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# PON1 status does not influence cholinesterase activity in Egyptian agricultural workers exposed to chlorpyrifos

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ABSTRACT

Animal studies have shown that paraoxonase 1 (PON1) genotype can influence susceptibility to the organophosphorus pesticide chlorpyrifos (CPF). However, Monte Carlo analysis suggests that PON1 genotype may not affect CPF-related toxicity at low exposure conditions in humans. The current study sought to determine the influence of PON1 genotype on the activity of blood cholinesterase as well as the effect of CPF exposure on serum PON1 in workers occupationally exposed to CPF. Saliva, blood and urine were collected from agricultural workers ($n = 120$) from Egypt's Menoufia Governorate to determine PON1 genotype, blood cholinesterase activity, serum PON1 activity towards chlorpyrifos-oxon (CPOase) and paraoxon (POase), and urinary levels of the CPF metabolite 3,5,6-trichloro-2-pyridinol (TCPy). The PON1 55 ($P \leq 0.05$) but not the PON1 192 genotype had a significant effect on CPOase activity. However, both the PON1 55 ($P \leq 0.05$) and PON1 192 ($P \leq 0.001$) genotypes had a significant effect on POase activity. Workers had significantly inhibited AChE and BuChE after CPF application; however, neither CPOase activity nor POase activity was associated with ChE depression when adjusted for CPF exposure (as determined by urinary TCPy levels) and stratified by PON1 genotype. CPOase and POase activity were also generally unaffected by CPF exposure although there were alterations in activity within specific genotype groups. Together, these results suggest that workers retained the capacity to detoxify chlorpyrifos-oxon under the exposure conditions experienced by this study population regardless of PON1 genotype and activity and that effects of CPF exposure on PON1 activity are minimal.



## Introduction

Organophosphorus pesticides (OPs) continue to be a human health concern due to their worldwide use and documented human exposures (Alexander et al., 2006; Farahat et al., 2010, 2011; Garabrant et al., 2009). Human and animal studies consistently identify neurotoxicity as the primary endpoint of concern (Bushnell and Moser, 2006; Costa, 2006). Determining the neurotoxic risks associated with occupational exposure to OPs requires an understanding of not only worker exposure levels, but also genetic susceptibility factors. With regard to the latter, genetic polymorphisms in enzymes that metabolize OPs are widely posited to influence susceptibility to OP toxicity. Phosphorothioate OPs undergo cytochrome P-450 (CYP) mediated metabolism to form an active, highly toxic, oxon intermediate metabolite (Ma and Chambers, 1994) which is the metabolite primarily responsible for the inhibition of not only AChE, but also other B-esterases such as butyrylcholinesterase (BuChE) and carboxylesterase (CE) (Sultatos, 1994). Detoxification of the active oxon metabolite primarily occurs by the A-esterase paraoxonase 1 (PON1) (Pond et al., 1998; Sultatos and Murphy, 1983), an enzyme expressed mainly in the liver and secreted into the blood. It has been proposed that inter-individual differences in PON1 can influence the rate of detoxification of OPs, resulting in differences in susceptibility to OP toxicity (Costa et al., 1999; Furlong et al., 2010).

Two common coding region polymorphisms in PON1 have been identified: (1) a leucine/methionine amino acid polymorphism at position 55 (PON1 L55M); and (2) a glutamine/arginine amino acid polymorphisms at position 192 (PON1 Q192R) (Adkins et al., 1993). The PON1 55 polymorphism affects PON1 mRNA (Leviev et al., 1997) resulting in lower PON1 serum protein concentrations in individuals with the M allele compared to the L allele (Garin et al., 1997). The

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PON1 192 polymorphism is functional, affecting PON1 enzyme activity towards OPs in a substrate specific manner (Davies et al., 1996). *In vitro*, the PON1 192R isoform hydrolyzes paraoxon and chlorpyrifos-oxon faster than the PON1 192Q isoform; in contrast, PON1 192R and 192Q hydrolyze diazoxon at similar rates (Furlong et al., 2005; Li et al., 2000). Similarly, *in vivo* studies demonstrate that intraperitoneal injection of PON1 192R or PON1 192Q into PON1 knockout mice confers a similar degree of protection against diazoxon intoxication, whereas PON1 192R provides better protection against chlorpyrifos-oxon than PON1 192Q (Li et al., 2000).

These observations have led to the proposal that PON1 status, which is determined by the amount of PON1 protein present (influenced by PON1 55 genotype) and the activity of the enzyme (influenced by PON1 192 genotype), impacts individual susceptibility to OP toxicity (Furlong et al., 2010; Hofmann et al., 2009; Li et al., 2000). The relationship between PON1 genotype and symptoms associated with chronic OP toxicity has been investigated in workers in the United Kingdom exposed to sheep dip containing primarily diazinon (Cherry et al., 2002; Mackness et al., 2003; Povey et al., 2005), farmers in India (Prabhavathy Das and Jamil, 2009), greenhouse workers in Spain (Hernandez et al., 2003) and South African workers exposed to pesticides (Lee et al., 2003). Collectively, these studies present conflicting results regarding an association between PON1 genotype and worker health and in those studies that did find an association, there are discrepancies as to which genotype is more sensitive to OP exposure. While these studies fail to provide a consensus view on the value of PON1 status as a biomarker of susceptibility, it is difficult to interpret what this means since OP exposures were determined largely by job classification and OP toxicity was based on symptoms associated with but not unique to chronic OP toxicity. However, two recent studies (Albers et al., 2010; Hofmann et al., 2009) that employed a more specific biomarker of OP effect, blood cholinesterase activity, to address the question of whether PON1 is a biomarker of susceptibility to OP neurotoxicity yielded conflicting conclusions as well. The Hofmann et al. (2009) study of agricultural pesticide applicators reported an inverse association between PON1 activity and butyrylcholinesterase (BuChE) activity; whereas the Albers et al. (2010) study of chlorpyrifos manufacturing workers failed to find an association between PON1 activity and either BuChE or acetylcholinesterase (AChE) activity. The discrepancy between these two studies may reflect differences in the OP exposure history between the two study populations, but the limited exposure data available from the Hofmann et al. (2009) study precludes rigorous assessment of this possibility.

We have been conducting a very detailed exposure assessment of the Ministry of Agriculture workers which apply pesticides in the cotton fields in Egypt's Menoufia Governorate. Data collected during the summer of 2007 (Farahat et al., 2010) and 2008 (Farahat et al., 2011) demonstrate significant exposures to the OP, chlorpyrifos (CPF), in this occupational cohort. In this study, we report data collected from a larger cohort ($n = 120$) recruited in 2009. Urinary 3,5,6-trichloro-2-pyridinol (TCPy) levels were measured as a CPF-specific biomarker of exposure; plasma BuChE and red blood cell (RBC) AChE activities were measured as biomarkers of effect; and PON1 genotype (both Q192R and L55M polymorphisms) and phenotype were investigated as potential biomarkers of susceptibility. Samples were collected prior to, during and after a cycle of daily CPF applications over 15 days, allowing us to not only rigorously test the controversial hypothesis that PON1 status influences human susceptibility to OP neurotoxicity in an occupational cohort with clearly defined exposures to a single OP, but to also test the novel hypothesis that repeated CPF exposure modulates PON1 activity.

## Materials and methods

*Study setting and population.* The study setting has been previously described (Farahat et al., 2010). In brief, the study took place in Menoufia, one of 29 governorates in Egypt, which is situated in the Nile River delta north of Cairo. The Ministry of Agriculture controls and oversees the use of pesticides and application procedures in cotton fields throughout Egypt. Pesticide application is performed by teams of workers employed by the Ministry of Agriculture consisting of applicators who apply CPF to the cotton field using backpack sprayers, technicians who direct the direction of spray and the rate at which applicators walk through the field, and engineers who oversee pesticide mixing and application to crops, often from the perimeter of the field. It should be noted that none of these workers used personal protective equipment to minimize dermal exposures. The current study took place during the summer of 2009 and included the first cycle of CPF application. CPF was the only OP used during this time period. All participants in the study were male Egyptians, indigenous to the Nile delta region, with the vast majority being born, raised and residing in Menoufia, Egypt at the time of the study. Participants were between 14 and 69 years of age and had an average body mass index of 26.9. Because certain disease states can adversely influence the metabolism and excretion of TCPy, all workers were questioned about prior diagnosis of diabetes mellitus and liver or kidney disease by a physician during the recruitment process. However, no exclusions for medical conditions were necessary.

Participants were asked to provide a urine and blood sample at two different time points to monitor individuals for CPF exposure and cholinesterase effects. The first sample was collected at baseline two to seven days prior to the period of daily CPF application. The second sample was collected post-exposure, one to two days after a period of up to 15 consecutive days of CPF application. For each time period, urine and blood samples were collected on the same day for an individual and all participant samples were collected within five days of each other. A total of 120 participants (96% agricultural workers) donated saliva at enrollment and were included in the present study. Of these 120 participants, cholinesterase activity was determined for 100 participants at baseline and 97 participants following CPF application. PON1 activity was determined for 67 participants who met the following criteria: (1) the individual provided a saliva sample for PON1 genotyping; (2) the individual provided a plasma sample at baseline and post CPF application; (3) the plasma sample was in good condition (i.e., remained frozen and had little/no hemolysis); and (4) all samples were clearly labeled. The protocol and consent forms used in this research were approved by the Oregon Health and Science University (USA) and Menoufia University (Egypt) Institutional Review Boards. Subjects gave written informed consent prior to enrollment in the study.

*Urine collection and TCPy analysis.* A single spot urine specimen was collected at the beginning of a given work day (2 pm) for assessing TCPy as a biomarker of exposure, since previous studies conducted in similar groups of Egyptian agriculture workers found that daily urinary TCPy levels for a given worker were very similar in specimens collected at the beginning (2–3 pm) and end (7–8 pm) of a given work day (Farahat et al., 2010, 2011). After collection, samples were placed on wet ice for transport to Menoufia University (Shebin El-Kom, Egypt). Urine samples were subsequently aliquotted and stored at −20 °C until shipped to the University at Buffalo (Buffalo, NY) on dry ice for analysis. All urine samples were in good condition (i.e., remained frozen) upon arrival to the University at Buffalo (Buffalo, NY). Urine samples were analyzed for TCPy (the primary metabolite of CPF) by negative-ion chemical ionization gas chromatography–mass spectrometry, with $^{13}C$-$^{15}N$-3,5,6-TCPy as an internal standard, as described previously (Farahat et al., 2010). Creatinine concentrations were measured using the Jaffe reaction (Fabiny and Ertingshausen, 1971). Urinary TCPy concentrations were expressed as μg TCPy/g creatinine. The within-run imprecision for TCPy analysis was very low as shown by a <2% coefficient of variation and an intraclass correlation coefficient between analytical replicates of 0.997 (Farahat et al., 2011).

*Blood collection and analysis of BuChE and AChE activity.* Blood samples were collected by venipuncture into 10 ml lavender top (15% $K_3$

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