphate exposures on neurological symptoms, vibration sense and tremor among South African farm workers. *Scand J Work Environ Health* 1998;24:18-29.

7 Delgado E, McConnell R, Miranda J et al. Central nervous system effects of acute organophosphate poisoning in a two-year follow-up. *Scand J Work Environ Health* 2004;30:362-70.

8 Keifer M, Rivas F, Moon JD, Checkoway H. Symptoms and cholinesterase activity among rural residents living near cotton fields in Nicaragua. *Occup Environ Med* 1996;53:726-9.

9 Ohayo-Mitoko GJ, Kromhout H, Simwa JM, Boleij JS, Heederik D. Self reported symptoms and inhibition of acetylcholinesterase activity among Kenyan agricultural workers. *Occup Environ Med* 2000;57:195-200.

10 Smit LA, van-Wendel-de-Joode BN, Heederik D, Peiris-John RJ, van der Hoek W. Neurological symptoms among Sri Lankan farmers occupationally exposed to acetylcholinesterase-inhibiting insecticides. *Am J Ind Med* 2003;44:254-64.

11 Farahat FM, Rohlman DS, Storzbach D, Ammerman T, Anger WK. Measures of short-term test-retest reliability of computerized neurobehavioral tests. *Neurotoxicology* 2003;24:513-21.

12 Beseler CL, Stallones L. A cohort study of pesticide poisoning and depression in Colorado farm residents. *Ann Epidemiol* 2008;18:768-74.

13 Ecobichon DJ. Pesticide use in developing countries. *Toxicology* 2001;160:27-33.

14 McCauley LA, Shapiro SE, Scherer JA, Lasarev MR. Assessing pesticide safety knowledge among Hispanic migrant farmworkers in Oregon. *J Agric Saf Health* 2004;10:177-86.

15 McCauley LA, Sticker D, Bryan C, Lasarev MR, Scherer JA. Pesticide knowledge and risk perception among adolescent Latino farmworkers. *J Agric Saf Health* 2002;8:397-409.

16 London L, Beseler C, Bouchard MF et al. Neurobehavioral and neurodevelopmental effects of pesticide exposures. *Neurotoxicology* 2012.

17 Costa LG, Li WF, Richter RJ, Shih DM, Lusis A, Furlong CE. The role of paraoxonase (PON1) in the detoxication of organophosphates and its human polymorphism. *Chem Biol Interact* 1999;119-120:429-38.

18 Costa LG, Cole TB, Vitalone A, Furlong CE. Measurement of paraoxonase (PON1) status as a potential biomarker of susceptibility to organophosphate toxicity. *Clin Chim Acta* 2005;352:37-47.

19 Abdel Rasoul GM, Abou Salem ME, Mechael AA, Hendy OM, Rohlman DS, Ismail AA. Effects of occupational pesticide exposure on children applying pesticides. *Neurotoxicology* 2008;29:833-8.

20 Ismail A, Rohlman D, Rasoul GA, Salem MA, Hendy O. Clinical and biochemical parameters of children and adolescents applying pesticides. *International Journal of Occupational and Environmental Medicine* 2010;1:132-143.

21 Rastogi SK, Tripathi S, Ravishanker D. A study of neurologic symptoms on exposure to organophosphate pesticides in the children of agricultural workers. *Indian J Occup Environ Med* 2010;14:54-7.

22 Nolan RJ, Rick DL, Freshour NL, Saunders JH. Chlorpyrifos: pharmacokinetics in human volunteers. *Toxicol Appl Pharmacol* 1984;73:8-15.

23 Farahat FM, Ellison CA, Bonner MR et al. Biomarkers of chlorpyrifos exposure and effect in Egyptian cotton field workers. *Environ Health Perspect* 2011;119:801-6.

24 Egeghy PP, Cohen Hubal EA, Tulve NS et al. Review of pesticide urinary biomarker measurements from selected US EPA children's observational exposure studies. *Int J Environ Res Public Health* 2011;8:1727-54.

25 Hofmann JN, Keifer MC, De Roos AJ et al. Occupational determinants of serum cholinesterase inhibition among organophosphate-exposed agricultural pesticide handlers in Washington State. *Occup Environ Med* 2010;67:375-86.

26 Fenske RA, Farahat FM, Galvin K, Fenske E, Olson JR. Contributions of inhalation and dermal exposure to chlorpyrifos dose in Egyptian cotton field workers *International Journal of Occupational and Environmental Health* 2012;(In Press).

27 Lundberg I, Hogberg M, Michelsen H, Nise G, Hogstedt C. Evaluation of the Q16 questionnaire on neurotoxic symptoms and a review of its use. *Occup Environ Med* 1997;54:343-50.

28 Kamel F, Engel LS, Gladen BC, Hoppin JA, Alavanja MC, Sandler DP. Neurologic symptoms in licensed pesticide applicators in the Agricultural Health Study. *Hum Exp Toxicol* 2007;26:243-50.

29 Fabiny DL, Ertingshausen G. Automated reaction-rate method for determination of serum creatinine with the CentrifiChem. *Clin Chem* 1971;17:696-700.

30 Liang K-Y, Zeger SL. Longitudinal Data Analysis Using Generalized Linear Models. *Biometrika* 1986;73:13-22.

31 Crane AL, Abdel Rasoul G, Ismail A et al. Longitudinal Assessment of Chlorpyrifos Exposure and Effect Biomarkers in Adolescent Egyptian Agricultural Workers. *Journal of Exposure Science and Environmental Epidemiology* 2012;(In Press).

32 Stallones L, Beseler C. Pesticide illness, farm practices, and neurological symptoms among farm residents in Colorado. *Environ Res* 2002;90:89-97.

33 Gomes J, Lloyd O, Revitt MD, Basha M. Morbidity among farm workers in a desert country in relation to long-term exposure to pesticides. *Scand J Work Environ Health* 1998;24:213-9.

34 Pilkington A, Buchanan D, Jamal GA et al. An epidemiological study of the relations between exposure to organophosphate pesticides and indices of chronic peripheral neuropathy and neuropsychological abnormalities in sheep farmers and dippers. *Occup Environ Med* 2001;58:702-10.

35 Steenland K, Dick RB, Howell RJ et al. Neurologic function among termiticide applicators exposed to chlorpyrifos. *Environ Health Perspect* 2000;108:293-300.

36 CDC. National Report on Human Exposure to Environmental Chemicals (Centers for Disease Control and Prevention). Available: http://www.cdc.gov/exposurereport [accessed 18 February 2012] 2009.

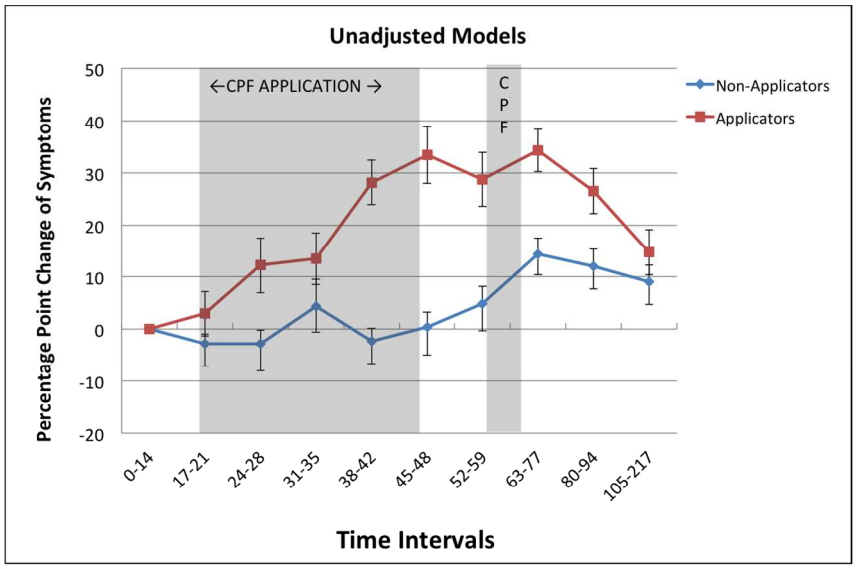
37 Eaton DL, Daroff RB, Autrup H et al. Review of the toxicology of chlorpyrifos with an emphasis on human exposure and neurodevelopment. *Crit Rev Toxicol* 2008;38 Suppl 2:1-125.

38 Miranda J, McConnell R, Wesseling C et al. Muscular strength and vibration thresholds during two years after acute poisoning with organophosphate insecticides. *Occup Environ Med* 2004;61:e4.

39 Quandt SA, Chen H, Grzywacz JG, Vallejos QM, Galvan L, Arcury TA. Cholinesterase depression and its association with pesticide exposure across the agricultural season among Latino farmworkers in North Carolina. *Environ Health Perspect* 2010;118:635-9.

40 Safi JM, Abu Mourad TA, Yassin MM. Hematological biomarkers in farm workers exposed to organophosphorus pesticides in the Gaza Strip. *Arch Environ Occup Health* 2005;60:235-41.

41 Kamel F, Engel LS, Gladen BC, Hoppin JA, Alavanja MC, Sandler DP. Neurologic symptoms in licensed private pesticide applicators in the agricultural health study. *Environ Health Perspect* 2005;113:877-82.



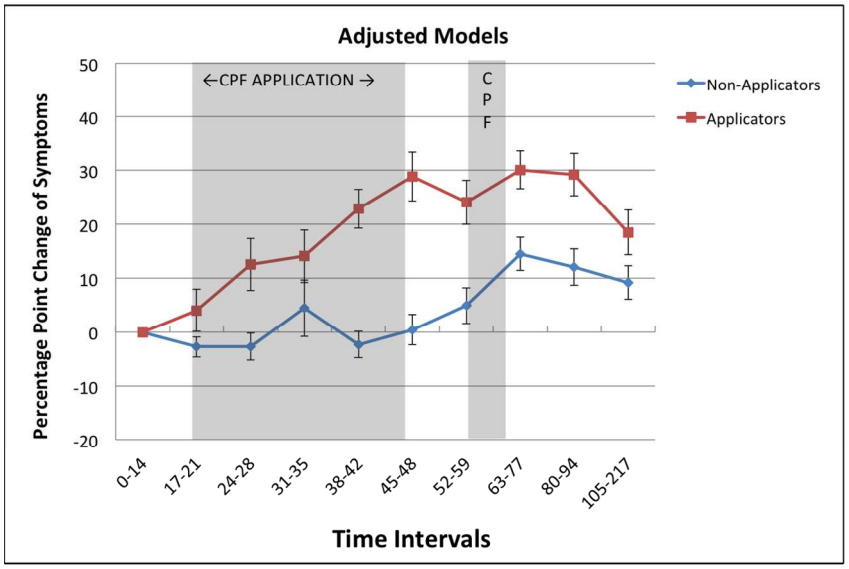
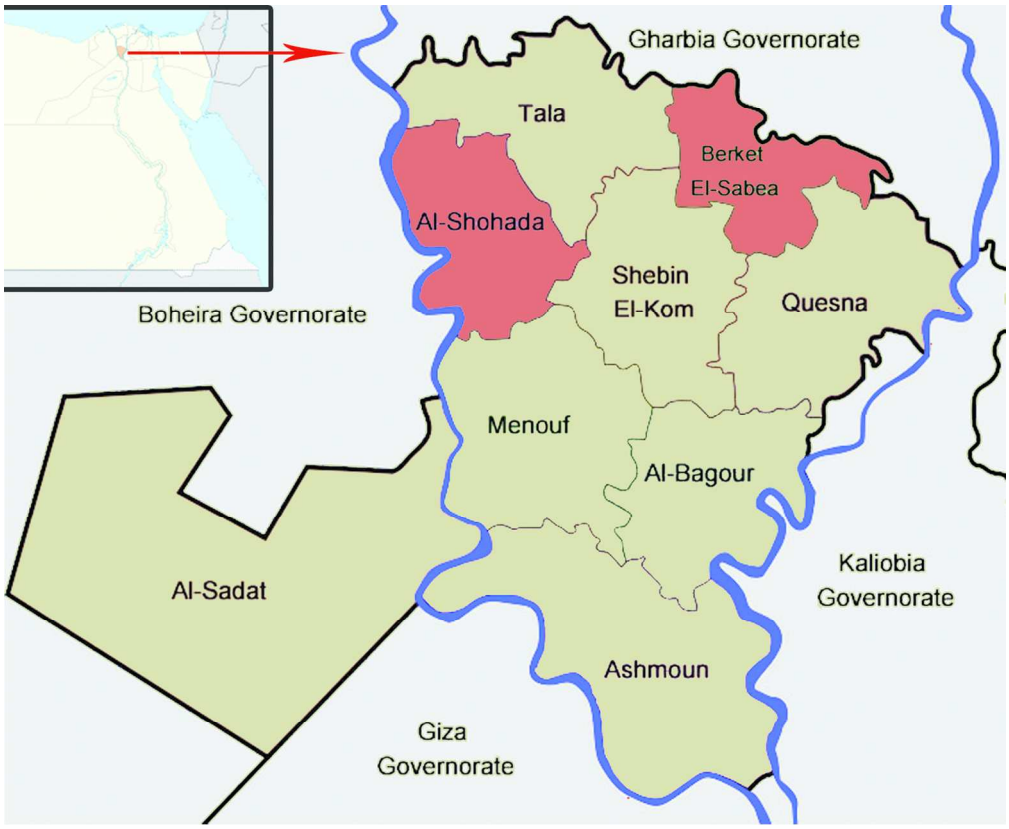


Figure 1. Difference, relative to baseline, in the percentage of symptoms reported at each of nine subsequent time intervals; error bars represent 95% confidence limits for the difference.

Supplementary Figure 1. Map of Menoufia governorate showing the study districts

Supplementary Figure 2. CPF application in the study area showing time intervals. Field stations 1 and 2 were located in Al-Shohada and Berket El-Sabea respectively.

Supplementary Figure 3. Scatter plots of (a) cumulative TCPy (ug/g creatinine) against percentage of symptoms (n=70), (b) log-transformed post AChE/pre AChE against percentage of symptoms (n=49) and (c) log-transformed post BChE/pre BChE against percentage of symptoms (n=50)



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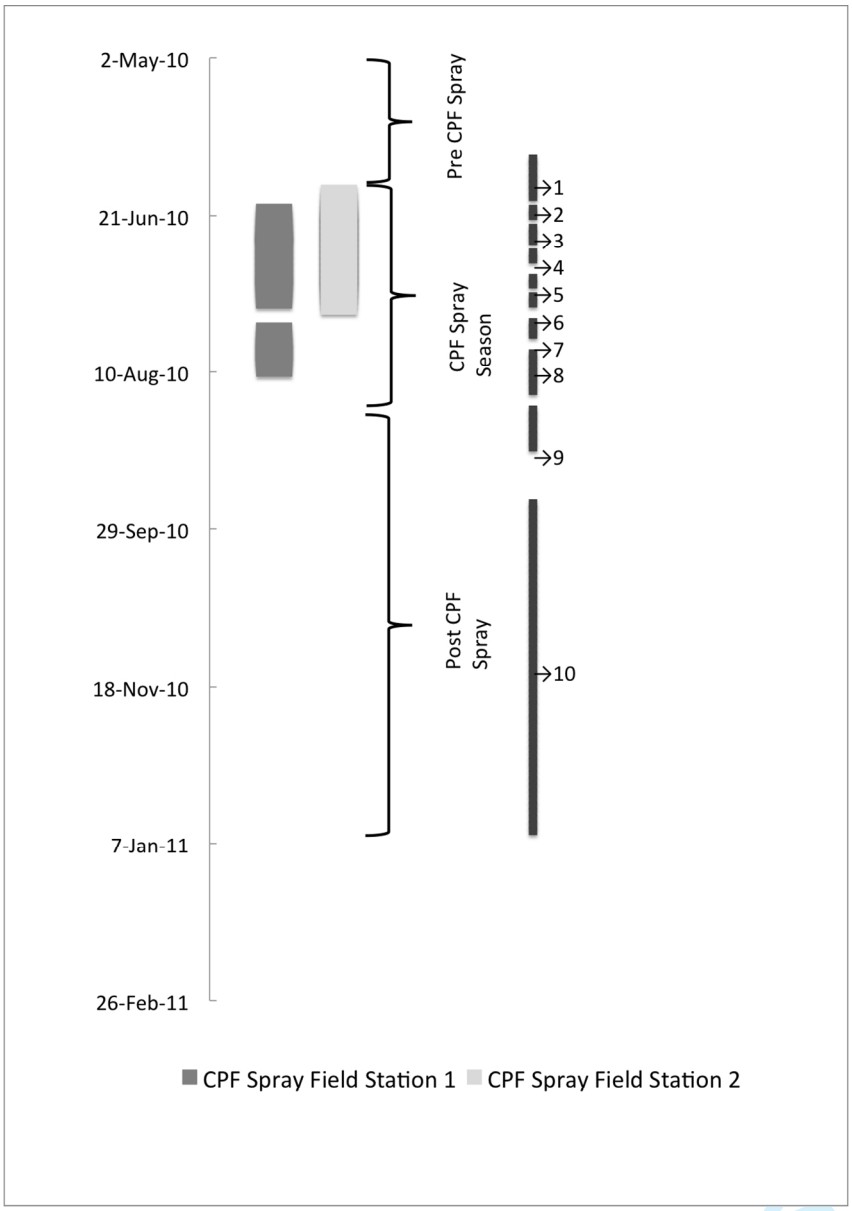
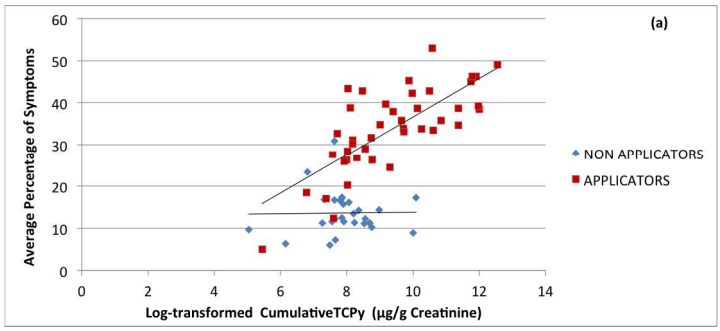
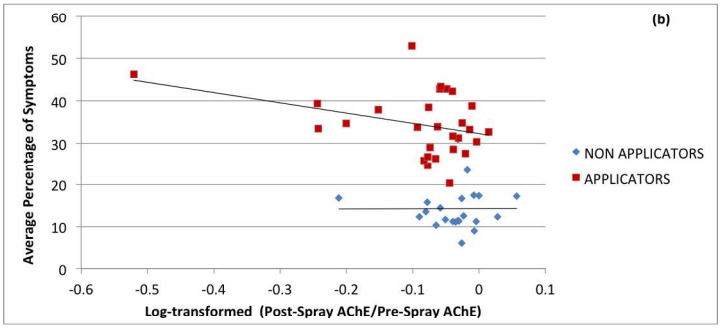
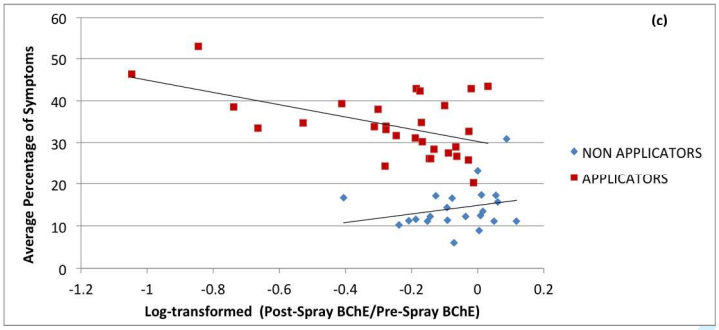

2-May-10
21-Jun-10
10-Aug-10
29-Sep-10
18-Nov-10
7-Jan-11
26-Feb-11
Pre CPF Spray
CPF Spray Season
Post CPF Spray
1
2
3
4
5
6
7
8
9
10
CPF Spray Field Station 1
CPF Spray Field Station 2


(a)
Average Percentage of Symptoms
Log-transformed CumulativeTCPy (µg/g Creatinine)
NON APPLICATORS
APPLICATORS


(b)
Average Percentage of Symptoms
Log-transformed (Post-Spray AChE/Pre-Spray AChE)
NON APPLICATORS
APPLICATORS


(c)
Average Percentage of Symptoms
Log-transformed (Post-Spray BChE/Pre-Spray BChE)
NON APPLICATORS
APPLICATORS

Supplementary Table 1. Summary of regression analysis for biomarkers of exposure and effect of chlorpyrifos predicting percentage of subclasses neurological symptoms among **Applicators**

	Unadjusted Model		**Adjusted Model**		**Unadjusted Model**		**Adjusted Model**		**Unadjusted Model**		**Adjusted Model**	
	Ln TCPy (mg/g Cr) (n=42)		Ln TCPy (mg/g Cr) (n=42)		Ln (Post AChE/Pre AChE) (n=28)		Ln (Post AChE/Pre AChE) (n=28)		Ln (Post BChE/Pre BChE) (n=29)		Ln (Post BChE/Pre BChE) (n=29)	
Outcome variable (Symptom Subclass)	**B (se)**	**p-value**	**B (se)**	**p-value**	**B (se)**	**p-value**	**B (se)**	**p-value**	**B (se)**	**p-value**	**B (se)**	**p-value**
Behavior & Affect	2.16 (0.57)	<0.001	2.80 (0.91)	**0.004**	-12.59 (10.86)	0.26	-16.34 (16.04)	0.33	-6.96 (4.09)	0.10	-12.26 (5.73)	**0.04**
Autonomic	5.69 (0.75)	<0.001	3.24 (0.99)	**0.002**	-38.45 (14.88)	0.02	-4.84 (17.22)	0.78	-19.65 (5.42)	0.001	-9.69 (6.40)	0.14
Cognitive	6.92 (0.87)	<0.001	5.27 (1.34)	**<0.001**	-46.22 (17.49)	0.01	1.73 (20.82)	0.94	-24.16 (6.03)	0.001	-13.56 (7.55)	**0.07**
Motor	2.48 (0.49)	<0.001	1.67 (0.74)	**0.03**	-6.27 (8.41)	0.46	4.50 (12.03)	0.37	-5.75 (3.15)	0.08	-3.90 (4.64)	0.41
Sensory	6.47 (0.90)	<0.001	4.94 (1.34)	**0.001**	-27.49 (17.64)	0.13	-0.75 (22.46)	0.97	-20.00 (6.41)	0.004	-13.88 (8.86)	0.13
Temporary Disability (Non-specific)	5.48 (0.61)	<0.001	3.64 (0.78)	**<0.001**	-41.13 (14.14)	0.007	-10.74 (16.61)	0.53	-21.80 (4.73)	0.001	-13.52 (5.73)	**0.03**

*Regression models adjusted for field stations, age, family income, home pesticide use and average number of hours of work in the field over the entire application season (for applicators only

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract: **Title indicates "Longitudinal Assessment……."**
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found: **Summary provided in the abstract**
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported: **Explained**
Objectives	3	State specific objectives, including any prespecified hypotheses: **Objectives specified**
Methods		
Study design	4	Present key elements of study design early in the paper: **Presented in the last paragraph of the Introduction section**
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection: **Described in the Method section**
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up: **Described in the Method section**
		(*b*) *Cohort study*—**This is not a matched study; Convenience sampling**
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable: **Described in Methods**
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group: **Provided**
Bias	9	Describe any efforts to address potential sources of bias
Study size	10	Explain how the study size was arrived at: **Explained in Method**
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why: **Described**
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding: **Described in statistical analysis section**
		(*b*) Describe any methods used to examine subgroups and interactions: **Described**
		(*c*) Explain how missing data were addressed: **No missing data**
		(*d*) *Cohort study*—**Not applicable**
		(*e*) Describe any sensitivity analyses- **Not applicable**

Continued on next page

Results		
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed: **Reported**
		(b) Give reasons for non-participation at each stage: **Not applicable**
		(c) Consider use of a flow diagram: **Not required as a figure in the result section explained the time-intervals when the participants were observed for the outcome**
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders: **Provided**
		(b) Indicate number of participants with missing data for each variable of interest: **Not applicable**
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount): **Summarized**
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time: **Reported**
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included: **Clear explanation of the estimates are provided**
		(*b*) Report category boundaries when continuous variables were categorized: **Not applicable**
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period: **Not relevant**
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses: **Not performed and therefore not reported**
Discussion		
Key results	18	Summarise key results with reference to study objectives: **Summary of key findings are presented in the Discussion section**
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias: **Limitations discussed in the last paragraph of the Discussion section**
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence: **Provided in the Discussion section**
Generalisability	21	Discuss the generalisability (external validity) of the study results: **Discussed in the paper**
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based: **Provided after the Discussion section just before the list of references**

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies: **Not applicable**

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

Longitudinal Assessment of Chlorpyrifos Exposure and Self-Reported Neurological Symptoms in Adolescent Pesticide Applicators

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Longitudinal Assessment of Chlorpyrifos Exposure and Self-Reported Neurological Symptoms in Adolescent Pesticide Applicators

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ABSTRACT

Objectives: Occupational exposure of organophosphorus pesticides (OPs), such as chlorpyrifos (CPF), in adolescents is of particular concern because of the potential vulnerability of the developing neurological system. The objectives of the study were to examine how neurological symptoms reported over the CPF application season vary across time, whether these effects are reversible post application and if there are any associations between CPF biomarkers and neurological symptoms in an adolescent study population.

Methods: Egyptian adolescent CPF applicators (n=57) and non-applicators (n=38) were recruited for a longitudinal study. Self-reported data for 25 neurological symptoms were collected at 32 time points over the 8-month period before, during and after CPF-application. Urine and blood samples were collected for CPF-specific biomarkers: urine trichloro-2-pyridinol (TCPy) and blood cholinesterase.

Results: When we compared reporting of symptoms between applicators and non-applicators at different time intervals over the 8-month study period, we observed both groups reporting the highest numbers of symptoms in the middle of the CPF application season. Applicators reported a greater percentage of neurological symptoms, relative to baseline, than the non-applicators after accounting for potential covariates. Only among the applicators, cumulative TCPy was positively and significantly associated with the average percentage of symptoms. Significant

associations between the change in butyrylcholinesterase (BChE) from pre to post application season and several domains of neurological symptoms were also found even after adjusting for potential covariates.

Conclusions: These observations reinforce the growing concern regarding the neurotoxic health effects of CPF in adolescent applicators in developing countries and the need for developing and implementing intervention programs through increased use of personal protective equipment.

STRENGTHS AND LIMITATIONS OF THE STUDY:

- This is the first longitudinal study demonstrating an association between CPF exposure and reporting of neurological symptoms in adolescent applicators.
- The study is also novel in its approach to include prospective measures of biomarkers of CPF exposure and effect and to examine their associations with neurological symptoms.
- The non-specific nature of many of the symptoms is a limitation of the current study.
- Small sample size of this study may have influenced the significance levels of exposure-outcome relationships.

What this paper adds

- Applicators are more likely to report increased symptoms compared to non-applicators.
- Repeated occupational exposure to CPF increases the reporting of acute neurological symptoms during the CPF application season; the symptoms persist for months after the cessation of exposure in both applicators and non-applicators.
- Cumulative biomarker of CPF exposure (TCPy) also demonstrates an association with neurological symptoms in applicators.
- Reduction of CPF exposure among the adolescent applicators and non-applicator residents of agricultural communities should be a public health priority since neurological symptoms remained elevated even after the cessation of CPF application.

INTRODUCTION

The high use of organophosphorus pesticides (OPs) has been recognized as a major global public health challenge for agriculture-based communities, due to their associations with adverse neurological outcomes. Immediate and short-term neurological signs and symptoms ranging from less severe (headache, dizziness, nausea etc.) to more severe (muscle weakness, bronchospasm, change in heart rate etc.) have all been reported after occupational exposure to OPs [1 2]. Although high levels of occupational OP exposure can be associated with symptoms persisting for several years [3], repeated, moderate to low exposures, can also produce chronic neurological symptoms and deficits in neurobehavioral performance [4]. Converging evidence regarding the associations between OP exposures and neurological symptoms is based on occupational studies with adults conducted in a wide range of settings; including comparisons between exposed and non-exposed farmworkers in the US [5], South Africa [6], Nicaragua [7 8], Kenya [9], Sri Lanka [10] and Egypt [11]. Additional evidence for the effect of pesticides on somatic and mood symptoms are also found in the literature [2 12].

Although it is illegal there have reports of involvement of US adolescents in mixing and applying pesticides in some agricultural communities [13 14]. The developing bodies of children and adolescents may not break down pesticide as effectively as adult and they may receive a larger dose per unit of body weight for a given exposure due to their smaller body size [15], making them more vulnerable to neurological effects. Animal and human studies have also suggested that paraoxonase PON-1, an organophosphate detoxifying enzyme, is less active in younger populations making them more vulnerable to OP toxicity[16 17]. A recent study has found association of environmental CPF exposure with structural changes in developing brain of the

children and adolescents [18]. In developing countries, children and adolescents are engaged in risky agricultural activities including the application of OPs[19]. In two epidemiological studies, Egyptian and Indian children and adolescents living in agricultural communities have demonstrated associations between occupational and environmental OP exposure and neurological and neuromuscular problems[20-22].

Biomarkers have been used to characterize OP exposure in epidemiological and occupational studies. Urinary trichloro-2-pyridinol (TCPy) is a relatively specific metabolite of CPF exposure, eliminated in the urine with a half-life of 27 hours following exposure [23]. Due to the ease and non-invasiveness of the collection of urine samples, TCPy is widely recognized as a useful biomarker of exposure, particularly in children and adolescents [24 25]. The classic mode of OP toxicity is manifested by the inhibition of cholinesterase. Both blood acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are biomarkers of effect, with BChE being more sensitive to inhibition by OP pesticides[26]. A small number of adult studies found associations between inhibition of cholinergic activities with self-reported symptoms [9 10]; however, this relationship has rarely been examined in adolescent studies.

Understanding the relationship between OP exposure and the change in neurological symptoms over time (temporal change) is important because application-related exposure follows a seasonal pattern in most areas. Two longitudinal studies with agricultural workers demonstrated that short-term neurological signs and symptoms were associated with initial acute episodes of exposure, which eventually advanced into long-term sequelae [7 12]. However, these

studies did not characterize exposure by identifying specific types of OPs that were related to the symptoms.

The primary objective of this study was to determine whether occupational exposure to CPF is associated with self-reported neurological symptoms in adolescents.Through a prospective study, we compared adolescent applicators exposed to CPF with adolescent non-applicators working and residing in agricultural communities in Egypt. Typically, CPF is the primary insecticide used by pesticide applicators in Egyptian cotton fields; offering us a unique exposure opportunity with well characterized occupational exposure. The possibility of potential confounding effects of other neurotoxic pesticides was minimal because of limited use of other pesticides in the study area. The goals of the study were to examine how neurological symptoms vary over time during the exposure season, if these effects could reverse at the cessation of exposure and whether there are any associations between CPF biomarkers and neurological symptoms in the adolescent study population.

METHODS

Study area and population

Two agricultural districts were selected from Menoufia Governorate, Egypt (Supplementary Figure 1) to conduct a prospective study from April 2010 to January 2011. In Egypt, adolescents are hired seasonally to apply pesticides to cotton fields and the schedule of pesticide applications to the cotton crop is regulated by the Ministry of Agriculture. The typical workday was from 8am-12pm and from 3pm-7pm, six days per week. During 2010, approximately 2100 liters of OPs were applied to 5700 acres of cotton fields (personal

communication with the Ministry of Agriculture). Chlorpyrifos is the primary OP applied to the cotton crop from mid-June to early August. Although there are slight variations in the timing of CPF application between the two districts (Figure 1), the application patterns are consistent across these two areas. Because there is no regulation in Egypt for mandatory use of personal protective equipment (PPE), dermal exposure and inhalation were both considered as the potential route of exposure in this population [20]. Recently, Fenske et al.[27] reported that dermal exposure and subsequent absorption through the skin accounted for 94-96% of the total dose of CPF in Egyptian pesticide applicators.

Recruitment and data collection

Fifty-eight male adolescents aged 12-21 that were hired seasonally by the Ministry of Agriculture to spray pesticides in the cotton fields, were recruited from two districts in the Menoufia governate. Forty adolescent non-applicators were recruited through convenience sampling (i.e., word of mouth, direct communication utilizing contacts through the staff from the local Ministry of Agriculture) from the same districts as the applicators for the cotton crop. These adolescents never worked in the cotton fields as pesticide applicators. One adolescent was excluded from the final analysis due to his inconsistency in participating in the study activities and two other participants were excluded for questionable sample integrity, resulting in a final sample size of 95 (57 applicators and 38 non-applicators). Written informed consent was obtained from all participants and their legal guardian (for those under 18). All the subjects were monetarily compensated for their time during the questionnaire survey and biological samples (~$5 per visit). The study was approved by the Oregon Health and Science University IRB in

June 2009, and by the Medical Ethics committee of the Faculty of Medicine, Menoufia University in July 2009.

Data collection, for both applicators and non-applicators, occurred at the primary field station for each district. Pesticides applicators and supervisors meet in the field stations, which also provides storage area for the pesticides and the equipment used for application.

Outcome assessment

We developed a 25-item, short-term neurological symptom questionnaire on the basis of the widely used Q16 questionnaire [28] and a modified version of the Q16 used in a previous study on licensed pesticide applicators [29]. The 25 symptoms were grouped into six domains: behavioral, autonomic, cognitive, sensory, motor and non-specific temporary disability (Table 1). The questionnaire had five response options (0-4) for each symptom ranging from "never" (coded as 0) to "everyday of the week" (coded as 4). Since more than 90% of the responses were between 0-2 (1=once a week and 2=once in every 2-3 days) we recoded each of the symptom response to "0" or "never" and "1" or "at least once a week or more." Self-reported neurological symptom counts were collected at 32 irregularly spaced dates over an eight-month period from early June 2010 through early January 2011. These time points ranged across three different time periods: pre-application, application, and post-application. For each time point, the number of positive responses (a response was considered positive and coded as "1" when the participant reported the frequency of the symptom "at least once a week or more") was totaled for each person to yield a score ranging from 0–25; division by 25 produced the proportion of symptoms endorsed at each of the 32 time points. This outcome variable was used to compare the change of symptoms over time between applicators and non-applicators. All these time points were

collapsed into 10 separate non-overlapping intervals lasting between one and four weeks in length (Figure 1). Symptom data during the pre-application period, including the first fifteen days of the study was collectively taken to represent the baseline time interval (or time interval 1). Symptom reporting from the other nine remaining time intervals was evaluated against time interval 1. The next 5 time intervals, between June 19 and July 21, were during the application period of CPF. The remaining 4 time intervals occurred between July 24, 2010 and January 5, 2011 and reflect the post-application period although a brief CPF application was reported in the district where field station 1 was located. The proportions of symptoms over all the 32 time points were averaged to produce a season-level average percentage of neurological symptoms over the entire study period. This outcome variable was used to examine the relationships between the biomarkers (TCPy, AChE and BChE) and symptoms. Participants also completed a questionnaire during baseline addressing their sociodemographic status, household and occupational use of pesticides.

Table 1. Domains of neurological symptoms

Domains	Symptoms
Behavioral Symptoms	1. Tense or anxious[#] 2. Excessively angry or irritable*[#] 3. Depressed or withdrawn*[#]
Autonomic Symptoms	4. Nausea[#] 5. Heavy sweating*[#] 6. Loss of appetite[#] 7. Fast heart rate*[#] 8. Excessive salivation
Cognitive Symptoms	9. Difficulty concentrating*[#] 10. Being absentminded and memory problem*[#]
Sensory Symptoms	11. Difficulty seeing at night[#] 12. Blurred or double vision[#] 13. Numbness in hands and feet[#] 14. Sense of smell or taste change[#] 15. Ringing in ears
Motor Symptoms	16. Difficulty with balance[#] 17. Weakness in arms and legs[#] 18. Involuntary movement of arms and legs[#] 19. Shaking in hands* 20. Difficulty speaking[#]
Temporary Disability (non-specific symptoms)	21. Dizziness[#] 22. Headache*[#] 23. Momentary loss of consciousness[#] 24. Fatigue*[#] 25. Insomnia[#]

*Symptoms used in Q16 [27]

#Symptoms used in Agricultural Health Study [28]

Urine collection and analysis

Spot urine samples were collected in new and individually wrapped cups at the beginning of the work shift at eight time points between April 2010 and January 2011. The cups were opened at the time of sample collection. Urine samples were subsequently transferred to the laboratory at Menoufia University in a cooler with wet ice. At the laboratory, 4 ml aliquots of urine were transferred into labeled 5 ml cryovials within hours of sampling and stored at –20 ºC. The banked urine samples were express mailed on dry ice to the University of Buffalo laboratory for analysis of pesticide metabolites; duplicate samples were retained in the –20 ºC freezer at Menoufia University. Urine samples in the field station 2 were collected one day after the collection date of the field station 1.

Creatinine concentrations were measured using the Jaffe reaction [30]. The method of urinary TCPy measurement (a primary metabolite of chlorpyrifos) has been described elsewhere [24]. Briefly, Samples were analyzed using gas chromatography–mass spectrometry (negative-ion chemical ionization) and utilized 13C-15N-3,5,6-TCPy as an internal standard. Samples were hydrolyzed with HCl, extracted with toluene, and derivatized using N-(tert-butyldimethylsilyl)-N-methyltrifluoro-acetamide (Sigma Aldrich, USA). A spiked quality control (QC) sample was routinely run with the analytical samples and the metabolite concentration was determined from a standard curve for the peak area for the selective ion. The QC samples consisted of lab samples that were first analyzed for TCPy and the levels were non-detectable. The TCPy standard curve was linear from 1-200 ng/ml with a correlation coefficient of 1.000. Samples spiked with 50ng of TCPy/mL (n=20) gave an average metabolite recovery of 94.8% (range = 92 - 98%; SD = 0.931;

RSD% = 1.965). A 1ng TCPy/ml spiked sample was run 10 times and the within series RSD% = 1.6. The minimum detection level was 0.5 ng/ml of urine.

Blood collection and ChE analysis

To establish the baseline ChE activity, pre-application blood draws occurred on April 11 and June 2, 2010, prior to the start of the official government-regulated CPF application season. As with the urine collection, blood draws occurred in the field station 2 one day later. Changes in both AChE and BChE levels from baseline to the end of CPF-application season (blood collected on September 4, 2010) were estimated. Blood samples were collected by venipuncture into 10mL lavender top (EDTA) vacutainer tubes and immediately placed on wet ice and transported to Menoufia University, where they were analyzed in duplicate for AChE and BChE activity using an EQM Test-Mate kit (EQM Research Inc., Cincinnati, OH, USA) as described previously [24].

Statistical analysis

We used SPSS version 18.0 and STATA (version 11; Stata Corporation, College Station, TX) for the statistical analysis. Sociodemographic variables were summarized and described using means and standard deviations for continuous responses and percentages for discrete outcomes; simple comparisons between applicators and non-applicators were completed using t-tests or chi-square tests. To calculate the value of cumulative TCPy for each participant we used STATA'a pharmacokinetic function (pkexamine) to employing the trapezoid rule to estimate the area under the curve for each participant over the study time. By definition, cumulative TCPy was the sum of the concentration at each time point multiplied by the duration between time

points. This variable reflects the total amount of TCPy excreted over the study period for which urine as collected and assayed. Concentrations of cumulative TCPy, AChE and BChE exhibited pronounced right skewed distribution and more than a 3-fold separation between the minimum and maximum observed values; consequently, these responses were log-transformed prior to analysis to improve symmetry. Both AChE and BChE were expressed as a log-transformed ratio of post-application activity relative to pre-application activity. Then the associations between the change of these cholinesterase markers from pre to post application seasons and self-reported symptoms were examined using linear regression models that took potential covariates into account. Similar regression analyses were used to examine the relationship between cumulative TCPy and neurological symptoms. All p-values are two-sided with significance judged relative to a 0.05 level.

Spearman correlation coefficients were used to estimate associations between urine and blood biomarkers and symptom scores. Generalized estimating equations (GEE)[31] were used to model the proportion of neurological symptoms reported in each time interval while controlling for number of days worked (within five days of the symptom reporting date), home use of pesticides, age, education and income levels. The one fitted model was used to estimate changes over time, relative to the first time interval (June 2–June 16), for applicators and non-applicators, as well as to examine whether changes relative to baseline differed between the two groups (via group-by-time interaction).

RESULTS

Sociodemographic Characteristics

Ninety-two of the participants (97%) were between 12 and 18 years old with the remaining three between 19 and 21. The two groups, non-applicators and applicators, did not differ significantly in terms of age, educational status, family income, number of people in house, years of pesticide use at home, and insecticides and rodenticides use at home (Table 2). Compared to non-applicators, a significantly higher number of applicators lived close to the field (within 25 meters), had carpet in their homes and applied herbicides at home. Applicators had significantly lower BMI than non-applicators. On average, applicators had been working in the field for a little over 3 years.

Table 2: Sociodemogrphic characteristics for participants at baseline

Variables	Non-applicators (n=38)	Applicators (n=57)
	Mean (SD)	Mean (SD)
Age	16.6 (2.4)	16.2 (1.6)
Education	9.8 (1.8)	9.9 (1.8)
Height (cm) #	166.3 (12.0)	163.4 (10.0)
Weight (kg)*	62.0 (15.4)	54.2 (8.6)
BMI (kg/m^2)*	22.1 (3.7)	20.2 (2.2)
Number of people in house	5.6 (1.1)	6.0 (1.8)
Home pesticide use (years)##*	1.6 (1.9)	2.5 (1.9)
Occupational application of pesticides (yrs)	-	3.1 (1.5)
Days/week of pesticide application	-	4.8 (1.3)
Hours/day of pesticide application	-	5.2 (0.7)
	% (n)	% (n)
Family Monthly Income (<500 E)	78.9 (30)	71.9 (41)
Applied pesticides in home in last 5 yrs (yes)*	47.4 (18)	78.9 (45)
Computer use (once a week or more)*	65.8 (25)	45.6 (26)
Carpet in house (yes)*	27.0 (10)	54.4 (31)
Live within 25m to agricultural field (yes)*	23.7 (9)	50.9 (29)
Types of pesticides applied at home###		
Herbicides*	13.0 (3)	44.9 (21)
Insecticides	83.3 (20)	93.9 (46)
Rodenticides	16.7 (4)	14.3 (7)

*p<0.05 for group difference; #30 applicators vs 34 non applicators, ##44 applicators vs 19 non applicators, #49 applicators vs 24 non applicators, E=Egyptian pound

Change in symptoms over time

We considered days 0-14 as the baseline time interval (time interval 1) when no application of CPF was reported. While examining symptoms reported over time among both applicators and non-applicators we took various potential confounders into account. These include occupational factors such as days worked per week in pesticide applications, number of years of pesticide use at home and socio-demographic factors such as age, education and income level of the participants. Applicators began increased reporting of neurological symptoms at the beginning of the CPF application season (at time interval 2 between days 17-21 of the study). The percentage of neurological symptoms continued to increase during the application season and reached the peak at time interval 6, representing days 45-48, the time when CPF application period ended. This was followed by a drop of symptom reporting indicating a small recovery due to the cessation of exposure in both districts. The highest peak of symptom reporting was observed at the time interval 8 representing days 63-77 (Table 3). This happened perhaps due to a small episode of CPF application in field station 1 (between time intervals 7 and 8). Similar to the applicators, the non-applicators also demonstrated the highest increase in the proportion of neurological symptoms during the time interval 8 although the magnitude of the change was smaller (14 percentage point increase of symptoms relative to baseline interval). The change of neurological symptoms relative to baseline declined over the next two time intervals (9 and 10) in both groups indicating a recovery phase during post-application. For applicators, the percentage of reported symptoms at each of the nine subsequent time intervals was always higher than the percentage observed at baseline; non-applicators, by contrast, had a pattern of reported symptoms that both increased and decreased relative to the baseline time interval (pre-application) over the course of the study (Figure 2).

When applicators and non-applicators are compared with respect to change in percentage of symptoms (relative to baseline), it was always the case that the change (percentage point change relative to baseline) for applicators was greater than the corresponding change for non-applicators even after adjusting for the covariates (Table 3).

Table 3. Estimated change (95% CI) from baseline in the percentage points of neurological symptoms reported at each of nine successive collected time points, shown separately for non-applicators and applicators.

	Time Intervals	Non-Applicators Adjusted Models*		Applicators Adjusted Models*		Change in difference between applicators and non-applicators relative to difference in baseline	
1	Days from Baseline (Day 0-14) June 2-June 14, 2010	b (% of Symptoms) (95% CI)	p-value	b (% of Symptoms) (95% CI)	p-value	b (% of Symptoms) (95% CI)	p-value
2	17-21 Jun 19-Jun 23	-2.74 (-4.61,-0.86)	0.004	4.08 (0.18,7.97)	**0.040**	6.81 (2.47, 11.15)	**0.002**
3	24-28 Jun 26-Jun 30	-2.68 (-5.18,0.17)	0.004	12.57 (7.75,17.38)	**<0.001**	15.25 (9.80, 20.69)	**<0.001**
4	31-35 Jul 3-Jul 7	4.45 (-0.80,9.71)	0.10	14.06 (9.21,18.90)	**<0.001**	9.60 (2.44, 16.77)	**0.009**
5	38-42 Jul 10-Jul 14	-2.28 (-4.75.0.17)	0.07	22.83 (19.25,26.40)	**<0.001**	25.11 (20.79, 29.43)	**<0.001**
6	45-48 Jul 17-Jul 21	0.46 (-2.37,3.30)	0.75	28.80 (24.27,33.35)	**<0.001**	28.35 (22.98, 33.72)	**<0.001**
7	52-59 Jul 24-Jul 31	4.95 (1.63,8.28)	**0.003**	24.01 (19.92,28.09)	**<0.001**	19.05 (13.80, 24.30)	**<0.001**
8	63-77 Aug 4-Aug 18	14.49 (11.45,17.55)	**<0.001**	30.10 (26.53,33.66)	**<0.001**	15.60 (10.95, 20.26)	**<0.001**
9	80-94 Aug 21-Sep 4	12.08 (8.72,15.44)	**<0.001**	29.17 (25.22,33.13)	**<0.001**	17.09 (11.93, 22.25)	**<0.001**
10	105-217 Sep 22-Jan 5[#]	9.22 (6.12,12.32)	**<0.001**	18.45 (14.30,22.59)	**<0.001**	9.22 (4.12,14.33)	**<0.001**

*Models adjusted for number of days worked for applying pesticides, years of pesticide use at home, age, education and income level. CPF application time intervals are shaded in grey.

Associations of neurological symptoms with biomarkers

TCPy was detected in 100% of the samples. Summary statistics for TCPy, AChE and BChE of the study samples have been already reported by Crane et al. (2013) [32], Mean creatinine concentration of the urine samples was reported to be 1696 μ g/ml with a maximum of 4199 and a minimum of 164 μ g/ml. In brief, the applicators had much higher mean and estimated median peak TCPy concentration than the non-applicators (mean: 719 vs 44.9 μ g/g creatinine; estimated median 137 vs 19.7 μ g/g creatinine). In our study sample, BChE was found to be more sensitive to CPF exposure than AChE, with median activity reduced by 37% from baseline in applicators and 13% in non-applicators during the CPF application period.

A scatter plot of cumulative TCPy (ug/g creatinine) against average percentage points of symptoms revealed distinct exposure-response gradients by pesticide application status (applicators vs non-applicators) (Figure 3). In addition, two other scatter plots of change in AChE activity and change in BChE activity from pre-application to post-application against percentage of symptoms also revealed effect measure modification by pesticide application status (Supplementary Figures 2a & 2b). Therefore, separate linear models for applicators and non-applicators were used to examine the associations of these three biomarkers with symptoms.

Log-transformed TCPy was positively associated with the average percentage of neurological symptoms in the regression models after adjusting for other covariates that may confound exposure-outcome relationship such as field stations, age, family monthly income, pesticide use at home and average number of hours worked in the field among applicators (b=2.68, p=0.007). However, non-applicators demonstrated positive,but statistically non-significant, associations between TCPy and symptoms. Among applicators, AChE and BChE

activity was negatively and significantly associated with the average percentage of neurological symptoms in the unadjusted models. In the adjusted models these associations remained negative but became non-significant (Table 4).

Table 4. Summary of regression analysis for biomarkers of exposure & effect of chlorpyrifos predicting average percentage of neurological symptoms over the entire study stratified by applicator status

	Unadjusted Models			Adjusted Models*		
Explanatory variables	**B (se)**	**95% CI**	**p-value**	**B (se)**	**95% CI**	**p-value**
For Non Applicators						
Ln TCPy (mg/g Cr) (n=28)	0.29 (0.76)	-1.26,1.84	0.71	0.57 (0.79)	-1.06,2.20	0.47
Ln (Post AChE/Pre AChE) (n=21)	-1.25 (16.41)	-35.59,33.1	0.94	-6.57 (18.80)	-43.64,33.50	0.73
Ln (Post BChE/Pre BChE) (n=21)	2.23 (7.04)	-12.51,16.98	0.76	2.50 (7.63)	-13.77,18.77	0.75
For Applicators						
Ln TCPy (mg/g Cr) (n=42)	4.56 (0.63)	3.29,5.84	<0.001	2.68 (0.93)	0.78,4.57	0.007
Ln (Post AChE/Pre AChE) (n=28)	-24.21 (12.79)	-50.50,2.09	0.07	-11.60 (12.44)	-37.46,14.25	0.36
Ln (Post BChE/Pre BChE) (n=29)	-14.52 (4.61)	-23.97,-5.07	0.004	-7.33(5.93)	-19.63,4.97	0.23

*Regression models adjusted for field stations, age, family monthly income, pesticide use at home and average number of hours of work in the field over the entire application season (for applicators only)

When we examined the biomarker-symptom relationship by domains of symptoms among the applicators, we observed significant positive associations of log-transformed TCPy with behavioral, autonomic, cognitive, motor and sensory problems after accounting for sociodemographic and occupational covariates (Table 5). The magnitudes of associations (adjusted betas) were greater for autonomic, cognitive and sensory symptoms than the two other domains. Although the log-transformed change in AChE activity was not associated with any of these subclasses, change in BChE activity demonstrated a significant association with average

percentage of behavioral symptoms (p=0.04) and a marginally significant association with average percentage of cognitive symptoms (p=0.07)

Table 5. Summary of regression analysis for biomarkers of exposure and effect of chlorpyrifos predicting average percentage of neurological symptoms by subclasses among **Applicators**

	Unadjusted Model Ln TCPy (mg/g Cr) (n=42)		**Adjusted Model** Ln TCPy (mg/g Cr) (n=42)		**Unadjusted Model** Ln (Post AChE/Pre AChE) (n=28)		**Adjusted Model** Ln (Post AChE/Pre AChE) (n=28)		**Unadjusted Model** Ln (Post BChE/Pre BChE) (n=29)		**Adjusted Model** Ln (Post BChE/Pre BChE) (n=29)	
Outcome variable (Symptom Subclass)	**B (se)**	**p-value**	**B (se)**	**p-value**	**B (se)**	**p-value**	**B (se)**	**p-value**	**B (se)**	**p-value**	**B (se)**	**p-value**
Behavior & Affect	2.16 (0.57)	<0.001	2.80 (0.91)	**0.004**	-12.59 (10.86)	0.26	-16.34 (16.04)	0.33	-6.96 (4.09)	0.10	-12.26 (5.73)	**0.04**
Autonomic	5.69 (0.75)	<0.001	3.24 (0.99)	**0.002**	-38.45 (14.88)	0.02	-4.84 (17.22)	0.78	-19.65 (5.42)	0.001	-9.69 (6.40)	0.14
Cognitive	6.92 (0.87)	<0.001	5.27 (1.34)	**<0.001**	-46.22 (17.49)	0.01	1.73 (20.82)	0.94	-24.16 (6.03)	0.001	-13.56 (7.55)	**0.07**
Motor	2.48 (0.49)	<0.001	1.67 (0.74)	**0.03**	-6.27 (8.41)	0.46	4.50 (12.03)	0.37	-5.75 (3.15)	0.08	-3.90 (4.64)	0.41
Sensory	6.47 (0.90)	<0.001	4.94 (1.34)	**0.001**	-27.49 (17.64)	0.13	-0.75 (22.46)	0.97	-20.00 (6.41)	0.004	-13.88 (8.86)	0.13
Temporary Disability (Non-specific)	5.48 (0.61)	<0.001	3.64 (0.78)	**<0.001**	-41.13 (14.14)	0.007	-10.74 (16.61)	0.53	-21.80 (4.73)	0.001	-13.52 (5.73)	**0.03**

*Regression models adjusted for field stations, age, family monthly income, pesticide use at home and average number of hours of work in the field over the entire application season (for applicators only)

DISCUSSION

The self-reported symptom questionnaire has been globally recognized as the primary method to capture symptom data in exposed populations. The most common questionnaire utilized is the extended or modified versions of Q16 [28], which has been used in many international studies including a study with Nicaraguans living close to cotton fields [8], Sri Lankan farmworkers [10] and Colorado agricultural communities [33]. However, time intervals between exposure and collection of symptom data in these studies varied from one month to twelve months [6 9 10 29 34-36]. Furthermore, the majority of studies have utilized cross-sectional design which lacks information about temporality.

To the best of our knowledge, this is the first longitudinal study with adolescents to examine the relationship between CPF and self-reported neurological symptoms. In this study, a gradual increase in neurological symptoms, relative to the baseline time interval, was observed among the applicators from during the CPF application period after accounting for the number of days worked during the week, home use of pesticides by the participant, age, education and family monthly income levels. A significant 30 percentage point increase in the neurological symptoms relative to the baseline time interval was observed on time interval 8 (days 63-77 of the study). This is perhaps due to a second short CPF application episode in the same season in field station 1. Self-reported symptoms among applicators remained significantly elevated from the pre-application period until day 217, approximately five months after the cessation of exposure showing evidence that despite discontinuation of CPF application, repeated exposure of this pesticide led to persistence of neurological health effects for several months. Compared with the applicators, the non-applicators showed relatively late reporting of neurological symptoms,

perhaps due to the environmental CPF exposure. It is interesting to note that the non-applicators still reported approximately 9 percentage point more symptoms relative to baseline at the last time interval (day 105-217). Residual CPF can survive in indoor environments for an extended period of time, can rapidly bind to soil and plants and has a half-life of several months in soil [37] [38]. We anticipate that because of these properties, CPF remained in the environment as a potential source of environmental exposure leading to increased symptom reporting among non-applicators.

The symptom reporting over time showed a recovery phase at time interval 10 (day 105-207) when percentage of symptom reporting relative to baseline declined substantially from the previous time intervals (Table 3, Figure 2). Using the same sample, we recently demonstrated that both the applicators and non-applicators experienced peak median BChE depression during the CPF application period but BChE returned to the baseline level by the end of the study (day 217/January 5, 2011) [32]. We anticipate that symptoms were following BChE activity pattern, i.e., as the BChE activity was returning back to the baseline level neurological symptoms were going through the recovery phase.

Prior to this study, a cross-sectional study on Egyptian cotton field workers reported associations between OP exposure and neurological symptoms [20 21]. Similar to an Indian study on occupationally exposed adolescents [22], the previous Egyptian adolescent study [20 21] presented descriptive statistics to show the difference between exposed and unexposed adolescents in terms of the prevalence of various neurological symptoms. However, these studies did not take potential sociodemographic confounders into account. Results of the present study were

consistent with several longitudinal studies conducted in adult populations. In one study of occupationally and non-occupationally OP pesticide-exposed farmers and fishermen, delayed persistence of neurological symptoms were found during the two-year follow-up[7]. Results from a clinical examination of the same cohort found that there were deficits related to sensory function [39]. Another study, conducted over three years with Colorado farm workers, reported an association between OP exposure and symptoms of depression [12]. Consistency in the results across studies indicate that a Q16 based self-reported questionnaire used in all of these studies is a reliable measure to estimate health effects resulting from OP (in this case CPF) exposure.

Our study is also novel in its approach of including prospective measures of biomarkers. First, instead of using single-time point biomarker data commonly used in cross-sectional studies, our study analyzed urinary TCPy levels at multiple time points. The collection of pre, during and post application samples resulted in a precise estimate of cumulative exposure from April 11 to January 5 [32]. This has enabled us to overcome a historical challenge in characterizing OP exposure and allows us to subsequently examine the association of cumulative exposure with neurological symptoms. An additional limitation often encountered by past studies was the absence of established baseline AChE and BChE levels. A recent adult study examining the variation of cholinesterase levels among OP pesticides and carbamate-exposed field-workers could not establish any baseline AChE/BChE due to the mobility of the migrant study population [40]. Another Egyptian adolescent study also reported greater reduction of AChE activity among the pesticide applicators compared to the controls [20 21]. By collecting blood samples prior to the start of the application season, baseline data were established, which allowed us to compute more

precise measures of change in activities of AChE and BChE from pre-exposure to post-exposure periods.

Two previous studies of Kenyan and Palestinian farm workers, which measured cholinesterase levels before and after exposure, found associations between cholinesterase inhibition and respiratory, eye and neurological symptoms [9 41]. Potential occupational confounding factors (e.g residential application of pesticides and number of days worked in agriculture) that are associated with neurological symptoms [20 42]were not taken into account while examining exposure-outcome associations in these past studies. These potential confounding variables were included in our study questionnaires and later examined during statistical analysis.

We identified a comparison group (non-applicators) who were similar in demographic characteristics to our applicators. It is often true that control groups in occupational settings may not be truly unexposed[1]. In our study, close proximity to the agricultural field (less than 25m) and application of pesticides at home were the two environmental factors offering potential exposure opportunities to the non-applicators as indicated by elevated urinary TCPy levels during the period of CPF application to cotton fields [32]. To encounter this potential confounder, all statistical models were adjusted for these two variables in addition to other sociodemographic variables.

It is difficult to explain why we found no relationship between TCPy and neurological symptoms among the non-applicators when a delayed effect of environmental or passive CPF

exposure on symptoms was evident among this subgroup in the corresponding GEE model (Table 3 & Figure 2). One possible explanation is that the range of cumulative exposure was much lower among the non-applicators (154 to 24,180 mg/g creatinine; median 2591 mg/g creatinine) compared to the applicators (232 to 28,260 mg/g creatinine; median 10318 mg/g creatinine). Small sample size and differences in cumulative exposure might have contributed to the non-significant association in the non-applicator subgroup. Some other undocumented environmental factors such as working during high temperatures along with carrying a heavy backpack during CPF application might have positively confounded the association among the applicators.

We acknowledge that we relied on self-reported outcome measure. Therefore, there was a possibility that the frequent completion of the neurological symptoms survey (32 times over 8 months) could itself have had an influence on the increase in symptoms during the CPF application season. This could partially explain why these symptoms were not associated with TCPy levels among non-applicators.

The non-specific nature of many of the symptoms is another limitation of the current study, the biological significance of these self-reported symptoms is unknown. However, the goal of the study was not to establish that more symptoms lead to development of any neurological disease. Rather we attempted to examine how repeated or cumulative exposure to CPF determined the pattern of neurological symptoms over the entire season. Five of the symptoms included in our questionnaire are considered non-specific, including, headache, dizziness, fatigue, loss of consciousness and insomnia. The remaining 20 symptoms were classified into more specific

neurological functions such as behavior, autonomic, sensory, cognitive or motor functions. When we excluded these non-specific symptoms from the summary measure, the estimated betas for the associations of TCPy, change in AChE and BChE activities with average percentage of 20 neurological symptoms were found to be 3.19 ($p<0.001$), -6.11 ($p=0.60$) and -9.49 ($p=0.05$) respectively after accounting for potential covariates.

CONCLUSION

Our study reinforces the need for the development and execution of intervention programs for the residents of agricultural communities, including pesticide applicators, in developing countries. Future interventions should include hygiene practices, behaviors and use of protective equipment, in both occupational and residential environments . Our study is the first to demonstrate that repeated occupational CPF exposure is an important determinant of neurological symptoms in adolescent applicators and non-applicators over time, with symptoms peaking during the exposure season and partly recovering in months following exposure. The study also showed a significant association between cumulative CPF exposure and symptoms, using cumulative urinary TCPy as a biomarker of exposure. Future studies are needed to assess the temporal and dose-dependent effects of repeated CPF exposure on neurological symptoms and neurobehavioral deficits in children, adolescents and adults to identify the most sensitive populations.

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Contributorship Statement

All authors have made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data in the study; Dr. Khalid Khan has taken the lead to draft the manuscript. Whereas other authors have revised the draft critically for important intellectual content; All of the authors have provided final approval of the version to be published. Dr. Diane Rohlman is the Principal Investigator of the study and has supervised each step of the manuscript development process. She has been listed as the Corresponding author.

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IRB Approval:

The study was approved by the OHSU IRB in June 2009 and by the Medical Ethics committee of the Faculty of Medicine, Menoufia University in July 2009.

Conflict of Interest Statement

None of the authors has any potential financial, personal or other conflict of interest, which could inappropriately influence this study.

Data Sharing Statement

Additional unpublished data from the study are available to Dr. Diane Rohlman (diane-rohlman@uiowa.edu), Dr. Ahmed Ismail (aa-ismail@hotmail.com) and Dr. James Olson (aa-ismail@hotmail.com) in Excel or SPSS datasets. They can be reached by email.

Abbreviations: CPF (chlorpyrifos); OP (organophosphorus); TCPy (3,5,6-trichloro-2-pyridinol); AChE (acetylcholinesterase); BChE (butyrylcholinesterase); CYP (cytochrome P450)

REFERENCES

1 Kamel F, Hoppin JA. Association of pesticide exposure with neurologic dysfunction and disease. *Environ Health Perspect* 2004;112:950-8.

2 Solomon C, Poole J, Palmer KT, et al. Acute symptoms following work with pesticides. *Occup Med (Lond)* 2007;57:505-11.

3 Wesseling C, van Wendel de Joode B, Keifer M,et al.. Symptoms of psychological distress and suicidal ideation among banana workers with a history of poisoning by organophosphate or n-methyl carbamate pesticides. *Occup Environ Med* 2010;67:778-84.

4 Alavanja MC, Hoppin JA, Kamel F. Health effects of chronic pesticide exposure: cancer and neurotoxicity. *Annu Rev Public Health* 2004;25:155-97.

5 Beseler C, Stallones L. Safety practices, neurological symptoms, and pesticide poisoning. *J Occup Environ Med* 2003;45:1079-86.

6 London L, Nell V, Thompson ML, et al. Effects of long-term organophosphate exposures on neurological symptoms, vibration sense and tremor among South African farm workers. *Scand J Work Environ Health* 1998;24:18-29.

7 Delgado E, McConnell R, Miranda J et al. Central nervous system effects of acute organophosphate poisoning in a two-year follow-up. *Scand J Work Environ Health* 2004;30:362-70.

8 Keifer M, Rivas F, Moon JD, et al. Symptoms and cholinesterase activity among rural residents living near cotton fields in Nicaragua. *Occup Environ Med* 1996;53:726-9.

9 Ohayo-Mitoko GJ, Kromhout H, Simwa JM, et al. Self reported symptoms and inhibition of acetylcholinesterase activity among Kenyan agricultural workers. *Occup Environ Med* 2000;57:195-200.

10 Smit LA, van-Wendel-de-Joode BN, Heederik D, et al. Neurological symptoms among Sri Lankan farmers occupationally exposed to acetylcholinesterase-inhibiting insecticides. *Am J Ind Med* 2003;44:254-64.

11 Farahat FM, Rohlman DS, Storzbach D, et al. Measures of short-term test-retest reliability of computerized neurobehavioral tests. *Neurotoxicology* 2003;24:513-21.

12 Beseler CL, Stallones L. A cohort study of pesticide poisoning and depression in Colorado farm residents. *Ann Epidemiol* 2008;18:768-74.

13 McCauley LA, Shapiro SE, Scherer JA, et al. Assessing pesticide safety knowledge among Hispanic migrant farmworkers in Oregon. *J Agric Saf Health* 2004;10:177-86.

14 McCauley LA, Sticker D, Bryan C, et al. Pesticide knowledge and risk perception among adolescent Latino farmworkers. *J Agric Saf Health* 2002;8:397-409.

15 London L, Beseler C, Bouchard MF et al. Neurobehavioral and neurodevelopmental effects of pesticide exposures. *Neurotoxicology* 2012.

16 Costa LG, Li WF, Richter RJ, et al. The role of paraoxonase (PON1) in the detoxication of organophosphates and its human polymorphism. *Chem Biol Interact* 1999;119-120:429-38.

17 Costa LG, Cole TB, Vitalone A, et al. Measurement of paraoxonase (PON1) status as a potential biomarker of susceptibility to organophosphate toxicity. *Clin Chim Acta* 2005;352:37-47.

18 Rauh VA, Perera FP, Horton MK et al. Brain anomalies in children exposed prenatally to a common organophosphate pesticide. *Proc Natl Acad Sci U S A* 2012;109:7871-6.

19 Ecobichon DJ. Pesticide use in developing countries. *Toxicology* 2001;160:27-33.

20 Abdel Rasoul GM, Abou Salem ME, Mechael AA, et al. Effects of occupational pesticide exposure on children applying pesticides. *Neurotoxicology* 2008;29:833-8.

21 Ismail A, Rohlman D, Rasoul GA, et al. Clinical and biochemical parameters of children and adolescents applying pesticides. *International Journal of Occupational and Environmental Medicine* 2010;1:132-143.

22 Rastogi SK, Tripathi S, Ravishanker D. A study of neurologic symptoms on exposure to organophosphate pesticides in the children of agricultural workers. *Indian J Occup Environ Med* 2010;14:54-7.

23 Nolan RJ, Rick DL, Freshour NL, et al. Chlorpyrifos: pharmacokinetics in human volunteers. *Toxicol Appl Pharmacol* 1984;73:8-15.

24 Farahat FM, Ellison CA, Bonner MR et al. Biomarkers of chlorpyrifos exposure and effect in Egyptian cotton field workers. *Environ Health Perspect* 2011;119:801-6.

25 Egeghy PP, Cohen Hubal EA, Tulve NS et al. Review of pesticide urinary biomarker measurements from selected US EPA children's observational exposure studies. *Int J Environ Res Public Health* 2011;8:1727-54.

26 Hofmann JN, Keifer MC, De Roos AJ et al. Occupational determinants of serum cholinesterase inhibition among organophosphate-exposed agricultural pesticide handlers in Washington State. *Occup Environ Med* 2010;67:375-86.

27 Fenske RA, Farahat FM, Galvin K, et al. Contributions of inhalation and dermal exposure to chlorpyrifos dose in Egyptian cotton field workers *International Journal of Occupational and Environmental Health* 2012;(In Press).

28 Lundberg I, Hogberg M, Michelsen H, et al. Evaluation of the Q16 questionnaire on neurotoxic symptoms and a review of its use. *Occup Environ Med* 1997;54:343-50.

29 Kamel F, Engel LS, Gladen BC, et al. Neurologic symptoms in licensed pesticide applicators in the Agricultural Health Study. *Hum Exp Toxicol* 2007;26:243-50.

30 Fabiny DL, Ertingshausen G. Automated reaction-rate method for determination of serum creatinine with the CentrifiChem. *Clin Chem* 1971;17:696-700.

31 Liang K-Y, Zeger SL. Longitudinal Data Analysis Using Generalized Linear Models. *Biometrika* 1986;73:13-22.

32 Crane AL, Abdel Rasoul G, Ismail A et al. Longitudinal Assessment of Chlorpyrifos Exposure and Effect Biomarkers in Adolescent Egyptian Agricultural Workers. *Journal of Exposure Science and Environmental Epidemiology* 2012;(In Press).

33 Stallones L, Beseler C. Pesticide illness, farm practices, and neurological symptoms among farm residents in Colorado. *Environ Res* 2002;90:89-97.

34 Gomes J, Lloyd O, Revitt MD, et al. Morbidity among farm workers in a desert country in relation to long-term exposure to pesticides. *Scand J Work Environ Health* 1998;24:213-9.

35 Pilkington A, Buchanan D, Jamal GA et al. An epidemiological study of the relations between exposure to organophosphate pesticides and indices of chronic peripheral neuropathy and neuropsychological abnormalities in sheep farmers and dippers. *Occup Environ Med* 2001;58:702-10.

36 Steenland K, Dick RB, Howell RJ et al. Neurologic function among termiticide applicators exposed to chlorpyrifos. *Environ Health Perspect* 2000;108:293-300.

37 CDC. National Report on Human Exposure to Environmental Chemicals (Centers for Disease Control and Prevention). Available: http://www.cdc.gov/exposurereport [accessed 18 February 2012] 2009.

38 Eaton DL, Daroff RB, Autrup H et al. Review of the toxicology of chlorpyrifos with an emphasis on human exposure and neurodevelopment. *Crit Rev Toxicol* 2008;38 Suppl 2:1-125.

39 Miranda J, McConnell R, Wesseling C et al. Muscular strength and vibration thresholds during two years after acute poisoning with organophosphate insecticides. *Occup Environ Med* 2004;61:e4.

40 Quandt SA, Chen H, Grzywacz JG, et al. Cholinesterase depression and its association with pesticide exposure across the agricultural season among Latino farmworkers in North Carolina. *Environ Health Perspect* 2010;118:635-9.

41 Safi JM, Abu Mourad TA, Yassin MM. Hematological biomarkers in farm workers exposed to organophosphorus pesticides in the Gaza Strip. *Arch Environ Occup Health* 2005;60:235-41.

42 Kamel F, Engel LS, Gladen BC, et al. Neurologic symptoms in licensed private pesticide applicators in the agricultural health study. *Environ Health Perspect* 2005;113:877-82.

Longitudinal Assessment of Chlorpyrifos Exposure and Self-Reported Neurological Symptoms in Adolescent Pesticide Applicators

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Word Count: 4510

Key Words: chlorpyrifos, neurological symptoms, TCPy, cholinesterase, occupational exposure

ABSTRACT

Objectives: Occupational exposure of organophosphorus pesticides (OPs), such as chlorpyrifos (CPF), in adolescents is of particular concern because of the potential vulnerability of the developing neurological system. The objectives of the study were to examine how neurological symptoms reported over the CPF application season vary across time, whether these effects are reversible post application and if there are any associations between CPF biomarkers and neurological symptoms in an adolescent study population.

Methods: Egyptian adolescent CPF applicators (n=57) and non-applicators (n=38) were recruited for a longitudinal study. Self-reported data for 25 neurological symptoms were collected at 32 time points over the ~~7~~8-month period before, during and after CPF-application. Urine and blood samples were collected for CPF-specific biomarkers: urine trichloro-2-pyridinol (TCPy)~~,~~ and blood cholinesterase.

Results: When we compared reporting of symptoms between applicators and non-applicators at different time intervals over the 8-month study period, we observed both groups reporting the highest numbers of symptoms in the middle of the CPF application season. ~~We observed the~~

greatestduring,increased reporting of neurological symptoms among both applicators and non-applicators after several weeks of repeated CPF application. Applicators demonstrated reported a greater percentage of neurological symptoms, relative to baseline, than the non-applicators after accounting for potential covariates. Similar models revealed that Only among the applicators, cumulative TCPy was positively and significantly associated with the average percentage of symptoms, but only among the applicators. Significant Associations associations of between the change in butyrylcholinesterase (BChE) from pre to post application season with and several subclasses domains of neurological symptoms were also found significant or marginally significanteven after adjusting for potential covariates.

Conclusions: These observations reinforce the growing concern regarding the neurotoxic health effects of CPF in adolescent applicators in developing countriess and the need for developing and implementing intervention programs the importance of exposure prevention during the application season.through increased use of personal protective equipment.

STRENGTHS AND LIMITATIONS OF THE STUDY:

- This is the first longitudinal study ~~showing the~~demonstrating an association between ~~specific organophosphorus pesticide~~CPF exposure and reporting of neurological symptoms in adolescent applicators.
- ~~Symptoms in applicators are compared with symptoms in non-applicator thus showing the effect of environmental CPF exposure in general population.~~
- The study is also novel in its approach to include prospective measures of biomarkers of CPF exposure and effect and to examine their associations with neurological symptoms.
- The non-specific nature of many of the symptoms is a limitation of the current study.
- Small sample size ~~is another limitation study that~~of this study may have influenced the significance levels of exposure-outcome relationships.

~~Results of the study may be generalizable only to agricultural communities with similar sociodemographic characteristics.~~

What this paper adds

- ~~It is not fully understood how neurological symptoms vary across time in adolescents exposed to specific organophosphorus pesticide.~~
- Applicators are more likely to report increased symptoms compared to non-applicators.
- Repeated occupational exposure to CPF increases the reporting of acute neurological symptoms during the CPF application season; ~~and~~ the symptoms ~~may~~ persist for months after the cessation of exposure in both applicators and non-applicators.
- Cumulative biomarker of CPF exposure (TCPy) also demonstrates an association with neurological symptoms in applicators.
- Reduction of CPF exposure among the adolescent applicators and non-applicator residents of agricultural communities should be a public health priority since neurological symptoms remained elevated even after the cessation of CPF application.

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INTRODUCTION

High The high prevalence use of agricultural use of organophosphorus pesticides (OPs) has been recognized as a major global public health challenge for agriculture-based communities, due to their associations with adverse neurological outcomes. Immediate or and short-term neurological signs and symptoms ranging from less severe (headache, dizziness, nausea etc.) to more severe (muscle weakness, bronchospasm, change in heart rate etc.) were have all been reported after occupational OP exposure to OPs [1]. These short-term symptoms were reported as early as 48 hours after acute exposure [2]. Although high levels of occupational OP exposure can be associated with symptoms persisting for several years [3], repeated, moderate to low exposures, can also produce chronic neurological symptoms and deficits in neurobehavioral performance [4]. Converging evidence regarding the associations between OP exposures and neurological symptoms is based on adult occupational studies with adults conducted in a wide range of study settings. These; includinge comparisons between exposed and non-exposed farmworkers in the US [5], South Africa [6], Nicaragua [7 8], Kenya [9], Sri Lanka [10] and Egypt [11]. These studies have used

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~~self reported questionnaire data containing non-specific neurological symptoms.~~ Additional evidence for the effect of pesticides on somatic and mood symptoms are also found in the literature [2 12]

Although it is illegal there have reports of involvement of US adolescents in mixing and applying pesticides in some agricultural communities [13 14]. The developing bodies of children and adolescents may not break down pesticide as effectively as adult and they may receive a larger dose per unit of body weight for a given exposure due to their smaller body size [15], making them more vulnerable to neurological effects. Animal and human studies have also suggested that paraoxonase PON-1, an organophosphate detoxifying enzyme, is less active in younger populations making them more vulnerable to OP toxicity [16 17]. ~~Although less commonly studied, OP exposures were also found to be associated with neurological symptoms in children and adolescents.~~ A recent study has found association of environmental CPF exposure with structural changes in developing brain of the children and adolescents [18]. In developing countries, children and adolescents are engaged in risky agricultural ~~work involving~~activities including ~~OP~~ the application of OPs, ~~and this presents~~presenting ~~a major public health concern~~ [19]. ~~Even in the US, adolescents can be involved in mixing and applying pesticides [15 16]. Because of their smaller body size, the biological doses of pesticides (for children and adolescents may be substantially higher than adults [17], making making them more vulnerable to neurological effects. Animal and human studies have also suggested that paraoxonase (PON-1) an organophosphate detoxifying enzyme is less active in younger populations making them more vulnerable to OP toxicity [18 19].~~ In two epidemiological studies, Egyptian and Indian children and adolescents living in agricultural communities have demonstrated associations between occupational and

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environmental OP exposure and neurological and neuromuscular problems[20-22]. ~~An Egyptian cross-sectional study found adolescent pesticide applicators reporting more neurological symptoms and neuromuscular problems than controls.[20,21] Association between environmental OP exposure and neurological symptoms was also demonstrated in children living in an Indian agricultural community.[22]~~

Biomarkers have been used to characterize OP exposure in epidemiological and occupational studies. Urinary trichloro-2-pyridinol (TCPy~~),~~) is a relatively specific ~~CPF~~ metabolite of CPF exposure~~, .~~ ~~which~~ It ~~is~~ eliminated in the urine with a half-life of 27 ~~hr~~ hours following exposure[23]. Due to the ease and non-invasiveness of the collection of urine samples, TCPy is widely recognized as a useful biomarker of exposure, particularly in children and adolescents[24,25]. The classic mode of OP toxicity is manifested by the inhibition of cholinesterase. Both blood acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are biomarkers of effect, with BChE being more sensitive to inhibition by OP pesticides[26]. A small number of adult studies found associations between inhibition of cholinergic activities with self-reported symptoms[9,10]; however, this relationship has rarely been examined in adolescent studies.

Understanding the relationship between OP exposure and the change in neurological symptoms ~~across~~ over time (temporal change) is important because application-related exposure follows a seasonal pattern in most areas. ~~Moreover, specific OP exposure is important to track the changes in symptom reporting over time.~~ Two longitudinal studies with agricultural workers

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demonstrated that short-term neurological signs and symptoms were associated with initial acute episodes of exposure, which eventually advanced into long-term sequelae[7 12]. However, these studies did not characterize exposure ~~and did not identify any specific OP that was being applied.~~by identifying specific types of OPs that were related to the symptoms.

The primary objective of this study was to determine whether occupational exposure to CPF is associated with self-reported neurological symptoms in adolescents.~~To investigate whether occupational exposure to CPF is associated with self-reported neurological symptoms,~~ Through a prospective study, we compared adolescent applicators exposed to CPF with adolescent non-applicators working and residing in agricultural communities in Egypt~~ through a prospective study~~. Typically, CPF is the primary insecticide used by pesticide applicators in Egyptian cotton fields; ~~, including adolescent applicators, and~~ offer~~ed~~ us a unique exposure ~~environment~~ opportunity with well characterized occupational exposure. The possibility of potential confounding effects of other neurotoxic pesticides was minimal because of limited use of other pesticides in the study area. ~~We attempted to answer the critical questions of how repeated exposures to OP determines reporting of neurological symptoms,~~ The goals of the study were to examine how neurological symptoms vary ~~across~~ over time during the exposure season, if these effects could reverse at the cessation of exposure and whether there are any associations between ~~OP~~ CPF biomarkers and neurological symptoms in the adolescent study population. ~~A questionnaire was administered pre , mid- and post-CPF application season to examine changes in self-reported symptoms across time.~~

METHODS

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Study area and population

Two agricultural districts were selected from ~~A prospective study was conducted in~~ Menoufia Governorate, Egypt (Supplementary Figure 1) to conduct a prospective study from April 2010 to January 2011~~. Two of the nine districts of Menoufia, Al-Shohada and Berket El-Sabea were chosen randomly to conduct the study (Supplementary Figure 1)~~. In Egypt, adolescents are hired seasonally to apply pesticides to cotton fields and the schedule of pesticide applications to the cotton crop is regulated by the Ministry of Agriculture. The typical workday was from 8am-12pm and from 3pm-7pm, six days per week. ~~In the year of~~During ~~2011~~2010, approximately 2100 liters of OPs were applied ~~on approximately~~to 5700 acres of cotton fields (personal communication with the Ministry of Agriculture). Chlorpyrifos is the primary OP applied ~~in the districts of Menoufia governorate~~to the cotton crop from mid-June to early August. Although there are slight variations in the timing of CPF application between the two districts (~~Supplementary Figure 2~~Figure 1), the application patterns are consistent across ~~field stations~~these two areas~~. The typical workday was from 8am-12pm and from 3pm-7pm, six days per week.~~ Because there is no regulation in Egypt for mandatory use of personal protective equipment (PPE), dermal exposure and inhalation were both considered as the potential route of exposure in this population[20]. Recently, Fenske et al.[27] reported that dermal exposure and subsequent absorption through the skin accounted for 94-96% of the total dose of ~~chlorpyrifos~~ CPF in Egyptian pesticide applicators.

Recruitment and data collection

Fifty-eight male adolescents aged 12-21~~,~~ that were hired seasonally by the Ministry of Agriculture to spray pesticides in the cotton fields, were recruited from two ~~field stations~~districts

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in the Menoufia governate ~~(i.e. Al Shohada and Berket El Sabea, field station 1 and field station 2, respectively)~~. Forty adolescent non-applicators were recruited through convenience sampling (i.e., word of mouth, direct communication ~~with~~ utilizing contacts through the staff from the local Ministry of Agriculture) from the same districts as the applicators for the cotton crop. These adolescents never worked in the cotton fields as pesticide applicators. ~~We excluded one~~One adolescent was excluded from the final analysis due to his inconsistency in participating in the study activities and two other ~~subjects~~ participants were excluded for questionable sample integrity, resulting in a final sample size of 95 (57 applicators and 38 non-applicators). Written informed consent was obtained from all participants and their legal guardian (for those under 18). All the subjects were monetarily compensated for their time during the questionnaire survey and biological samples (~$5 per visit). The study was approved by the ~~OHSU~~ Oregon Health and Science University IRB in June 2009, and by the Medical Ethics committee of the Faculty of Medicine, Menoufia University in July 2009.

Data collection, for both applicators and non-applicators, occurred at the primary field station for each district. Pesticides applicators and supervisors meet in the field stations, which also provides storage area for the pesticides and the equipment used for application.

Outcome assessment

We developed a ~~multiple time point,~~ 25-item, short-term neurological symptom questionnaire on the basis of the widely used Q16 questionnaire [28] and a modified version of the Q16 used in a previous study ~~with~~ on licensed pesticide applicators [29]. The 25 symptoms were grouped into six domains: behavioral, autonomic, cognitive, sensory, motor and non-specific temporary disability (Table 1). The questionnaire had ~~There were~~ five ~~frequency choices~~response

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options (0-4) for each symptom ranging from "never" (coded as 0) to "everyday of the week" (coded as 4). Since more than 90% of the responses were between 0-2 (1=once a week and 2=once in every 2-3 days) we recoded each of the symptom response to "0" or "never" and "1" or "at least once a week or more." Self-reported neurological symptom counts were collected at 32 irregularly spaced dates over an eight-month period ~~spanning~~ from early June 2010 through early January 2011.~~Beginning on June 2 of 2010 through January 2011 participants reported symptoms occurring in the past week~~ These time points ~~spanned through this symptom questionnaire administered 32 separate times, at least once per week) and~~ ranged across three ~~spanning over all relevant application periods in the season (~~different time periods: pre-application, ~~during~~ application, and post-application~~)~~. For each time point, ~~The~~ the number of positive responses (a response was considered positive and coded as "1" when the participant reported the frequency of the symptom "at least once a week or more") ~~-~~was totaled for each person to yield a score ranging from 0–25; division by 25 produced the proportion of symptoms endorsed at each of the 32 time points. This outcome variable was used to compare the change of symptoms over time between applicators and non-applicators. ~~These sample periods were~~All these time points were collapsed into 10 separate non-overlapping intervals lasting between one and four weeks in length (~~Supplementary Figure 2~~Figure 1). Symptom data during the pre-application period, including the ~~from the first three dates (i.e~~ first fifteen days of the study ~~from June 2 to June 16), when no CPF was applied,~~ was collectively taken to represent the baseline time interval (or time interval 1)~~,~~ ~~against which symptom~~Symptom reporting from the other nine remaining time intervals was evaluated against time interval 1. The next 5 time intervals, ~~In five of these nine time intervals (~~between June 19 and July 21~~)~~, were during the application period of CPF~~ was reported in both field stations~~. The remaining ~~XX~~4 time intervals occurred between

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~~XX~~July 24, 2010 and ~~XX~~January 5, 2011 and reflect the post-application period although a brief CPF application was reported in the district where field station 1 was located. ~~and this~~The proportions of symptoms over all the 32 time points ~~was averaged~~were averaged ~~across the 32 collection points~~ to produce a season-level average percentage of neurological symptoms over the entire study period~~mean proportion of self-reported symptoms~~. This outcome variable was used to examine the relationships between the biomarkers (TCPy, AChE and BChE) and symptoms. Participants also completed a questionnaire ~~at~~ during baseline addressing their socio-demographic status, household and occupational use of pesticides~~, including such as number of days of pesticide application or mixing, medical history, safety practices and lifestyle activities including smoking status, hours of sleep at night, number of drinks containing caffeine~~.

Table 1. Domains of neurological symptoms

Domains	Symptoms

Behavioral Symptoms	1. Tense or anxious[#] 2. Excessively angry or irritable*[#] 3. Depressed or withdrawn*[#]
Autonomic Symptoms	4. Nausea[#] 5. Heavy sweating*[#] 6. Loss of appetite[#] 7. Fast heart rate*[#] 8. Excessive salivation
Cognitive Symptoms	9. Difficulty concentrating*[#] 10. Being absentminded and memory problem*[#]
Sensory Symptoms	11. Difficulty seeing at night[#] 12. Blurred or double vision[#] 13. Numbness in hands and feet[#] 14. Sense of smell or taste change[#] 15. Ringing in ears
Motor Symptoms	16. Difficulty with balance[#] 17. Weakness in arms and legs[#] 18. Involuntary movement of arms and legs[#] 19. Shaking in hands* 20. Difficulty speaking[#]
Temporary Disability (non-specific symptoms)	21. Dizziness[#] 22. Headache*[#] 23. Momentary loss of consciousness[#] 24. Fatigue*[#] 25. Insomnia[#]

*Symptoms used in Q-16 [27]

#Symptoms used in Agricultural Health Study [28]

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Urine collection and analysis

Spot urine samples were collected in new and individually wrapped cups at the beginning of the work shift ~~Urine was collected in new wide mouth plastic cups~~ at eight time points between April 2010 and January 2011. The cups were opened at the time of sample ~~We collected spot urine samples at the field station at the beginning of the work shift. The~~collection. Urine ~~cups~~ samples were subsequently transferred to the laboratory at Menoufia University in a cooler with wet ice. At the laboratory, 4 ml aliquots of urine were transferred into labeled 5 ml cryovials within hours of sampling and stored at –20 ºC. The banked urine samples were express mailed on dry ice to the University of Buffalo laboratory for analysis of pesticide metabolites; duplicate samples were retained in the –20 ºC freezer at Menoufia University. Urine samples in the field station ~~at Berket El-Sabea district~~2 were collected one day after the collection date of the field station ~~at Al-Shohada~~1.

Creatinine concentrations were measured using the Jaffe reaction[30]. The method of urinary TCPy measurement (a primary metabolite of chlorpyrifos) has been described elsewhere [24]. Briefly, Samples were analyzed using gas chromatography–mass spectrometry (negative-ion chemical ionization) and utilized 13C-15N-3,5,6-TCPy as an internal standard. Samples were hydrolyzed with HCl, extracted with toluene, and derivatized using N-(tert-butyldimethylsilyl)-N-methyltrifluoro-acetamide (Sigma Aldrich, USA). A spiked quality control (QC) sample was routinely run with the analytical samples and the metabolite concentration was determined from a standard curve for the peak area for the selective ion. The QC samples consisted of lab samples that were first analyzed for TCPy and the levels were non-detectable. The TCPy standard curve

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was linear from 1-200 ng/ml with a correlation coefficient of 1.000. Samples spiked with 50ng of TCPy/mL (n=20) gave an average metabolite recovery of 94.8% (range = 92 - 98%; SD = 0.931; RSD% = 1.965). A 1ng TCPy/ml spiked sample was run 10 times and the within series RSD% = 1.6. The minimum detection level was 0.5 ng/ml of urine. ~~Briefly, negative ion chemical ionization gas chromatography–mass spectrometry was used that utilized 13C-15N-3,5,6-TCPy as an internal standard. Jaffe reaction was used for colorimetric analysis of creatinine.[30] The within-run imprecision of this assay is very low (< 2% coefficient of variation and an intra-class correlation coefficient of 0.997). The quality control (QC) samples consisted of lab samples that were first analyzed for TCPy levels; these levels were non-detectable. Twenty aliquots were then spiked with 50ng of TCPy/mL of urine; these were then extracted and analyzed as per protocol. The recovery rates ranged from 92% - 98% with the average being 94.8%, SD = 0.931 and the CV% = 1.965, minimum detection level was 0.0501 ng. QC replicates had 94.75% recovery. Finally, cumulative urinary TCPy for each participant was determined by calculating the area under the curve for the plotted values for eight time intervals.~~

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Blood collection and ChE analysis

To establish the baseline ChE activity, pre-application blood draws occurred on April 11 and June 2, 2010, prior to the start of the official government-regulated CPF application season. As with the urine collection, blood draws occurred in the field station ~~at Berket El Sabea~~2 ~~were performed~~ one day later. Changes in both AChE and BChE levels from baseline to the end of CPF-application season (blood collected on September 4, 2010) were estimated. Blood samples were collected by venipuncture into 10mL lavender top (EDTA) vacutainer tubes and

immediately placed on wet ice and transported to Menoufia University, where they were analyzed in duplicate for AChE and BChE activity using an EQM Test-Mate kit (EQM Research Inc., Cincinnati, OH, USA) as described previously [24]

Statistical analysis

We used SPSS version 18.0 and STATA (version 11; Stata Corporation, College Station, TX) for the statistical analysis. Sociodemographic variables were summarized and described using means and standard deviations for continuous responses and percentages for discrete outcomes; simple comparisons between applicators and non-applicators were completed using t-tests ~~(continuous measures)~~ or chi-square tests ~~(discrete outcomes)~~. To calculate the value of cumulative TCPy for each participant we used STATA'a pharmacokinetic function (pkexamine) to employing the trapezoid rule to estimate the area under the curve for each participant over the study time. By definition, cumulative TCPy was the sum of the concentration at each time point multiplied by the duration between time points. This variable reflects the total amount of TCPy excreted over the study period for which urine as collected and assayed. Concentrations of cumulative TCPy, AChE and BChE exhibited pronounced right skewed distribution~~ness~~ and more than a 3-fold separation between the minimum and maximum observed values; consequently, these responses were log–transformed prior to analysis to improve symmetry. Both AChE and BChE were expressed as a log-transformed ratio of post-application activity relative to pre-application activity. Then the associations between the change of these cholinesterase markers from pre to post application seasons and self-reported symptoms were examined using linear regression models that took potential covariates into account. Similar regression analyses were used to examine the relationship between cumulative TCPy and

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neurological symptoms. ~~prior to investigation of associations with average percentage of self-reported symptoms.~~ All p-values are two-sided with significance judged relative to a 0.05 level.

Spearman correlation coefficients were used to estimate associations between urine and blood biomarkers and symptom scores. Generalized estimating equations (GEE)[31] were used to model the proportion of neurological symptoms reported in each time interval while controlling for number of days worked (within five days of the symptom reporting date), home use of pesticides, age, education and income levels. The one fitted model was used to estimate changes over time, relative to the first time interval (June 2–June 16), for applicators and non-applicators, as well as to examine whether changes relative to baseline differed between the two groups (via group-by-time interaction).

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RESULTS

Sociodemographic Characteristics

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Ninety-two of the participants (97%) were between 12 and 18 years old with the remaining three between 19 and 21. The two groups, non-applicators and applicators, did not differ significantly in terms of age, educational status, family income, number of people in house, years of pesticide use at home, and insecticides and rodenticides use at home (Table 2). Compared to non-applicators, a significantly higher number of applicators lived close to the field (within 25 meters), had carpet in their homes and applied herbicides at home. Applicators had

significantly lower BMI than non-applicators. On average, applicators had been working in the field for a little over 3 years.

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Table 2: Sociodemogrphic characteristics for participants at baseline

Variables	Non-applicators (n=38) Mean (SD)	Applicators (n=57) Mean (SD)
Age	16.6 (2.4)	16.2 (1.6)
Education	9.8 (1.8)	9.9 (1.8)
Height (cm) ~~*(34 non-applicators vs 30 app)*~~[#]	166.3 (12.0)	163.4 (10.0)
Weight (kg) ~~*(34 non-applicators vs 30 app)*~~*	62.0 (15.4)	54.2 (8.6)
BMI (kg/m^2) ~~(34 non-applicators vs 30 app)~~*	22.1 (3.7)	20.2 (2.2)
Number of people in house	5.6 (1.1)	6.0 (1.8)
Home pesticide use (years)[##]* ~~(19 Non-applicators & 44 App)*~~	1.6 (1.9)	2.5 (1.9)
Occupational application of pesticides (yrs)	-	3.1 (1.5)
Days/week of pesticide application	-	4.8 (1.3)
Hours/day of pesticide application	-	5.2 (0.7)
	% (n)	**% (n)**
Family Monthly Income (<500 E)~~(Low)~~	78.9 (30)	71.9 (41)
Applied pesticides in home in last 5 yrs (yes)*	47.4 (18)	78.9 (45)
Computer use (once a week or more)*	65.8 (25)	45.6 (26)
Carpet in house (yes)*	27.0 (10)	54.4 (31)
Live ~~close~~ within 25m to agricultural field (yes)*	23.7 (9)	50.9 (29)
Types of pesticides applied at home ~~(24 vs 49)~~[###]		
Herbicides*	13.0 (3)	44.9 (21)
Insecticides	83.3 (20)	93.9 (46)
Rodenticides	16.7 (4)	14.3 (7)

*p<0.05 for group difference, ~~#30 applicators vs 34 non applicators, ##44 applicators vs 19 non applicators, #49 applicators vs 24 non~~ applicators, E=Egyptian pound

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Change in symptoms over time

We considered days 0-14 as the baseline time interval (~~1st~~ time interval 1) when no application of CPF was reported. While examining symptoms reported over time among both applicators and non-applicators we took various potential confounders into account. These include occupational factors such as days worked per week in pesticide applications, number of years of pesticide use at home and socio-demographic factors such as age, education and income level of the participants. Applicators began increased reporting of neurological symptoms at the beginning of the ~~chlorpyrifos~~ CPF application season (at ~~the 2nd~~ time interval 2 between days 17-21 of the study). The percentage of neurological symptoms ~~increased~~ continued to increase during the application season and reached the peak at ~~the 6th~~ time interval 6, representing days 45-48, the time when ~~the chlorpyrifos~~CPF application period ended. This was followed by a drop of symptom reporting indicating a small recovery due to the cessation of exposure in both districts. The highest peak of symptom reporting was observed at the ~~8th~~ time interval 8 representing days 63-77 (Table 3). This happened perhaps due to a small episode of CPF application in field station 1 (between time intervals 7 and 8). Similar to the applicators, the non-applicators also demonstrated the highest increase in the proportion of neurological symptoms during the ~~8th~~ time interval 8 although the magnitude of the change was smaller (14 percentage point increase of symptoms relative to baseline interval). The change of neurological symptoms relative to baseline declined over the next two time intervals (9th and 10th) in both groups indicating a recovery phase during post-application. For applicators, the percentage of

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reported symptoms at each of the nine subsequent time intervals was always higher than the percentage observed at baseline; non-applicators, by contrast, had a pattern ~~of percentage~~ of reported symptoms that both increased and decreased relative to the baseline time interval (pre-application) over the course of the study (Figure ~~1~~2).

When applicators and non-applicators are compared with respect to change in percentage of symptoms (relative to baseline), it was always the case that the change (percentage point change relative to baseline) for applicators was greater than the corresponding change for non-applicators even after adjusting for the covariates (Table 3).

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Table 3. Estimated change (95% CI) from baseline in the percentage points of neurological symptoms reported at each of nine successive collected time points, shown separately for non-applicators and applicators.

~~DOES GREY AREA INDICATE WHEN CPF WAS APPLIED?~~

	Time Intervals	Non-Applicators Adjusted Models*		Applicators Adjusted Models*		Change in difference between applicators and non-applicators relative to difference in baseline	
1	Days from Baseline (Day 0-14) June 2-June 14, 2010	b (% of Symptoms) (95% CI)	p-value	b (% of Symptoms) (95% CI)	p-value	b (% of Symptoms) (95% CI)	p-value
2	17-21 Jun 19-Jun 23	-2.74 (-4.61,-0.86)	0.004	4.08 (0.18,7.97)	**0.040**	6.81 (2.47, 11.15)	**0.002**
3	24-28 Jun 26-Jun 30	-2.68 (-5.18,0.17)	0.004	12.57 (7.75,17.38)	**<0.001**	15.25 (9.80, 20.69)	**<0.001**
4	31-35 Jul 3-Jul 7	4.45 (-0.80,9.71)	0.10	14.06 (9.21,18.90)	**<0.001**	9.60 (2.44, 16.77)	**0.009**
5	38-42 Jul 10-Jul 14	-2.28 (-4.75.0.17)	0.07	22.83 (19.25,26.40)	**<0.001**	25.11 (20.79, 29.43)	**<0.001**
6	45-48	0.46 (-2.37,3.30)	0.75	28.80 (24.27,33.35)	**<0.001**	28.35 (22.98, 33.72)	**<0.001**

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	Jul 17-Jul 21							
7	52-59	4.95 (1.63,8.28)	**0.003**	24.01 (19.92,28.09)	**<0.001**	19.05 (13.80, 24.30)	**<0.001**	
	Jul 24-Jul 31							
8	63-77	14.49 (11.45,17.55)	**<0.001**	30.10 (26.53,33.66)	**<0.001**	15.60 (10.95, 20.26)	**<0.001**	
	Aug 4-Aug 18							
9	80-94	12.08 (8.72,15.44)	**<0.001**	29.17 (25.22,33.13)	**<0.001**	17.09 (11.93, 22.25)	**<0.001**	
	Aug 21-Sep 4							
10	105-217	9.22 (6.12,12.32)	**<0.001**	18.45 (14.30,22.59)	**<0.001**	9.22 (4.12,14.33)	**<0.001**	
	Sep 22-Jan 5[#]							

*Models adjusted for number of days worked for applying pesticides, years of pesticide use at home, age, education and income level. CPF application time intervals are shaded in grey. ~~*Estimates have been adjusted for number of days worked applying pesticides, home use of pesticides, age, education and income level. CPF application time intervals are shaded in grey.~~

Associations of neurological symptoms with biomarkers

TCPy was detected in 100% of the samples. Summary statistics for TCPy, AChE and BChE of the study samples have been already reported by Crane et al. (2013)[32]. Mean creatinine concentration of the urine samples was reported to be 1696 μ g/ml with a maximum of 4199 and a minimum of 164 μ g/ml. In brief, the applicators had much higher mean and estimated median peak TCPy concentration than the non-applicators (mean: 719 vs 44.9 μ g/g creatinine; estimated median 137 vs 19.7 μ g/g creatinine). In our study sample, BChE was found to be more sensitive to CPF exposure than AChE, with median activity reduced by 37% from baseline in applicators and 13% in non-applicators during the CPF application period.

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A scatter plot of cumulative TCPy (ug/g creatinine) against average percentage points of symptoms revealed distinct exposure-response gradients by pesticide application status (applicators vs non-applicators) (~~Supplementary Figure 3a2a~~Figure 3). In addition, two other scatter plots of change in AChE activity and change in BChE activity from pre-application to post-application against percentage of symptoms also revealed effect measure modification by pesticide application status (Supplementary Figures ~~3b 2ba~~ & ~~3c2eb~~). Therefore, separate linear models for applicators and non-applicators were used to examine the associations of these three biomarkers with ~~the outcome measures~~symptoms.

Log-transformed TCPy was positively associated with the average percentage of neurological symptoms in the regression models after adjusting for other covariates that may confound exposure-outcome relationship such as field stations, age, family monthly income, ~~home~~ pesticide use at home and average number of hours worked in the field among applicators (b=2.68, p=0.007). However, non-applicators demonstrated positive, but statistically non-significant, associations between TCPy and symptoms. Among applicators, AChE and BChE activity was negatively and significantly associated with the average percentage of neurological symptoms in the unadjusted models. In the adjusted models these associations remained negative but became non-significant (Table 4).

Table 4. Summary of regression analysis for biomarkers of exposure & effect of chlorpyrifos predicting average percentage of neurological symptoms over the entire study stratified by applicator status

	Unadjusted Models			Adjusted Models*		
Explanatory variables	**B (se)**	**95% CI**	**p-value**	**B (se)**	**95% CI**	**p-value**
For Non Applicators						

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Ln TCPy (mg/g Cr) (n=28)	0.29 (0.76)	-1.26,1.84	0.71	0.57 (0.79)	-1.06,2.20	0.47
Ln (Post AChE/Pre AChE) (n=21)	-1.25 (16.41)	-35.59,33.1	0.94	-6.57 (18.80)	-43.64,33.50	0.73
Ln (Post BChE/Pre BChE) (n=21)	2.23 (7.04)	-12.51,16.98	0.76	2.50 (7.63)	-13.77,18.77	0.75
For Applicators						
Ln TCPy (mg/g Cr) (n=42)	4.56 (0.63)	3.29,5.84	<0.001	2.68 (0.93)	0.78,4.57	0.007
Ln (Post AChE/Pre AChE) (n=28)	-24.21 (12.79)	-50.50,2.09	0.07	-11.60 (12.44)	-37.46,14.25	0.36
Ln (Post BChE/Pre BChE) (n=29)	-14.52 (4.61)	-23.97,-5.07	0.004	-7.33(5.93)	-19.63,4.97	0.23

*Regression models adjusted for field stations, age, family monthly income, ~~home~~ pesticide use at home and average number of hours of work in the field over the entire application season (for applicators only)

When we examined the biomarker-symptom relationship by ~~subclasses~~ domains of symptoms among the applicators, we observed significant positive associations of log-transformed TCPy with behavioral, autonomic, cognitive, motor and sensory problems after accounting for sociodemographic and occupational covariates (~~Supplementary~~ Table ~~1~~5). The magnitudes of associations (adjusted betas) were greater for autonomic, cognitive and sensory symptoms than the two other ~~subclasses~~domains. Although the log-transformed change in AChE activity was not associated with any of these subclasses, change in BChE activity demonstrated a significant association with average percentage of behavioral symptoms (p=0.04) and a marginally significant association with average percentage of cognitive symptoms (p=0.07) ~~(~~

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~~Supplementary Table 1).~~

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Table 5. Summary of regression analysis for biomarkers of exposure and effect of chlorpyrifos predicting average percentage of neurological symptoms by subclasses among **Applicators**

	Unadjusted Model Ln TCPy (mg/g Cr) (n=42)		Adjusted Model Ln TCPy (mg/g Cr) (n=42)		Unadjusted Model Ln (Post AChE/Pre AChE) (n=28)		Adjusted Model Ln (Post AChE/Pre AChE) (n=28)		Unadjusted Model Ln (Post BChE/Pre BChE) (n=29)		Adjusted Model Ln (Post BChE/Pre BChE) (n=29)	
Outcome variable (Symptom Subclass)	**B (se)**	**p-value**	**B (se)**	**p-value**	**B (se)**	**p-value**	**B (se)**	**p-value**	**B (se)**	**p-value**	**B (se)**	**p-value**
Behavior & Affect	2.16 (0.57)	<0.001	2.80 (0.91)	**0.004**	-12.59 (10.86)	0.26	-16.34 (16.04)	0.33	-6.96 (4.09)	0.10	-12.26 (5.73)	**0.04**
Autonomic	5.69 (0.75)	<0.001	3.24 (0.99)	**0.002**	-38.45 (14.88)	0.02	-4.84 (17.22)	0.78	-19.65 (5.42)	0.001	-9.69 (6.40)	0.14
Cognitive	6.92 (0.87)	<0.001	5.27 (1.34)	**<0.001**	-46.22 (17.49)	0.01	1.73 (20.82)	0.94	-24.16 (6.03)	0.001	-13.56 (7.55)	**0.07**
Motor	2.48 (0.49)	<0.001	1.67 (0.74)	**0.03**	-6.27 (8.41)	0.46	4.50 (12.03)	0.37	-5.75 (3.15)	0.08	-3.90 (4.64)	0.41
Sensory	6.47 (0.90)	<0.001	4.94 (1.34)	**0.001**	-27.49 (17.64)	0.13	-0.75 (22.46)	0.97	-20.00 (6.41)	0.004	-13.88 (8.86)	0.13
Temporary Disability (Non-specific)	5.48 (0.61)	<0.001	3.64 (0.78)	**<0.001**	-41.13 (14.14)	0.007	-10.74 (16.61)	0.53	-21.80 (4.73)	0.001	-13.52 (5.73)	**0.03**

*Regression models adjusted for field stations, age, family monthly income, pesticide use at home and average number of hours of work in the field over the entire application season (for applicators only)

DISCUSSION

~~A~~ The self-reported symptom questionnaire has been globally recognized as the primary method to capture symptom data in exposed populations. The most common questionnaire utilized is the extended or modified versions of Q-16 [28], which has been used in many international studies including a study with Nicaraguans living close to cotton fields [8], Sri Lankan farmworkers [10] and Colorado agricultural communities [33]. However, time intervals between exposure and collection of symptom data in these studies varied from one month to twelve months [6 9 10 29 34-36]. Furthermore, the majority of studies have utilized cross-sectional design which lacks information about temporality.

To the best of our knowledge, this is the first longitudinal study ~~on~~ with adolescents to ~~look~~ examine ~~into~~ the relationship between CPF and self-reported neurological symptoms. In this study, a gradual increase in neurological symptoms, relative to the baseline time interval, was observed among the applicators from ~~the 2nd to the 8th time intervals (days 24-77 of the study during June 26 to August18, 2010)~~during the CPF application period after accounting for the number of days worked during the week, home use of pesticides by the participant, age, education and family monthly income levels. A significant 30 percentage point increase in the neurological symptoms relative to the baseline time interval was observed on ~~the 8th~~ time interval 8 (days 63-77 of the study). This is perhaps due to a second short CPF application episode in the same season in ~~the~~ field station 1~~at Al Shohada~~. Self-reported symptoms among applicators remained significantly elevated from the ~~baseline time interval~~pre-application period until day 217, approximately five months after the cessation of exposure showing evidence that despite discontinuation of CPF application, repeated exposure of this pesticide led to persistence of

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neurological health effects for several months. Compared with the applicators, the non-applicators showed relatively late reporting of neurological symptoms, perhaps due to the ~~low level~~ environmental ~~chlorpyrifos~~ CPF exposure. It is interesting to note that the non-applicators still reported approximately 9 percentage point more symptoms relative to baseline ~~in~~ at the last time ~~point~~ interval (day 105-217). Residual CPF can survive in indoor environments for an extended period of time, can rapidly bind to soil and plants and has a half-life of several months in soil [37 38]. We anticipate that because of these properties, CPF remained in the environment as a potential source of environmental exposure leading to increased symptom reporting among non-applicators.

The symptom reporting ~~across~~ over time showed a recovery phase at ~~the 10~~th time interval 10 (day 105-207) when percentage of symptom reporting relative to baseline declined substantially from the previous time intervals (Table 3, Figure ~~1~~2). Using the same sample, we recently demonstrated that both the applicators and non-applicators experienced peak median BChE depression during the CPF application period but BChE returned to the baseline level by the end of the study (day 217/January 5, 2011) [32]. We anticipate that symptoms were following BChE activity pattern, i.e., as the BChE activity was returning back to the baseline level~~, recovery from the~~ neurological symptoms ~~was taking place.~~ were going through the recovery phase.

Prior to this study, a cross-sectional study on Egyptian cotton field workers reported associations between OP exposure and neurological symptoms [20 21]. Similar to an~~other~~ Indian study on occupationally exposed adolescents [22], the previous Egyptian adolescent study [20 21] presented descriptive statistics to show the difference between exposed and unexposed

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adolescents in terms of the prevalence of various neurological symptoms. However, these studies did not take potential ~~without taking other~~ sociodemographic confounders into account. Results of the present study were consistent with several longitudinal studies conducted in adult populations. In one study of occupationally and non-occupationally OP pesticide-exposed farmers and fishermen, delayed persistence of neurological symptoms were found during the two-year follow-up[7]. Results from a clinical examination of the same cohort found that there were deficits related to sensory function[39]. Another study, conducted over three years with Colorado farm workers, reported an association between OP exposure and symptoms of depression[12]. Consistency in the results across studies indicate that a Q-16 based self-reported questionnaire used in all of these studies is a reliable measure to estimate health effects resulting from OP (in this case ~~chlorpyrifos~~CPF) exposure.

Our study is also novel in its approach ~~to include~~of including prospective measures of biomarkers. First, instead of using single-time point biomarker data ~~(urinary TCPy)~~ commonly used in cross-sectional studies, our study analyzed urinary TCPy levels at multiple time points. The collection of pre, during and post ~~exposure~~ application samples resulted~~ing~~ in a precise estimate of cumulative exposure from April 11 to January 5[32]. This has enabled us to overcome a historical challenge in characterizing OP exposure and allows us to subsequently examine the association of cumulative exposure with neurological symptoms. An additional limitation often encountered by past studies was the absence of established baseline AChE and BChE levels. A recent adult study examining the variation of cholinesterase levels among OP pesticides and carbamate-exposed field-workers could not establish any baseline AChE/BChE due to the mobility of the migrant study population[40]. Another Egyptian adolescent study also reported

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greater reduction of AChE~~acetylcholinesterase~~ activity among the pesticide applicators compared to the controls [20 21]. By collecting blood samples prior to the start of the application season, baseline data were established, which allowed us to compute more precise measures of change in activities of AChE and BChE from pre-exposure to post-exposure periods.

Two previous studies of Kenyan and Palestinian farm workers, which measured cholinesterase levels before and after exposure, found associations between cholinesterase inhibition and respiratory, eye and neurological symptoms [9 41]. Potential occupational confounding factors (e.g residential application of pesticides and number of days worked in agriculture ~~into account~~) that are associated with neurological symptoms [20 42] were not taken into account while examining exposure-outcome associations in these past studies~~-~~. These potential confounding variables were included in our study questionnaires and later examined during statistical analysis.

We identified a comparison group (non-applicators) who were similar in demographic characteristics to our applicators. It is often true that control groups in occupational settings may not be truly unexposed[1]. In our study, close proximity to the agricultural field (less than 25m) and application of pesticides at home were the two environmental factors offering ~~some degree of OP exposure~~potential exposure opportunities to the non-applicators as indicated by elevated urinary TCPy levels during the period of ~~chlorpyrifos~~ CPF application to cotton fields [32]. To encounter this potential confounder, all statistical models were adjusted for these two variables in addition to other sociodemographic variables.

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It is difficult to explain why we found no relationship between TCPy and neurological symptoms among the non-applicators when a delayed effect of environmental or passive CPF exposure on symptoms was evident among this subgroup in the corresponding GEE model (Table 3 & Figure ~~1~~2). One possible explanation is that the range of cumulative exposure was much lower among the non-applicators (154 to 24,180 mg/g creatinine; median 2591 mg/g creatinine) compared to the applicators (232 to 28,260 mg/g creatinine; median 10318 mg/g creatinine). Small sample size and differences in cumulative exposure might have contributed to the non-significant association in the non-applicator subgroup. Some other undocumented environmental factors such as working during high temperatures along with carrying a heavy backpack during CPF application might have positively confounded the association among the applicators.

We acknowledge that we relied on self-reported outcome measure. Therefore, there was a possibility that the frequent completion of the neurological symptoms survey (32 times over 8 months) could itself have had an influence on the increase in symptoms during the CPF application season. This could partially explain why these symptoms were not associated with TCPy levels among non-applicators.

The non-specific nature of many of the symptoms is another limitation of the current study~~.~~ ~~In addition,~~, the biological significance of these self-reported symptoms is unknown. However, the goal of the study was not to establish that more symptoms lead to development of any neurological disease. Rather we attempted to examine how repeated or cumulative exposure to ~~chlorpyrifos~~ CPF determined the pattern of neurological symptoms over the entire season. Five

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of the symptoms included in our questionnaire are considered non-specific, including, headache, dizziness, fatigue, loss of consciousness and insomnia. The remaining 20 symptoms were classified into more specific neurological functions such as behavior, autonomic, sensory, cognitive or motor functions. When we excluded ~~the five~~these non-specific symptoms from the summary measure, t~~T~~he estimated betas for the associations of ~~exposure variables cumulative~~ TCPy, change in AChE and BChE activities with average percentage of 20 neurological symptoms were found to be 3.19 ($p<0.001$), -6.11 ($p=0.60$) and -9.49 ($p=0.05$) respectively after accounting for potential covariates.

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~~Our study was conducted in an agricultural community in Egypt, which is relatively where families are primarily middle class to lower middle class. Results of our study may be generalizable only to agricultural communities with similar sociodemographic characteristics.~~

CONCLUSION

Our study reinforces the need for the development and execution of intervention programs for the residents of agricultural communities, including pesticide applicators, in developing countries. Future interventions ~~should~~should include ~~address~~ hygiene practices, behaviors and use of protective equipment, in ~~addressing~~ both occupational and ~~environmental~~residential environments ~~exposures~~. Our study is the first to demonstrate that repeated occupational CPF exposure is an important determinant of neurological symptoms in adolescent applicators and non-applicators ~~across~~ over time, with symptoms peaking during the exposure season and partly recovering in months following exposure. The study also showed a significant association between cumulative CPF exposure and symptoms, using cumulative urinary TCPy as a

biomarker of exposure. Future studies are needed to assess the temporal and dose-dependent effects of repeated CPF exposure on neurological symptoms and neurobehavioral deficits in children, adolescents and adults to identify the most sensitive populations. ~~Similar prospective studies with a larger populations are also needed to assess the relationship between these endpoints and biomarkers of exposure, effect and susceptibility, ultimately identifying biomarkers, which may help protect sensitive population.~~

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Contributorship Statement

All authors have made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data in the study; Dr. Khalid Khan has taken the lead to draft the manuscript. Whereas other authors have revised the draft critically for important intellectual content; All of the authors have provided final approval of the version to be published. Dr. Diane

Rohlman is the Principal Investigator of the study and has supervised each step of the manuscript development process. She has been listed as the Corresponding author.

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IRB Approval:

The study was approved by the OHSU IRB in June 2009 and by the Medical Ethics committee of the Faculty of Medicine, Menoufia University in July 2009.

Conflict of Interest Statement

None of the authors has any potential financial, personal or other conflict of interest, which could inappropriately influence this study.

Data Sharing Statement

Additional unpublished data from the study are available to Dr. Diane Rohlman (diane-rohlman@uiowa.edu), Dr. Ahmed Ismail (aa-ismail@hotmail.com) and Dr. James Olson (aa-ismail@hotmail.com) in Excel or SPSS datasets. They can be reached by email.

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Abbreviations: CPF (chlorpyrifos); OP (organophosphorus); TCPy (3,5,6-trichloro-2-pyridinol); AChE (acetylcholinesterase); BChE (butyrylcholinesterase); CYP (cytochrome P450)

REFERENCES

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1 Kamel F, Hoppin JA. Association of pesticide exposure with neurologic dysfunction and disease. *Environ Health Perspect* 2004;112:950-8.

2 Solomon C, Poole J, Palmer KT, Peveler R, Coggon D. Acute symptoms following work with pesticides. *Occup Med (Lond)* 2007;57:505-11.

3 Wesseling C, van Wendel de Joode B, Keifer M, London L, Mergler D, Stallones L. Symptoms of psychological distress and suicidal ideation among banana workers with a history of poisoning by organophosphate or n-methyl carbamate pesticides. *Occup Environ Med* 2010;67:778-84.

4 Alavanja MC, Hoppin JA, Kamel F. Health effects of chronic pesticide exposure: cancer and neurotoxicity. *Annu Rev Public Health* 2004;25:155-97.

5 Beseler C, Stallones L. Safety practices, neurological symptoms, and pesticide poisoning. *J Occup Environ Med* 2003;45:1079-86.

6 London L, Nell V, Thompson ML, Myers JE. Effects of long-term organophosphate exposures on neurological symptoms, vibration sense and tremor among South African farm workers. *Scand J Work Environ Health* 1998;24:18-29.

7 Delgado E, McConnell R, Miranda J et al. Central nervous system effects of acute organophosphate poisoning in a two-year follow-up. *Scand J Work Environ Health* 2004;30:362-70.

8 Keifer M, Rivas F, Moon JD, Checkoway H. Symptoms and cholinesterase activity among rural residents living near cotton fields in Nicaragua. *Occup Environ Med* 1996;53:726-9.

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9 Ohayo-Mitoko GJ, Kromhout H, Simwa JM, Boleij JS, Heederik D. Self reported symptoms and inhibition of acetylcholinesterase activity among Kenyan agricultural workers. *Occup Environ Med* 2000;57:195-200.

10 Smit LA, van-Wendel-de-Joode BN, Heederik D, Peiris-John RJ, van der Hoek W. Neurological symptoms among Sri Lankan farmers occupationally exposed to acetylcholinesterase-inhibiting insecticides. *Am J Ind Med* 2003;44:254-64.

11 Farahat FM, Rohlman DS, Storzbach D, Ammerman T, Anger WK. Measures of short-term test-retest reliability of computerized neurobehavioral tests. *Neurotoxicology* 2003;24:513-21.

12 Beseler CL, Stallones L. A cohort study of pesticide poisoning and depression in Colorado farm residents. *Ann Epidemiol* 2008;18:768-74.

13 McCauley LA, Shapiro SE, Scherer JA, Lasarev MR. Assessing pesticide safety knowledge among Hispanic migrant farmworkers in Oregon. *J Agric Saf Health* 2004;10:177-86.

14 McCauley LA, Sticker D, Bryan C, Lasarev MR, Scherer JA. Pesticide knowledge and risk perception among adolescent Latino farmworkers. *J Agric Saf Health* 2002;8:397-409.

15 London L, Beseler C, Bouchard MF et al. Neurobehavioral and neurodevelopmental effects of pesticide exposures. *Neurotoxicology* 2012.

16 Costa LG, Li WF, Richter RJ, Shih DM, Lusis A, Furlong CE. The role of paraoxonase (PON1) in the detoxication of organophosphates and its human polymorphism. *Chem Biol Interact* 1999;119-120:429-38.

17 Costa LG, Cole TB, Vitalone A, Furlong CE. Measurement of paraoxonase (PON1) status as a potential biomarker of susceptibility to organophosphate toxicity. *Clin Chim Acta* 2005;352:37-47.

18 Rauh VA, Perera FP, Horton MK et al. Brain anomalies in children exposed prenatally to a common organophosphate pesticide. *Proc Natl Acad Sci U S A* 2012;109:7871-6.

19 Ecobichon DJ. Pesticide use in developing countries. *Toxicology* 2001;160:27-33.

20 Abdel Rasoul GM, Abou Salem ME, Mechael AA, Hendy OM, Rohlman DS, Ismail AA. Effects of occupational pesticide exposure on children applying pesticides. *Neurotoxicology* 2008;29:833-8.

21 Ismail A, Rohlman D, Rasoul GA, Salem MA, Hendy O. Clinical and biochemical parameters of children and adolescents applying pesticides. *International Journal of Occupational and Environmental Medicine* 2010;1:132-143.

22 Rastogi SK, Tripathi S, Ravishanker D. A study of neurologic symptoms on exposure to organophosphate pesticides in the children of agricultural workers. *Indian J Occup Environ Med* 2010;14:54-7.

23 Nolan RJ, Rick DL, Freshour NL, Saunders JH. Chlorpyrifos: pharmacokinetics in human volunteers. *Toxicol Appl Pharmacol* 1984;73:8-15.

24 Farahat FM, Ellison CA, Bonner MR et al. Biomarkers of chlorpyrifos exposure and effect in Egyptian cotton field workers. *Environ Health Perspect* 2011;119:801-6.

25 Egeghy PP, Cohen Hubal EA, Tulve NS et al. Review of pesticide urinary biomarker measurements from selected US EPA children's observational exposure studies. *Int J Environ Res Public Health* 2011;8:1727-54.

26 Hofmann JN, Keifer MC, De Roos AJ et al. Occupational determinants of serum cholinesterase inhibition among organophosphate-exposed agricultural pesticide handlers in Washington State. *Occup Environ Med* 2010;67:375-86.

27 Fenske RA, Farahat FM, Galvin K, Fenske E, Olson JR. Contributions of inhalation and dermal exposure to chlorpyrifos dose in Egyptian cotton field workers *International Journal of Occupational and Environmental Health* 2012;(In Press).

28 Lundberg I, Hogberg M, Michelsen H, Nise G, Hogstedt C. Evaluation of the Q16 questionnaire on neurotoxic symptoms and a review of its use. *Occup Environ Med* 1997;54:343-50.

29 Kamel F, Engel LS, Gladen BC, Hoppin JA, Alavanja MC, Sandler DP. Neurologic symptoms in licensed pesticide applicators in the Agricultural Health Study. *Hum Exp Toxicol* 2007;26:243-50.

30 Fabiny DL, Ertingshausen G. Automated reaction-rate method for determination of serum creatinine with the CentrifiChem. *Clin Chem* 1971;17:696-700.

31 Liang K-Y, Zeger SL. Longitudinal Data Analysis Using Generalized Linear Models. *Biometrika* 1986;73:13-22.

32 Crane AL, Abdel Rasoul G, Ismail A et al. Longitudinal Assessment of Chlorpyrifos Exposure and Effect Biomarkers in Adolescent Egyptian Agricultural Workers. *Journal of Exposure Science and Environmental Epidemiology* 2012;(In Press).

33 Stallones L, Beseler C. Pesticide illness, farm practices, and neurological symptoms among farm residents in Colorado. *Environ Res* 2002;90:89-97.

34 Gomes J, Lloyd O, Revitt MD, Basha M. Morbidity among farm workers in a desert country in relation to long-term exposure to pesticides. *Scand J Work Environ Health* 1998;24:213-9.

35 Pilkington A, Buchanan D, Jamal GA et al. An epidemiological study of the relations between exposure to organophosphate pesticides and indices of chronic peripheral neuropathy and neuropsychological abnormalities in sheep farmers and dippers. *Occup Environ Med* 2001;58:702-10.

36 Steenland K, Dick RB, Howell RJ et al. Neurologic function among termiticide applicators exposed to chlorpyrifos. *Environ Health Perspect* 2000;108:293-300.

37 CDC. National Report on Human Exposure to Environmental Chemicals (Centers for Disease Control and Prevention). Available: http://www.cdc.gov/exposurereport [accessed 18 February 2012] 2009.

38 Eaton DL, Daroff RB, Autrup H et al. Review of the toxicology of chlorpyrifos with an emphasis on human exposure and neurodevelopment. *Crit Rev Toxicol* 2008;38 Suppl 2:1-125.

39 Miranda J, McConnell R, Wesseling C et al. Muscular strength and vibration thresholds during two years after acute poisoning with organophosphate insecticides. *Occup Environ Med* 2004;61:e4.

40 Quandt SA, Chen H, Grzywacz JG, Vallejos QM, Galvan L, Arcury TA. Cholinesterase depression and its association with pesticide exposure across the agricultural season among Latino farmworkers in North Carolina. *Environ Health Perspect* 2010;118:635-9.

41 Safi JM, Abu Mourad TA, Yassin MM. Hematological biomarkers in farm workers exposed to organophosphorus pesticides in the Gaza Strip. *Arch Environ Occup Health* 2005;60:235-41.

42 Kamel F, Engel LS, Gladen BC, Hoppin JA, Alavanja MC, Sandler DP. Neurologic symptoms in licensed private pesticide applicators in the agricultural health study. *Environ Health Perspect* 2005;113:877-82.

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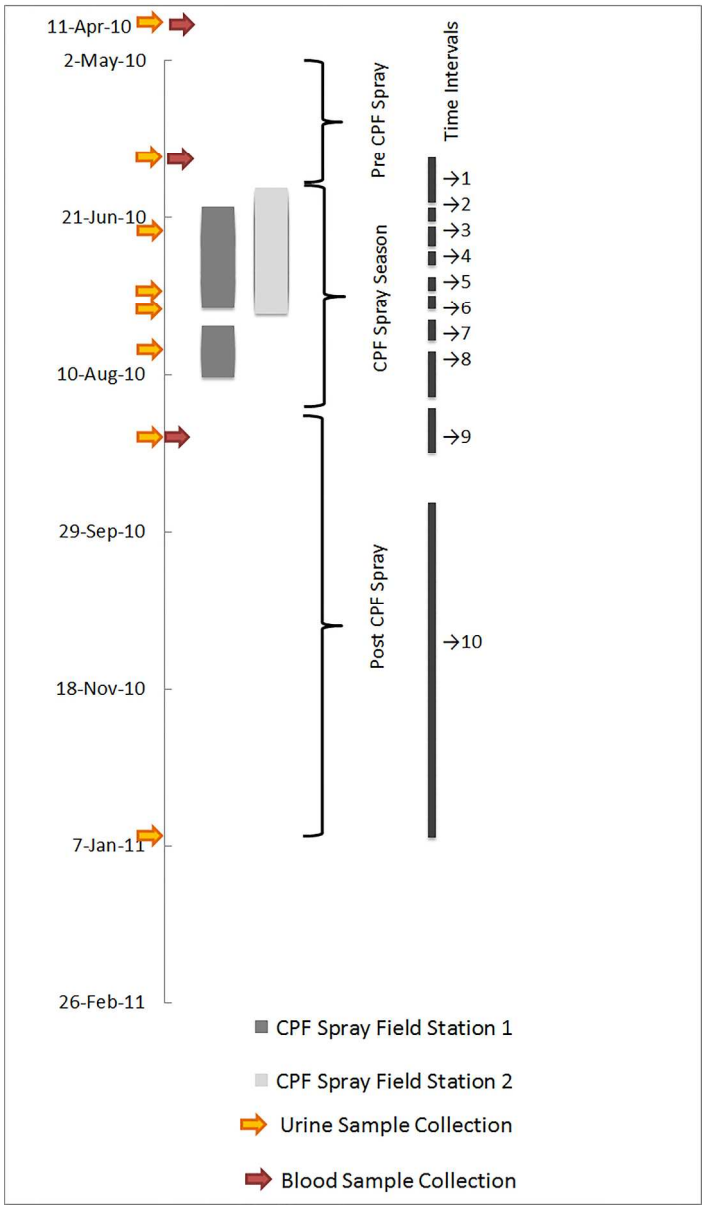

Figure 1. CPF application in the study area showing time intervals in both field stations 1 and 2.

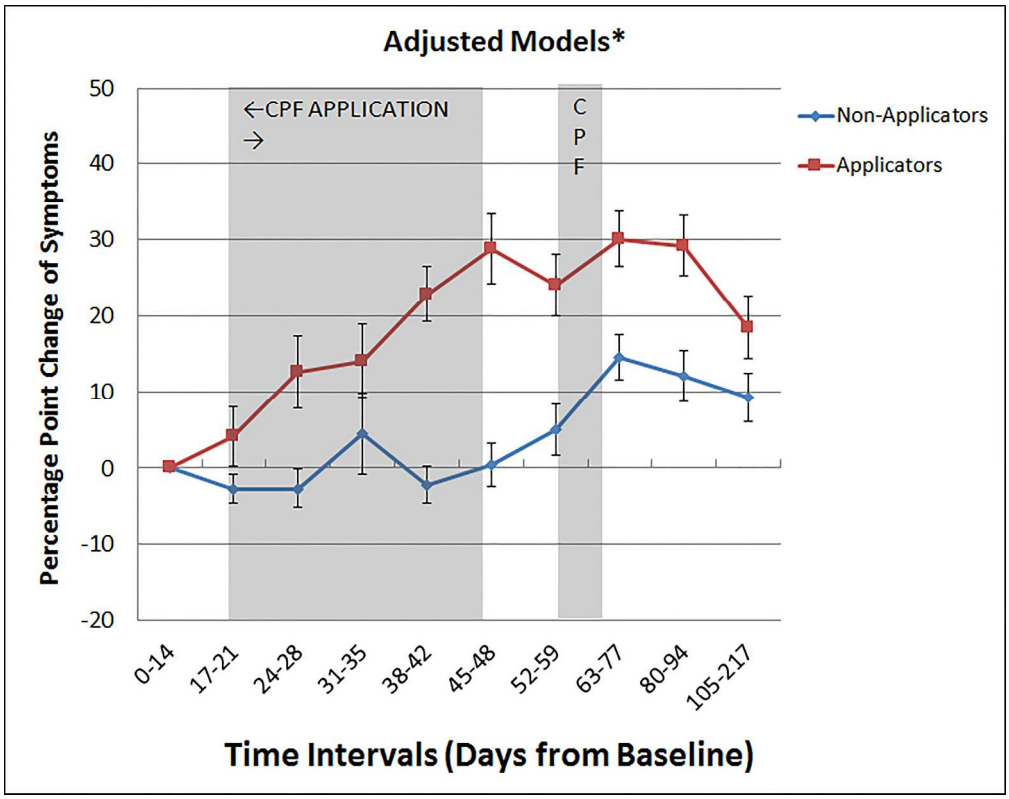

Figure 2. Difference, relative to baseline, in the percentage of symptoms reported at each of nine subsequent time intervals; error bars represent 95% confidence limits for the difference.

Footnote (Figure 2): *Models adjusted for number of days worked for applying pesticides, years of pesticide use at home, age, education and family monthly income. CPF application time intervals are shaded in grey.

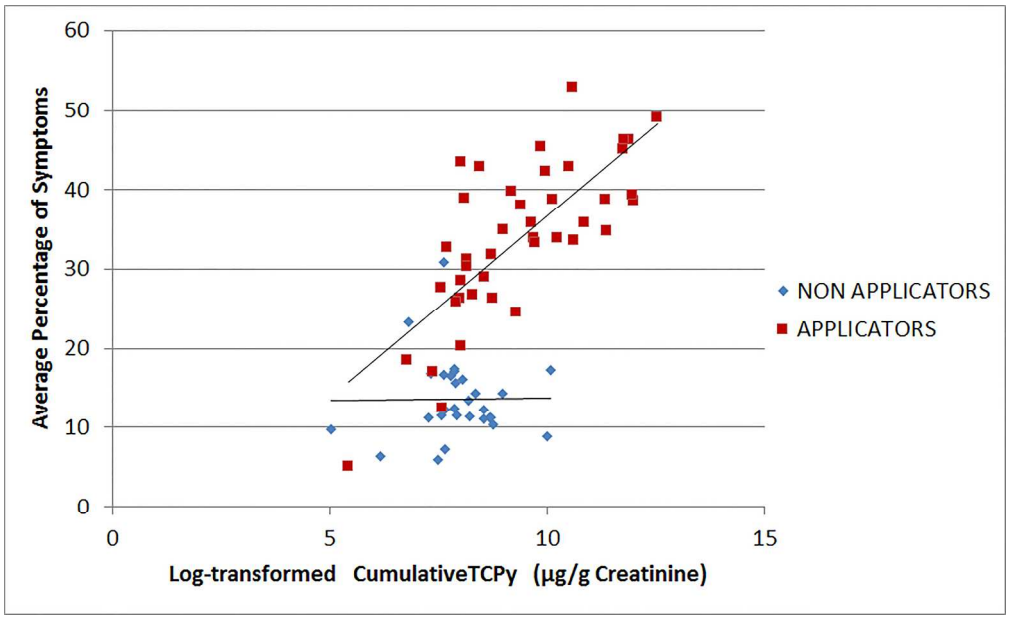

Figure 3. Scatter plots of cumulative TCPy (ug/g creatinine) against percentage of symptoms (n=70)

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract: **Title indicates "Longitudinal Assessment……."**
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found: **Summary provided in the abstract**
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported: **Explained**
Objectives	3	State specific objectives, including any prespecified hypotheses: **Objectives specified**
Methods		
Study design	4	Present key elements of study design early in the paper: **Presented in the last paragraph of the Introduction section**
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection: **Described in the Method section**
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up: **Described in the Method section**
		(*b*) *Cohort study*—**This is not a matched study; Convenience sampling**
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable: **Described in Methods**
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group: **Provided**
Bias	9	Describe any efforts to address potential sources of bias
Study size	10	Explain how the study size was arrived at: **Explained in Method**
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why: **Described**
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding: **Described in statistical analysis section**
		(*b*) Describe any methods used to examine subgroups and interactions: **Described**
		(*c*) Explain how missing data were addressed: **No missing data**
		(*d*) *Cohort study*—**Not applicable**
		(*e*) Describe any sensitivity analyses- **Not applicable**

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Results		
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed: **Reported**
		(b) Give reasons for non-participation at each stage: **Not applicable**
		(c) Consider use of a flow diagram: **Not required as a figure in the result section explained the time-intervals when the participants were observed for the outcome**
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders: **Provided**
		(b) Indicate number of participants with missing data for each variable of interest: **Not applicable**
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount): **Summarized**
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time: **Reported**
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included: **Clear explanation of the estimates are provided**
		(*b*) Report category boundaries when continuous variables were categorized: **Not applicable**
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period: **Not relevant**
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses: **Not performed and therefore not reported**
Discussion		
Key results	18	Summarise key results with reference to study objectives: **Summary of key findings are presented in the Discussion section**
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias: **Limitations discussed in the last paragraph of the Discussion section**
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence: **Provided in the Discussion section**
Generalisability	21	Discuss the generalisability (external validity) of the study results: **Discussed in the paper**
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based: **Provided after the Discussion section just before the list of references**

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies: **Not applicable**

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.