The Role of Early Life Pesticide Exposure, Genetic Susceptibility, and Thyroid Function in Childhood Neurodevelopment

by

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DEDICATION

To my family, who never told me what I couldn’t do but always what I could do.

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U.S.	United States
ADHD	Attention Deficit Hyperactivity Disorder
TCPY	3, 5, 6-trichloro-2-pyridinol
CPF	Chlorpyrifos
OP	Organophosphate
ACHE	Acetylcholinesterase
PON1	Paraoxonase
PDD	Pervasive Developmental Disorder
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders
DSM-IV-TR	Diagnostic and Statistical Manual of Mental Disorders-Text Revised
ICC	Interclass Correlation
TH	Thyroid Hormones
TSH	Thyroid-stimulating hormone
T4	Thyroxin
FT3	Free Triiodothyronine
T3	Triiodothyronine
NHANES	National Health and Nutrition Examination Surveys
LOD	Limit of Detection

ABSTRACT

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Globally, organophosphate (OP) pesticide usage and exposure is widespread. Studies have found that fetuses and infants are more sensitive than adults to environmental toxicants and that prenatal exposure to low levels of OPs has been associated with an Attention Deficit Hyperactivity Disorder-like phenotype (ADHD-LP). ADHD is the most commonly studied and diagnosed cognitive and behavioral disorder in school-age children. The etiology of ADHD is unclear, but genetic and environmental factors, such as pesticide exposure, have been hypothesized. Numerous animal studies have demonstrated that *in utero* exposure to OP pesticides adversely affects neurodevelopment, but human studies remain limited. Using a prospective cohort study design, this research investigates the relationship between *in utero* exposure to chlorpyrifos and ADHD-LP in low to middle income, school-age, children from a Mexican birth cohort. Maternal, third trimester, second-morning void urine samples were

analyzed for 3, 5, 6-trichloro-2-pyridinol (TCPY), a urinary metabolite of chlorpyrifos and chlorpyrifos-methyl. Maternal and child Paraoxonase 1 (PON1) polymorphisms, $PON1_{G192R}$ and $PON1_{L55M}$ were analyzed to explore their relationship with ADHD-LP and as a modifying factor in the relationship between chlorpyrifos (or chlorpyrifos-methyl) exposure and ADHD-LP. We assessed ADHD-LP for children 6-13 years old using subscales of Conners' Parental Rating Scales-Revised, Conners' Continuous Performance Test, and the parental scores for Behavior Assessment System for Children-2. Lastly, we assessed whether urinary concentrations of TCPY were associated with circulating thyroid hormone (TH) levels as a potential non-cholinergic mechanism of action of OP neurotoxicity using publicly available data from the National Health and Nutrition Examination Survey (NHANES). Results of this work suggest fetal exposure to chlorpyrifos, chlorpyrifos-methyl, or TCPY and maternal PON1 polymorphisms may influence the display of ADHD characteristics in childhood. We also found that urinary TCPY concentrations altered THs. Considering the continued widespread agricultural and possible residential use of chlorpyrifos and chlorpyrifos-methyl in Mexico, this research has added to the current knowledge on environmental predictors of child neurodevelopment and behavior, and provided additional insights into proposed biological mechanisms, susceptibility factors, and biomarker utility.

Chapter I

Introduction

Attention-Deficit Hyperactivity Disorder

Attention-deficit hyperactivity disorder (ADHD) is the most commonly diagnosed and studied cognitive and behavioral disorder among school-age children (Banerjee et al., 2007; Rowland et al., 2002). In the United States (U.S.), parents reported approximately 9.5% of children aged 4 to 17 years were diagnosed with ADHD as of 2007 (CDC, 2010) which reportedly has an annual societal economic impact between $12,005-17,458 per individual (Pelham et al., 2007). Yearly, the societal cost of illness, which focuses on tangible resource consequences (i.e. costs associated with education of ADHD-diagnosed individuals and impact on peers/school personnel, quality of life of parents and children, ADHD treatment/health care, etc.), for ADHD in children and adolescents is estimated to range between $36-52.5 billion (based on ADHD prevalence estimates between 2-9%) (Pelham et al., 2007).

ADHD is primarily characterized by a persistent pattern of inattentiveness, hyperactivity, and impulsiveness that is more severe than those at comparable age and developmental stages (Diagnostic and Statistical Manual of Mental Disorders-IV-Text Revised (4th ed., text rev.); *DSM–IV–TR*; American Psychiatric Association, 2000). Since there is no single core symptom associated with ADHD, different symptoms of ADHD

present differently in each diagnosed person and have been categorized into the following subtypes: ADHD-Inattentive Type, ADHD-Hyperactivity Type, and ADHD-Combined. Those diagnosed with ADHD-Inattentive or ADHD-Hyperactivity Types display six or more symptoms from their respective subtypes but fewer symptoms from the other subtype for at least six months. ADHD-Combined Type diagnosed display six or more symptoms of inattention and hyperactivity/impulsivity for at least six months (4th ed., text rev.; *DSM–IV–TR*; American Psychiatric Association, 2000). Notable symptoms of inattention include: often fails to pay close attention to details or makes careless mistakes in schoolwork, often has difficulty sustaining attention in tasks, does not seem to listen when spoken to directly, and is often easily distracted by extraneous stimuli (4th ed., text rev.; *DSM–IV–TR*; American Psychiatric Association, 2000). Notable symptoms of hyperactivity/impulsivity includes: fidgeting with hands or feet, runs about or climbs excessively in inappropriate situations, often talks excessively, and has difficulty awaiting turn (4th ed., text rev.; *DSM–IV–TR*; American Psychiatric Association, 2000). The DSM-IV also states that characteristics of ADHD must present before the age of 7 and must occur in at least two settings (i.e. home, school, or other). Development of appropriate social, academic, or occupational functioning must be clearly affected and symptoms must not occur as a result of other mental disorders such as Pervasive Developmental Disorder (PDD), Schizophrenia, or other Psychotic Disorders (4th ed., text rev.; *DSM–IV–TR*; American Psychiatric Association, 2000). However, ADHD does not often present alone but as a co-morbidity with other conditions (Rowland et al., 2002). It has been found that some disorders mimic ADHD, such as

learning, anxiety, and conduct disorders, and causes in some cases false diagnosis (Rowland et al., 2002).

Increasing rates of ADHD diagnosis are of public health concern. In the U.S., between years 1997 and 2008, ADHD diagnosis prevalence increased 6.69% in children aged 3-17 years with non-Hispanic whites and non-Hispanic blacks having significantly higher prevalence than Hispanics in the U.S. (Boyle et al., 2011). Also, studies have shown that boys are greater than two times more likely to be diagnosed with ADHD (deHaas et al., 1986; CDC, 2010; Boyle et al., 2011). This occurrence may be due to the presentation of the disorder as girls typically exhibit less impulsivity symptoms and may be less likely to come to clinical attention (Boyle et al., 2011). Girls and racial minority children are less likely to receive ADHD treatment than boys and Caucasian children, respectively (Bussing et al., 2003). Other factors affecting ADHD treatment include having regular pediatric visits and socioeconomic status (Bussing et al., 2003). The likelihood of parents recognizing problems in school-aged children that are at high risk for ADHD is relatively high at 88% (Bussing et al., 2003). Diagnosis and treatment are important because untreated children are at increased risk for alcohol and drug abuse, criminal behavior, self-inflicted injury, and other social problems as adults (Monastra et al., 2005).

The etiology of ADHD is not clearly understood, but genetics has been strongly linked and some studies have reported risk factors such as low social class, marital distress, small head circumference, low birth weight, and preterm birth (Rowland et al., 2002; Berkowitz et al., 2004). Several other environmental factors have also been

proposed as risk factors for ADHD, including lead, alcohol exposure, maternal smoking during pregnancy, and organophosphate (OP) pesticide exposure (Rowland et al., 2002; Eskenazi et al., 2007; Marks et al., 2010; Bouchard et al., 2010). Many of the environmental risk factors for ADHD occur early in development, which is consistent with the idea that ADHD is a neurodevelopmental condition (Rowland et al., 2002). The fetus' brain is vulnerable to adverse impacts from some environmental toxicants during its developmental process (Woodruff et al., 2004). *In utero* exposure to OPs has been found to contribute to the development of ADHD and like behaviors in children (Eskenazi et al., 2007; Marks et al., 2010; Bouchard et al., 2010). However, these studies have either not used a specific pesticide biomarker of exposure, (for example, urinary 3, 5, 6-trichloro-2-pyridinol (TCPY) as a measure of chlorpyrifos exposure), or have not directly assessed measures of ADHD in school-age children. Also, these studies have been conducted in U.S. populations where exposure sources and concentrations may be different than a population from a different country/region.

Chlorpyrifos Exposure

In the United States (U.S.), a billion pounds or more of conventional pesticides are used annually with 85% of households storing at least one pesticide in their homes (Bradman et al., 2005; Adgate et al., 2001; EPA, 2009). Worldwide, chlorpyrifos, a non-persistent broad-spectrum insecticide, is the most widely used OP pesticide (Ye et al., 2008;Bradman et al., 2005; Timchalk et al., 2007) and is currently registered for use in 100 countries including Mexico (Dow, 2013). It has been used in both residential and

agricultural settings as an insecticide that inhibits the widespread growth and proliferation of insects such as cockroaches, termites, grasshoppers, maggots, and other pests (Timchalk et al., 2007; Dow, 2013). Chlorpyrifos is used on the following crops: alfalfa, citrus, cotton, grapes, soybeans, pome fruits, and wheat (Dow, 2013). OPs, including chlorpyrifos, are widely used in Mexico for animal husbandry, alfalfa, and crops for animal feed production (Salas et al., 2003). Environmental human exposure is widespread and occurs through inhalation of vapors or aerosols from spray drift, ingestion of residuals on food and house dust/soil, and dermal absorption following skin contact (Panuwet et al., 2008) and is usually metabolized and excreted within hours and/or days in urine (Bradman et al., 2005).

Chlorpyrifos was cancelled for residential use in the U.S. in December 2000, except for preconstruction termite application, mainly due to suspected effects on neurodevelopment in humans (U.S. EPA, 2002). In February 2001, residential formulation production was stopped, and in December 2001, all retail sales for residential application were terminated (U.S. EPA, 2002). However, its use is not restricted in many other countries, and it is still registered for other uses in the U.S. Thus, the potential for exposure among the general population remains. In countries that engage in non-agricultural uses of chlorpyrifos, inhalation exposure from indoor air likely contributes significantly to total daily exposure concentrations and body burden (Pang et al., 2002). According to some studies, OPs and pyrethroid pesticides are largely applied in residences through high-exposure spray cans and pest bomb applications (Williams et al., 2008). With an indoor half-life of several months, carpet dust is a major

pathway of exposure due to lack of degradation sources such as sunlight, water, and/or soil microorganisms (Pang et al., 2002). Diet is also thought to be a primary route of environmental exposure (Barr et al., 2006).

Maternal exposure likely leads to exposure for fetuses because chlorpyrifos can readily pass through the placenta, amniotic fluid, and blood-brain barrier to potentially cause adverse health effects to the developing fetus, specifically adverse neurodevelopment (Perera et al., 2005). The primary target organ for acute chlorpyrifos toxicity is the central and peripheral nervous systems, due to the ability of the chlorpyrifos-oxon metabolite to inhibit the enzyme activity of acetylcholinesterase (AChE) (Eaton et al., 2008). AChE hydrolyzes acetylcholine (Ach) into acetate and choline, thereby terminating neurotransmission at cholinergic synapses (Eaton et al., 2008). Due to the inhibition of AChE by chlorpyrifos, neurodevelopmental delays or effects have been observed in both animal and human studies (Timchalk et al., 2007). Human and experimental studies also have shown that the fetus and infant are more sensitive than adults to many environmental toxicants including chlorpyrifos (Timchalk et al., 2007).

Unlike classic teratogens cases, in which the greatest sensitivity is seen during the first trimester, the window of vulnerability for organophosphates is likely to extend from the embryonic period into postnatal life and is likely permanent due to non-cholinergic mechanisms (Whitney et al., 1995; Alhbom et al., 1995; Slotkin et al., 1999; Rauh et al., 2006). Exposures during the spurt in brain growth, which occurs during the third trimester in human pregnancies, may be particularly harmful (Rauh et al., 2006).

This leads to the hypothesis that exposure during human pregnancy, particularly during the third trimester when there is rapid development of the fetal central nervous system, could potentially result in adverse effects on the central and peripheral nervous system. Several animal studies have demonstrated that gestational and/or postnatal exposures to chlopyrifos induce neurobehavioral alterations, especially at doses that inhibit AChE activity (De Angelis et al., 2009). As a result, behavioral changes such as hyperactivity and memory errors occur (Eaton et al., 2008).

Additionally, OPs may disrupt brain development through non-cholinergic mechanisms, at doses that cause minimal or no AChE inhibition (Whitney et al., 1995; Alhbom et al., 1995; Dam et al., 1998; Slotkin et al., 1999; Garcia et al., 2003; Rauh et al., 2006). Studies in humans have also shown that *in utero* exposure to OPs, including chlorpyrifos, at low levels are significantly associated with adverse neurodevelopmental outcomes in children such as mental development, IQ, and ADHD (Perera et al., 2005; Eskenaki et al., 2007; Marks et al., 2010; Brouchard et al., 2010; Raul et al., 2012). The actual mechanisms by which low level chlorpyrifos exposure perturbs neural development remain unclear and complicated (Garcia et al., 2003), but *in vitro* studies have identified thyroid hormone disruption as a potential molecular target (Eaton et al., 2008). Nevertheless, it is clear that chlorpyrifos exposure is far more toxic in immature animals than in adults.

Biomarkers of Exposure:

A biomarker of exposure is a xenobiotic substance or its metabolites that is measured in the body and can be related to an environmental exposure (Sly et al., 2009). One advantage of using biomarkers of exposures is that it accounts for all pathways and routes of exposure, which is important for pesticides since exposure is multi-pathway and multi-route. The choice of matrix for biomonitoring depends on pharmacokinetics, access to samples, and analytical capability (Barr et al., 2002). Urine is the most frequently used matrix for human studies of non-persistent pesticides because of the availability of large amounts of sample, relatively higher concentrations of urinary metabolites compared to those of the parent compound in blood (Barr et al., 2002); (Ye et al., 2008), and laboratory methods that are more developed with the ability to measure multiple metabolites (Barr et al., 2006; Bradman et al.,2005; Olsson et al., 2004). Also, urinary biomarkers are typically more stable compared to blood markers (Barr et al., 2006). Diethylphosphate (DEP), diethylthiophosphate (DETP) and 3, 5, 6-trichloro-2-pyridinol (TCPY) are products of metabolism and of environmental degradation of chlorpyrifos and are routinely measured in urine as biomarkers of exposure (Timchalk et al., 2007), usually reflecting variable, low-level exposure (Bradman et al., 2005). Chlorpyrifos undergoes cytochrome-p450 (CYP450) metabolism to form chlorpyrifos-oxon or TCPY and DETP (Timchalk et al., 2007). Then liver enzymes such as paraoxonase (PON1) and tissue B-esterases (cholinesterase) further metabolize chloryprifos-oxon to form TCPY and DEP (Timchalk et al., 2007). TCPY is a specific biomarker of exposure to chlorpyrifos and chlorpyrifos-methyl (or exposure to TCPY

itself resulting from environmental degradation of either of these chemicals), while dialkyphosphates (DAPs), DEP and DETP, are non-specific markers of OP exposure (Barr et al., 2006; Timchalk et al., 2007).

Biomarker of Susceptibility (Gene-Environment Interaction): Paraoxonase (PON1)

Paraoxonase (PON1) is an arylesterase, primarily synthesized in the liver, which metabolizes OP compounds through hydrolysis after the bioactivation of the parent compound by CYP450 (Albers et al., 2010). PON1 hydrolyzes the active metabolites of several OP insecticides including diazoxon, paraoxon, and chlorpyrifos oxon as well as nerve agents such as soman and sarin (Costa et al., 2005). The catalytic efficiency of hydrolysis is one of the most important factors in preventing OP-induced neurotoxicity (Albers et al., 2010). PON1 can detoxify chlorpyrifos oxon before it inhibits AChE in the peripheral and central nervous systems (Berkowitz et al., 2004). PON1 activity in individuals, in terms of metabolism of OP pesticides, is based on quantity of circulating PON1 in their plasma, which can be influenced by amino acids (glutamine or arginine) at position 192 and (methionine or leucine) at position 55 (Brophy et al., 2000; Berkowitz et al., 2004). In animal (mice) studies, PON1 knockout mice were more vulnerable to chlorpyrifos exposure than those with PON1 plasma activity (Furlong et al., 2005). Exposure to levels of chlorpyrifos oxon that produced no symptoms of cholinergic effects and minimal inhibition of brain cholinesterase in knockout mice proved to be lethal (Furlong et al., 2005).

Single nucleotide polymorphisms (SNPs) do occur in human PON1. Those of particular interest for this research affect the coding region that results in amino acid substitutions at Q192R, which is responsible for PON1 activity and hydrolysis of PON1 substrates (Cole et al., 2005). Substitutions at L55M can also impact plasma PON1 protein levels (Leviev et al., 1997) where circulating PON1 is lower in individuals with the M allele compared to the L allele (Garin et al., 1997). However, the PON1 L55M polymorphism does not affect catalytic activity. It has been found that the efficiency of hydrolysis between PON1 Q192 and PON1 R192 dictates sensitivity or protection from chlorpyrifos exposure (Cole et al., 2005). PON1 knockout mice expressing human $PON1_{R192}$ were significantly less sensitive to the acute cholinergic toxicity of chlorpyrifos-oxon than $PON1_{Q192}$ or PON1 knockout mice (wild type) (Costa et al., 2005; Androutsopoulos et al., 2011). The catalytic efficiency for hydrolysis of PON1 polymorphisms is substrate-dependent. Contrary to the observation that R192 is protective for chlorpyrifos exposure, the PON1 Q192 alloform was found to hydrolyze diazoxon, sarin, and soman more rapidly than PON1 R192 *in vitro* (Costa et al., 2005).

The gene frequencies for specific alleles of PON1 genes vary by ethnicity, implying differential susceptibility to pesticides among different ethnic groups and geographical regions (Allebrandt et al. 2002; Brophy et al. 2001). Noticeable differences in haplotype frequency have been observed between ethnic groups in several studies (Chen et al. 2004; Holland et al 2006; D'Amelio et al., 2005; Lopez-Flores et al., 2009; CDC, 2013; Eskenazi et al., 2010). Allele frequencies of the Q192 allele range from about 0.5 for Mexican American populations to about 0.3 for Caucasian populations in the U.S.

(CDC, 2013). Allele frequencies of the M55 allele range from about 0.3 for Mexican Americans to about 0.4 for Caucasian populations (CDC, 2013).

Newborns in general have very low PON1 levels until mature PON1 levels are expressed (Cole et al., 2005), thus maternal PON1 activity can serve as a proxy for fetal sensitivity to OP exposure and it is reasonable to assume that PON1 levels are even lower in fetuses than in newborns. This leads to concern about exposure of the fetuses of mothers with low PON1 status or non-protective genotypes to chlorpyrifos exposure (Furlong et al., 2005). Clinical evidence for the role of PON1 in OP toxicity supports the implication of this enzyme in pesticide-associated disease (Androutsopoulos et al., 2011). In human studies examining the effect modification of PON1 levels and/or genotypes on the relationship between *in utero* OP exposure and disease outcomes, investigators have reported significantly decreased head circumference (Berkowitz et al., 2004), decreased MDI scores in 12 month olds with mothers with $PON1_{Q192}$ genotype (Engel et al., 2011), and decrements in perceptual reasoning in early childhood (Engel et al., 2011). Trends have also been found between increased OP exposure and decreased neurodevelopment within those with $PON1_{192Q}$ genotypes (Engel et al., 2011).

Thyroid Function as a Potential Non-Cholinergic Mechanism for Chlorpyrifos Neurotoxicity

Thyroid hormones (THs) are involved in numerous physiological processes as regulators of metabolism, bone remodeling, cardiac function, and mental status (Boas et al., 2012). Thyroid hormones are also especially important for normal brain

development in animals and humans and even slight perturbations in hormone levels can cause deleterious impairments (Porterfield et al., 2000). The absence of thyroid hormones reduces neuronal growth and differentiation in the cerebral cortex, hippocampus, and cerebellum (Auso et al., 2004; Lavado-Autric et al., 203; Nicholoson and Altman, 1972; Boas et al., 2012). Studies have demonstrated that maternal thyroid hormones (TH) are important for fetal brain development during the period before the fetus is able to produce its own THs. TH synthesis begins at 10 – 12 weeks gestation and the hypothalamic-pituitary-thyroid axis is functional by the latter half of gestation (Porterfield et al., 2000; Porterfield et al., 1993). Exposure to certain chemicals could potentially affect maternal and/or fetal TH levels and, subsequently, affect cognitive functions of offspring (Ghisari et al., 2005; Porterfield et al., 2000; Zoeller et al., 2007).

Environmental exposures to chemicals have been found to alter thyroid hormone levels (Blount et al., 2006; De Angelis et al., 2009; Lacasana et al., 2010; Fortenberry et al., 2012) even at low-levels of exposure. Environmental chemicals may interfere in thyroid signaling through different mechanisms of action, e.g. at the receptor level, in binding to transport proteins, in cellular uptake mechanisms, or in modifying the metabolism of thyroid hormones (Boas et al., 2009; Lacasana et al., 2010), which is especially important in pregnant women (Boas et al., 2009). A few animal and human models suggest that chlorpyrifos may target the thyroid gland (DeAngelis et al., 2009; Ghisari et al., 2005) and in turn may influence neurological development and function causing lower IQ, learning disabilities, and ADHD in children (Porterfield et al., 2000).

Summary

Because increasing rates of ADHD diagnosis are of public health concern and exposure to chlorpyrifos has been implicated as a risk factor for characteristics of ADHD and ADHD diagnosis, this research adds to the current knowledge about environmental predictors of child neurodevelopment and behavior. With chlorpyrifos exposure being widespread, globally, this study examines the distribution and temporal variability of the specific biomarker of exposure, urinary TCPY, in a Mexican population. As stated previously, TCPY is a specific metabolite and environmental degradant of chlorpyrifos (and chlorpyrifos-methyl) and are routinely measured in urine as biomarkers of exposure (Timchalk et al., 2007). This study also explores the relationship between urinary TCPY measured among mothers during the third trimester and ADHD and related outcomes among their children between ages 6 to 13 years.

Single nucleotide polymorphisms (SNPs) occur in human PON1, which has a role in metabolizing OPs. Polymorphisms in the PON1 coding region, $PON1_{R192Q}$ and $PON1_{L55M}$, have been found to impact circulating PON1 activity in humans and sensitivity to adverse effects from chlorpyrifos exposure in animals. In this study, gene-environment interactions are examined to assess PON1 genotypes as biomarkers of OP exposure susceptibility. The relationship of PON1 genotypes with characteristics of ADHD is examined. Additionally, it has been found that thyroid hormones are important for normal brain development in animals and humans and even slight changes in hormone levels can cause impairments (Porterfield et al., 2000). Thus, another goal of this project is to provide additional insight on the influence of chlorpyrifos exposure on

thyroid function, which is a proposed non-cholinergic mechanism of action of chlorpyrifos neurotoxicity.

This doctoral research project utilizes data and archived samples from a prospective birth cohort, Early Life Exposures in Mexico to Environmental Toxicants (ELEMENT), based in Mexico City, Mexico through the collaboration of investigators at the University of Toronto, University of Michigan, Harvard University, and National Institute of Public Health in Mexico. Participants were low-to-moderate income, pregnant women from three sequentially enrolled, prospective birth cohorts during: 1994-1997 (cohort 1), 1997-2000 (cohort 2), and 2001-2005 (cohort 3). This collaboration has primarily focused on the effect of early life exposure to heavy metals on developmental and birth outcomes including neurodevelopment, preterm birth, and birth anthropometry. Thus, this study represents a new direction in the exposure and health endpoints of focus, and additionally considers potential individual susceptibility factors. This project also utilizes the U.S. National Health and Nutrition Examination Survey (NHANES), which is a cross-sectional study designed to be representative of the civilian, non-institutionalized population of the United States.

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Chapter II

Urinary 3, 5, 6-trichloro-2-pyridinol (TCPY) in pregnant women from Mexico City: distribution, temporal variability, and relationship with child attention and hyperactivity

Abstract

Attention Deficit Hyperactivity Disorder (ADHD) is the most commonly diagnosed and studied cognitive and behavioral disorder in school-age children. The etiology of ADHD and ADHD-Like Phenotypes (LP) is unclear, but genetic and environmental factors, such as pesticides, have been hypothesized. The objective of this study was to investigate the relationship between *in utero* exposure to chlorpyrifos, chlorpyrifos-methyl, and/or 3, 5, 6-trichloro-2-pyridinol (TCPY) and ADHD-LP in school-age Mexican children. The temporal reliability of repeated maternal urinary TCPY concentrations across trimesters was also explored. From a prospective birth cohort, third trimester urinary TCPY concentrations in 187 mother-child pairs were measured. Child neurodevelopment in children 6-11 years of age was assessed using Conners' Parental Rating Scales-Revised (CRS-R), Conners' Continuous Performance Test (CPT), and Behavior Assessment System for Children-2 (BASC-2). Multivariable linear regression models were used to test relationships for all children combined and also stratified by sex. Intraclass correlation coefficients (ICC) calculations were based on a random effects model. The ICC was 0.41 for uncorrected TCPY, and ranged from 0.29 to 0.32 for specific gravity-corrected TCPY.

We did not observe any statistically significant associations between tertiles of maternal TCPY concentrations and ADHD-LP in children. However, compared to the lowest tertile we found suggestive evidence for increased ADHD index in the highest TCPY tertile in boys (β= 5.55 points; 95% CI(-0.19, 11.3); p=0.06) and increased attention problems for the middle tertile in girls (β=5.81 points; 95% CI(-0.75, 12.4); p=0.08). Considering the continued widespread agricultural and possible residential use of chlorpyrifos and chlorpyrifos-methyl in Mexico and the educational implications of cognitive and behavior deficits, these relationships deserve further study. [1]

Introduction

[1] Fortenberry, G.Z., Meeker, J.D., Sánchez, B. N., Barr, D. B., Panuwet, P., Bellinger, D., Schnaas,

Attention Deficit Hyperactivity Disorder (ADHD) is the most commonly diagnosed and studied cognitive and behavioral disorder in school-age children with parents reporting 9.5% or 5.4 million children 4-17 years of age ever being diagnosed (CDC, 2011; Banerjee et al 2007; Rowland et al 2002). ADHD is characterized by a persistent pattern of inattention and/or hyperactivity-impulsivity that is more severe than is typically observed in individuals at comparable levels of development (CDC, 2011). Boys are about three times more likely than girls to be diagnosed with ADHD (CDC, 2011). The etiology of ADHD and ADHD-Like Phenotype, specifically inattention and hyperactivity, remains unclear, but genetic and environmental factors, including pesticides, have been hypothesized (Braun et al., 2006; Ernst et al., 2001; Garcia et al., 2003). Studies have shown that organophosphate (OP) exposures at low levels are associated with ADHD and ADHD-related symptoms (Rauh et al., 2006; Bouchard et al 2010; Marks et al., 2010).

Globally, over $30 billion is spent annually on pesticides with one third spent in the developing world (Handal et al., 2008; Karlsson et al., 2004). Chlorpyrifos (CPF), in particular, is the most widely used OP pesticide worldwide (Bradman et al., 2005; Timchalk et al., 2007; Ye et al., 2008). According to the most recent data available, approximately 10 million pounds of CPF are used annually in the U.S. (U.S. EPA, 2011) on crops and sprayed aerially to kill mosquitoes, mites, and termites (CDC, 2012). Human exposure is widespread and occurs through inhalation of vapors and aerosols from spray drift (Pang et al., 2002; Whyatt et al., 2009), ingestion of residuals on food and house dust/soil (Pang et al., 2002; Salas et al., 2003), and dermal absorption following skin

contact (Panuwet et al., 2008) and is usually excreted within hours or days in urine (Bradman et al., 2005; Timchalk et al., 2007).

Indoor residential use of CPF was banned in the U.S. in 2001, and pre- and post-construction applications for termite control was phased out by 2005 (U.S. EPA, 2002) due to suspected effects on neurodevelopment in humans. However, it is still registered for agricultural use in the U.S. and all-purpose use in over 100 countries, including Mexico (Dow, 2012). In fact, CPF was one of the more highly used OPs in Mexico in 2000 (Salas et al., 2003). In Mexico, residuals of CPF have been found in raw and commercial pasteurized milk due to its use on crops destined for animal feed such as alfalfa, sorghum, soy, and maize (Salas et al., 2003). However, data on non-occupational human exposure to CPF in Mexico is lacking.

Human and experimental studies have found that the fetus and infant are more sensitive than adults to many environmental toxicants, including CPF (Timchalk et al., 2007) and that a mother's exposure is a potential source of fetal exposure (Berkowitz et al., 2004). There is evidence that CPF readily passes through the blood-brain barrier, placenta, and amniotic fluid to potentially cause adverse health effects to developing fetuses, specifically, adverse neurodevelopment (Bradman et al., 2003; Whyatt et al., 2005; Perera et al., 2005). Studies have shown children prenatally exposed to OPs display adverse neurodevelopment, including: IQ, mental development, psychomotor development, and attention problems (Engel et al., 2011; Eskenazi et al., 2007; Bouchard et al., 2010; Marks et al., 2010). A study of 3-year old children in New York City found children with high prenatal exposure to CPF, assessed using cord blood, also

showed problems with attention, attention-deficit/hyperactivity disorder, and pervasive developmental disorder (Rauh et al., 2006; Rauh et al., 2011). Also in the New York City study, significant morphological abnormalities in the cerebral surface of the brain that affects cognitive and behavioral processes, notably, attention and inhibitory control, was found in children prenatally exposed to higher levels of CPF compared to children with lower exposures (Rauh et al., 2012).

Several animal studies have demonstrated that gestational and/or postnatal exposures to CPF induce neurobehavioral alterations, even at doses that do not elicit measurable cholinergic responses (Dam et al., 2000; De Angelis et al., 2009; Garcia et al., 2005; Levin et al., 2001; Venerosi et al., 2009). As a result, behavioral changes, such as hyperactivity and memory errors, occur (Eaton et al., 2008) and impaired cognitive and behavioral functions may continue into adolescence (Icenogle et al., 2004).

The present study was conducted to explore the presence of urinary concentrations of 3, 5, 6-trichloro-2-pyridinol (TCPY; a metabolite of CPF and chlorpyrifos-methyl) in a study of pregnant women in Mexico City, and to explore relationships between urinary TCPY and measures of child neurodevelopment, specifically Attention Deficit Hyperactivity Disorder-Like Phenotypes (ADHD-LP). We also explored the temporal reliability of repeated urinary TCPY concentrations across trimesters among a subset of the women. The degree of temporal variability and reliability in an exposure measure has important implications for epidemiology study design and interpretation, especially for chemicals such as CPF and chlorpyrifos-methyl, which are rapidly metabolized.

Methods

Study Population

Participants in the present study were from three sequentially enrolled, prospective birth cohorts conducted in Mexico City, Mexico during: 1994-1997 (cohort 1), 1997-2000 (cohort 2), and 2001-2005 (cohort 3). All three cohorts enrolled homogenous, low-to-moderate income, pregnant women recruited from the National Institute of Perinatology, Hospital General Dr. Manuel Gea Gonzalez, or clinics affiliated with the Mexican Social Security Institute (Braun et al., 2012). Mother-child pairs from the three cohorts (N=827) were re-invited between 2007-2011 to examine childhood and adolescent neurodevelopmental characteristics. Exclusion characteristics for all cohorts included: plans to leave the area within the next 5 years; daily consumption of alcoholic beverages; addiction to illegal drugs; continuous use of prescription drugs; diagnosis of multiple pregnancy, pre-eclampsia, renal or heart disease, gestational diabetes; a history of infertility, diabetes, or psychosis; diagnosis of high risk pregnancy; or suffering from seizures requiring medical treatment (Hu et al., 2006). Specific objectives of each cohort have been previously published (Hernandez-Avila et al., 2003; Tellez-Rojo et al., 2004; Ettinger et al., 2009). Exposure, outcome, and demographic characteristics were collected from all eligible participants by the same group of investigators and field staff. Participants were informed of the study, associated aims, and uses of biological samples/data and written consent was obtained before enrollment. The Institutional Review Boards of the National Institute of Public Health

(Mexico), Harvard School of Public Health, University of Michigan, and participating hospitals approved all study materials and procedures.

Participants provided second morning void urine samples at each trimester of pregnancy. In this study, we utilized third trimester urine samples from mother-child pairs that had completed psychometric assessments for children (N=187) from cohorts 2 and 3. In a subset of women from both cohorts 2 and 3 (N=21), we measured urinary TCPY in samples collected during all three trimesters of pregnancy.

Psychometric Instruments

We used three psychometric assessments to evaluate behavioral characteristics of children 6 – 11 years of age: Conners' Parental Rating Scales-Revised (CPRS-R), Behavior Assessment System for Children – Parental Rating Scales (BASC-PRS), and Conners' Continuous Performance Test (CPT). Scores from these psychometric instruments assess ADHD-LP and are not designed as diagnostic tools, but rather for screening. The instructions and prompts were translated into Spanish by a researcher in our group who also trained and supervised the personnel who administered the assessments. Standardization and quality control checks were conducted by reviews of videotaped evaluations.

Conners' Parental Rating Scales-Revised (CPRS-R)

CPRS-R is a 27-question assessment tool for parents that are used to determine a child's behavior for children and adolescents 3-17 years (Conner, et al. 1998). Most questions are based on behavioral characteristics that are described in the Diagnostic

and Statistical Manual of Mental Disorders-IV (DSM-IV) diagnostic guidelines for ADHD (American Psychiatric Association, 2000; Deb et al., 2008). In this study, we used the following subscales: ADHD Index, DSM-IV Hyperactivity/Impulsivity, DSM-IV Inattention, DSM-IV Total, and Global Restless/Impulsivity Index. For these scales, the higher the score typically indicates an elevated level of concern with a score of 40-59 considered average and <40 displaying even fewer concerns. The ADHD index identifies children/adolescents that are at risk for ADHD while Global Restless/Impulsivity Index indicates tendencies toward hyperactivity as well as inattention. DSM-IV subscales yield scores between 0-9; scores of 6 and over suggest possible DSM-IV diagnosis (Conner Profile, 2012). DSM-IV Hyperactivity/Impulsivity and DSM-IV Inattention correspond to a diagnostic type of ADHD. DSM-IV Total represents the diagnostic criteria for the combined type of ADHD.

Behavior Assessment System for Children (BASC) – Parental Rating Scales (PRS)

BASC-PRS is used to measure adaptive and problem child behaviors in the community and home setting (Pearson Assessment, 2012). PRS for 6-11 year olds are used to assess attention problems and hyperactivity. For these scales, the higher the score typically indicates an elevated level of concern. Scores of 41-59 were considered average with children displaying typical levels of attention/hyperactivity problems for a child at that age.

Continuous Performance Test (CPT)

The CPT is a 14-minute computer test that measures sustained attention and impulsivity (Wilmshurst et al., 2009) and compares the participant's answers to a

reference group and results in a confidence index of clinical and non-clinical ADHD profiles. CPT has a high sensitivity (83-90%), but is poorer (59-61%) when measured against clinical ADHD diagnosis (Linnet et al, 2003). The clinical index, which measures symptoms in which the participant will have a higher likelihood of ADHD diagnosis, is presented in this article. The CPT also provides a series of error and variability measures s such as omissions, commissions, hit reaction time (hit rt), hit rt standard error (hit rt se), and hit rt block change that provide information on specific characteristics of ADHD. Particularly, hit rt block change is the variability of reaction time for correct responses across blocks or sections of the test and has been indicated as a measure of vigilance or sustained attention (Conners', 2000). Within the CPT, vigilance is a measure of consistency across the test (Conners', 2000).

TCPY concentrations in urine

Maternal urine samples (2 mL) were transported on dry ice to Emory University for analysis of TCPY in urine. Samples were spiked with stable isotopically-labeled TCPY and then subjected to an enzyme hydrolysis. Hydrolysates were extracted using mixed-polarity solid-phase extraction cartridges (CDC, 2006; Olsson et al., 2004). Elutes were concentrated and analyzed using HPLC/tandem MS (Olsson et al., 2004) with both quantification and confirmation ions monitored. Metabolites were quantified using isotope dilution calibration. The LOD was 0.10 ng/mL for TCPY. Values below the LOD were assigned a value of LOD divided by the square root of two. Urinary specific gravity (SG) was determined using a handheld digital refractometer (ATAGO Company Ltd.,

Tokyo, Japan). SG-corrected urinary TCPY concentrations were calculated for use in certain statistical analyses.

Data Analysis

Statistical Analysis Software (SAS) (version 9.2; SAS Institute Inc., Cary, NC, USA) was used for most analyses. Descriptive statistics for demographic information were calculated along with the distribution of TCPY and the psychometric scales. ELEMENT third trimester geometric mean TCPY concentration was compared to the geometric mean among pregnant females aged 18-40 years from the U.S. National Health and Nutrition Examination Survey (NHANES), years 1999-2002, using a two-sample *t*-test.

Bivariate analyses between the dependent variables (psychometric assessments-ADHD Index, DSM IV Hyperactivity/Impulsivity, DSM IV Inattention, DSM IV Total, and Global Restless/Impulsivity Index, BASC attention, BASC hyperactivity, CPT clinical index, and CPT Hit Reaction Time Block Change), the primary independent variable of interest (urinary TCPY), as well as other covariates (maternal IQ, maternal education, socioeconomic status, specific gravity, season, breastfeeding, maternal blood lead, child age at testing, child sex, birth length, and head circumference at birth). Spearman correlations were used for continuous variables and Wilcoxon tests for categorical variables.

The intraclass correlation coefficient (ICC) was used to evaluate temporal variability and reliability of TCPY levels within individuals across all three trimesters of pregnancy among a subset of women. Calculations were based on a random effects

model using PROC Mixed in SAS. ICC represents the reliability of repeated measures over time and is defined as the ratio of between-subject variance to total variance (between-subject plus within-subject) (Meeker et al. 2005). If a measure is highly reliable between time points, the ICC would be near 1.0, whereas a measure with low reliability would have an ICC closer to zero.

Multivariable regression models were created using variables found to be associated with ADHD-LP and/or TCPY in bivariate analyses ($p<0.05$). Variables were also considered from *a priori* suspicion of potential confounders of the association between prenatal CPF/chlorpyrifos-methyl exposure and psychometric outcome. Covariates included: maternal IQ, maternal education, income, maternal urine specific gravity, breastfeeding, maternal blood lead one month after delivery, season of sample collection, child's age at testing, child's sex, birth length, and head circumference at birth. Birth length and head circumference at birth were determined by a nurse at the time of delivery and were used as continuous variables. Maternal IQ was calculated on the basis of the mother's scores on the Information, Comprehension, Similarities, and Block Designs scales of the Spanish Wechsler Adult Intelligence Scale (Téllez-Rojo et al., 2006; Weschler, 1968). A continuous variable was created to capture socioeconomic status and income based on material possessions. Maternal education was the cumulative number of years that the mother attended school. Breastfeeding (yes/no) from a questionnaire administered to the mother during the child's infancy was included in the model and used categorically. Maternal blood lead was measured one-month post-partum and was used continuously. Season was categorized as either rainy

(June – October) or dry (November – May) based on the month of sample collection. Using Generalized Additive Models (GAM) adjusted for previously mentioned variables, we assessed overall model fitness by adding splines to continuous variables and examining the structure of the relationship between the variables and the psychometric outcome.

TCPY was categorized into tertiles for the multivariable models. This decision was based on the ICC analysis and the observation in our previous work that found that classifying TCPY concentrations into broad categories might be more robust to temporal within-person variability and measurement error (Meeker et al. 2005). Models were explored for all children combined and also stratified by sex.

Results

A total of 230-second morning void urine samples were analyzed for TCPY. These samples consisted of 187 third trimester samples from women who had children with data on at least one of the neurodevelopmental outcome measures of interest. The remaining samples were from the subset of women for whom we also measured urinary TCPY variability across all three semesters. Characteristics of the women are presented in Table 1. The median maternal age at delivery was 26 years, with 11 years of schooling and a median IQ of 96. Most mothers were married (74%), did not smoke during pregnancy (99%), and breastfed their baby (90%). Of those infants that were not breastfed, 8 were male and 11 were female. Median blood lead measures (5.4 ug/dl) in moms were slightly above the recommended CDC threshold of acceptable levels in

pregnant and lactating women of 5.0 ug/dL (CDC, 2012). The distributions of TCPY concentrations (uncorrected for SG) in the present study and among pregnant women from NHANES 1999-2002 are presented in Table 2. TCPY was detected in over 90% of urine samples in the present study. Geometric mean TCPY concentration among ELEMENT women was significantly higher than pregnant women in NHANES (p-value =0.03).

Among the subset of women with repeated measures, there were no significant differences in geometric mean TCPY concentrations between trimesters (Figure 1). However, there was significant within-woman variability across trimesters (Figure 2). ICCs for uncorrected and SG-corrected TCPY concentrations are shown in Table 3, calculated when considering only women in the variability subset as well as when including all women with TCPY measures. The ICC was 0.41 for uncorrected TCPY, and ranged from 0.29 to 0.32 for SG-corrected TCPY.

Table 4 describes characteristics of the children at birth and at the time of the psychometric assessments. In this population, 52% of children were female and the median age of the child at testing was 7.5 years. Results from the multivariable regression analysis are presented in Table 5. No statistically significant associations were observed in models that considered all children combined and models stratified by sex. However, we found a suggestive trend for increasing Hit RT Block Change in relation to increasing TCPY tertiles (p-value=0.09). In models stratified by sex, this relationship appeared to be stronger among boys than girls. In the sex-stratified analysis, we also found a suggestive trend for increased ADHD Index in relation to TCPY tertiles (p-

value=0.06). In this model, the highest TCPY tertile was associated with an ADHD index score that was 5.55 points higher than children in the lowest tertile (p-value=0.06). Among girls, there was a suggestive increase in attention problems score in relation to exposure, but only for the middle TCPY tertile (p=0.08).

Discussion

The objectives of this study were to define the distribution of TCPY concentrations among a population of pregnant women in Mexico, to assess between- and within-individual variability of urinary TCPY levels over the course of pregnancy, and to explore the relationship between third trimester maternal urinary TCPY concentrations and child neurodevelopment, particularly attention and hyperactivity, using subscales from psychometric assessments. This is the first study to assess urinary TCPY concentrations in a Mexican population, as well as the first to explore associations with attention and hyperactivity, using urinary TCPY as a biomarker of exposure to CPF, chlorpyrifos-methyl or TCPY.

We found that TCPY levels were somewhat higher in our study population compared to U.S. pregnant women from NHANES investigations in overlapping years. While the ICC we report here indicates a fair level of reliability between TCPY measures across pregnancy (Landis et al. 1977; Portney et al. 2000), prior studies have also documented larger within-person variability relative to between-person variability in TCPY concentrations measured in repeat spot urine samples from the same individual. In a study in New York City, the ICC was 0.43 for TCPY in repeated urine samples from

the third trimester of pregnancy (with one sample post-delivery) in participants enrolled 2001-2002 (Whyatt et al. 2009). This is quite similar to the ICC reported in our study (0.41). Studies in non-pregnant women such as a study in Maryland found an ICC of 0.40 in urinary TCPY after collecting six repeat first morning void urine samples from 80 participants over the course of a year (Egeghy et al., 2005). In an additional study, that collected nine repeat urine samples from 10 men over three months, ICCs ranged from 0.15 to 0.21 for uncorrected and corrected (for creatinine and specific gravity) TCPY (Meeker et al. 2005). However, the authors did report that a single urine measure of TCPY was able to adequately predict tertiles of exposure based on the average TCPY levels in repeated samples collected over three months (Meeker et al. 2005).

In the present study, we did not observe any statistically significant associations between tertiles of maternal third trimester urinary TCPY and measures of attention and hyperactivity in children. However, we found suggestive evidence for increases in the ADHD index in relation to TCPY tertiles among boys. This suggests that fetal exposure to increased quantities of chlorpyrifos, chlorpyrifos-methyl, or TCPY during the last trimester of pregnancy may influence the display of ADHD characteristics in childhood. Similarly, an animal study examining behavioral alterations after CPF exposure during neurolation found that Sprague-Dawley rats injected with 5 mg/kg of CPF during gestational days 9-12 compared to the control group (0 mg/kg) were more affected by adverse neurobehavior, particularly working and reference memory which involves attentional processes (Icenogle et al., 2004). There are limited human studies examining urinary TCPY and neurodevelopment and even fewer examining ADHD and

ADHD-LP. One study in California did, however, utilize prenatal urinary TCPY, but did not find any association with attention or ADHD-related problems in 2-year old children (Eskenazi et al., 2007), however, such behaviors may not be apparent at that age. In another California study, where the association between prenatal urinary dialkyl phosphates (DAPs), a non-specific measure of OPs, and ADHD assessments at 3.5 and 5 years of age was examined, a stronger association with ADHD was found in boys than in girls and generally stronger associations in 5 year olds compared to the younger age children (Mark et al., 2010). In another study in New York City (N=228), it was found that 3 year olds more highly exposed to chlorpyrifos in utero, as measured in cord blood, had lower scores on the Bayley Scales of Infant Development Psychomotor Development (PDI) and Mental Development (MDI) Indices (Bayley, 1993) when compared to lower levels of exposure (Rauh et al., 2006). At the 7-year follow-up of the New York City study, significant associations were also found with working memory skills, thus consistent with previous findings (Rauh et al., 2011).

In this study, we found a suggestive association for increased attention problems when middle TCPY tertile was compared to the lowest tertile in girls. This is in contrast to the previously mentioned study in California of 348 mother-child pairs where urinary DAPs were associated with attention problems and ADHD-LP that were stronger in 5 year-old boys than girls (Marks et al., 2010). We did, however, observe a 5.6-point increase in attention problems among boys in the highest TCPY tertile compared to the lowest (p=0.16). Animal studies have found that sex differences in the impacts on neurodevelopment occur when CPF exposure occurs in late pregnancy (17-20

gestational days in Sprague-Dawley rats) or soon after birth; however, CPF exposures throughout pregnancy may cause non-sex modified adverse neurodevelopment (Icenogle et al., 2004; Levin et al., 2001; Levin et al., 2002). It has been found that prenatal CPF exposure in rats, as early as neurolation or gestational days 9-12, resulted in increased locomotor activities, a characteristic of ADHD and impaired cognitive function in adolescence and adulthood (Icenogle et al., 2004). Other animal studies suggested that CPF may interfere with the development of sex differences in behavioral patterns that are dependent on the timing of exposure (Levin et al., 2001; Levin et al., 2002). However, there is not a clear mechanism to explain this occurrence. Studies have found that in the clinical presentation of attention deficit disorder, girls display more inattentive-type problems while boys display more hyperactive and impulsive behaviors (Biederman et al., 2002; Marks et al., 2010; Staller et al., 2006). In general, more boys are more likely to be diagnosed with ADHD than girls (CDC, 2011).

Our study had a number of strengths and limitations. Some imitations include a relatively modest sample size for exploring relationships between urinary TCPY and childhood neurodevelopment and the use of a single urinary measure to estimate exposure in those analyses. Despite our modest sample size, our study provides a foundation for a larger study and tentatively supports the limited previously published literature regarding urinary TCPY and childhood neurodevelopment. Strengths of our study included the use of urinary TCPY as a biomarker of exposure and the use of validated ADHD psychometric assessments by trained and experienced research team. Using urinary TCPY as a biomarker of chlorpyrifos exposure allowed the specific measure

of chlorprifos and chlorprifos-methyl, whereas similar studies have measured DAPs, which examined OPs as a class. Also, urinary measures of chlorpyrifos are likely to be more reliable over time in comparison to blood given the rapid metabolism and low detection rates of non-persistent pesticides in blood. However, the presence of TCPY in urine may also reflect environmental or dietary exposure to TCPY following environmental degradation of CPF of CPF-methyl. To our knowledge, this is the first study to assess the association between urinary TCPY and a direct assessment of ADHD and ADHD-LP in school-aged children. Previous studies have not directly assessed ADHD and ADHD-LP in school-age children based on early life CPF, chlorpyrifos-methyl, and/or TCPY exposure. While previous studies have assessed OP exposure in relation to attention and ADHD in pre-school children, prenatal urinary TCPY exposure has not been previously examined with these outcomes in school-age children. Also, this is the first study to assess *in utero* CPF, chlorpyrifos-methyl, and TCPY exposure in relation to neurodevelopment during childhood in a Mexico City, Mexico population.

In summary, these results are important considering the continued widespread agricultural and possibly residential use of chlorpyrifos and chlorpyrifos-methyl in Mexico and the educational implications of cognitive and behavioral deficits. Exactly how prenatal CPF and chlorpyrifos-methyl exposure might affect the onset of clinically diagnosed ADHD is unclear, but this study provides interesting information on CPF, CPF-methyl, and TCPY exposures in pregnancy and deserves further study in a larger population

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Table 2.1 Descriptive maternal characteristics (N=187) and for the subset with all three urine samples available (N=20)

	Full (N=187)		Sub-set (N=20)		
Characteristics	N (%)	Median (25th, 75th)	N (%)	Median (25th, 75th)	p*
Maternal Age	187	26 (22, 30)	20	28(23, 31)	0.59
Maternal Education	187	11 (9, 11)	20	12(9,12)	0.71
Maternal IQ	187	96 (88, 103)	20	96(89, 107)	0.72
Socioeconomic Status	178	8.0 (6.0, 11)	18	7.5(6.0, 10.0)	0.98
Blood Lead (ug/dl)	187	5.4(3.3, 7.8)	20	5.9(2.6, 7.7)	0.51
Marital Status					0.71
Married	139(74)		16(80)		
Divorced	1(0.5)		0		
Separated	1(0.5)		1(5)		
Never married	15(8)		2(15)		
Living with partner	31(17)		0		
Smoking during Pregnancy					
Some days	2(1)		1(10)		0.80
Not at all	185(99)		9(90)		
Season of Sample Collection					
rainy- June to October	89 (48)		5 (25)		0.04
dry - November to May	98 (52)		15(75)		
Breastfed					
Yes	167(90)		18(90)		0.98
No	19(10)		2(10)		
Trimester of Pregnancy					
1st Trimester	10(5)		10(5)		
2nd Trimester	11(6)		11(6)		
3rd Trimester	187(100)		187(100)		

*Two sample t-test

Table 2.2 Distribution of uncorrected urinary TCPY concentrations among women in ELEMENT and NHANES

	N	Geomean (95% CI)	10th	25th	50th	75th	90th	95th	Max
ELEMENT TCPY[a,c]	187	1.76 (1.55, 2.02)	0.45	0.91	1.78	3.57	6.40	11.6	44.8
NHANES TCPY (Pregnant Women)	177	1.41(1.23, 1.61)	<LOD	0.61	1.60	3.05	5.00	6.85	15.2

[a]TCPY=3,5,6-trichloro-2-pyridinol
[b]Females are of reproductive age, 18-40 years
[c,d]LOD-limit of detection; ELEMENT TCPY LOD=0.10 ng/ml; NHANES TCPY LOD=0.40 ug/L
Two sample t-test: p=0.03

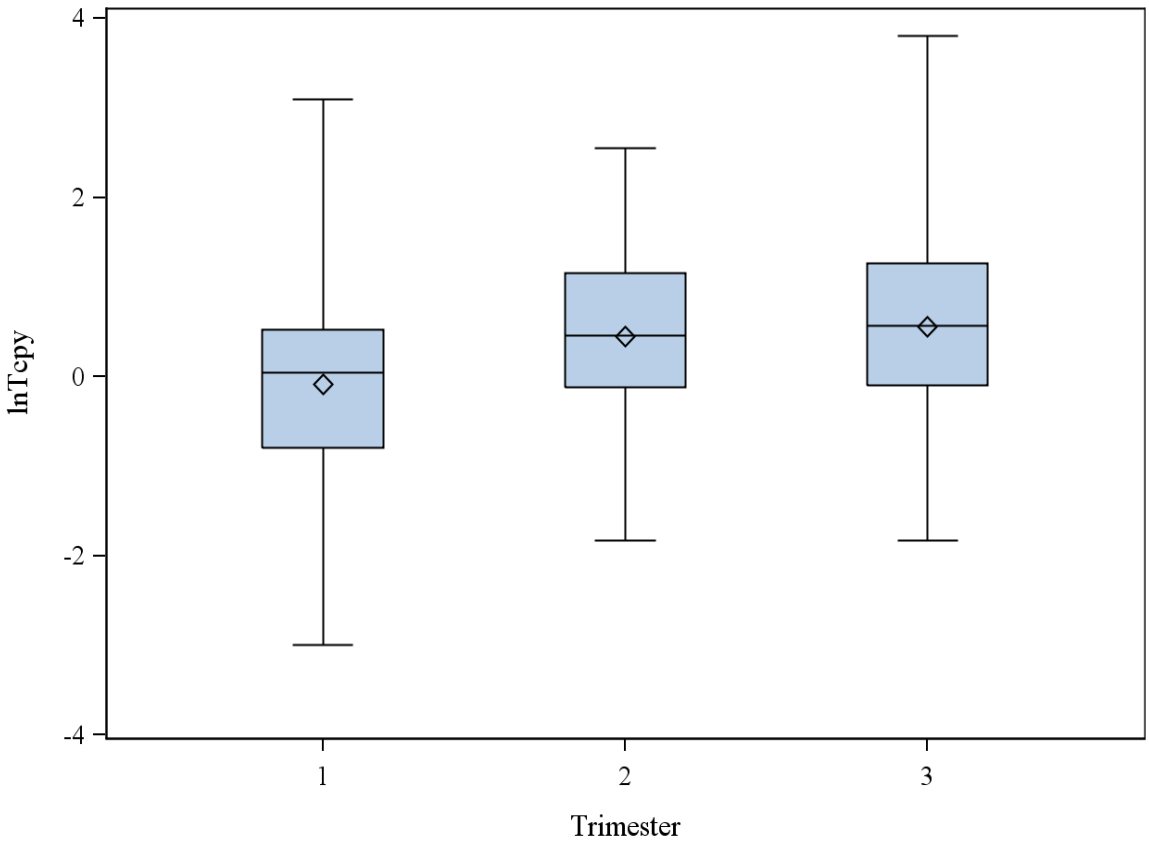

Figure 2.1 Natural log-transformed TCPY concentrations in maternal spot urine samples by trimester of pregnancy

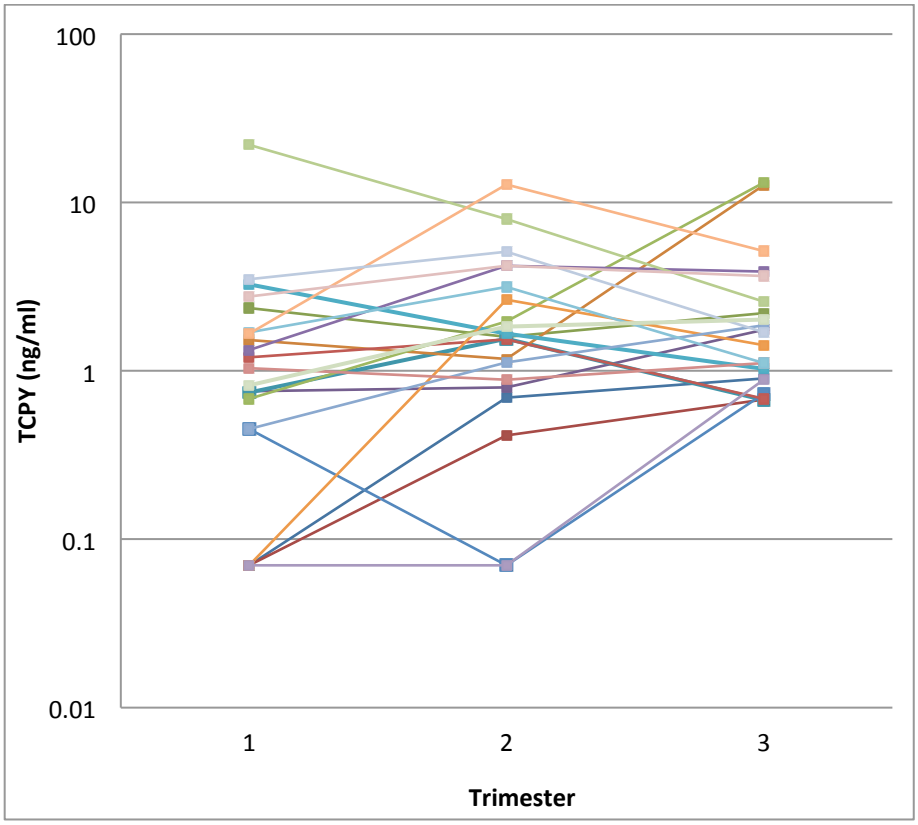

Figure 2.2 TCPY concentrations in maternal spot urine samples collected in each trimester of pregnancy (N=20). Each color represents repeated samples collected from the same woman.

Table 2.3 Variance estimates and intraclass correlation coefficients (ICC) for uncorrected and SG-corrected ln-transformed TCPY across trimesters of pregnancy

	Uncorrected (ng/ml) (N=63)				Uncorrected (ng/ml) (N=230)		
	Variance Estimate	Std. error of Variance	ICC		Variance Estimate	Std. error of Variance	ICC
Between-subject	0.54	0.25	0.41	Between-subject	0.54	0.17	0.41
Within-subject	0.78	0.17		Within-subject	0.75	0.15	
	Specific Gravity-Corrected (ng/ml)				Specific Gravity-Corrected (ng/ml)		
Between-subject	0.31	0.18	0.29	Between-subject	0.30	0.11	0.32
Within-subject	0.74	0.16		Within-subject	0.63	0.11	

Table 2.4 Child characteristics at birth and psychometric assessments in childhood

	N (%)	10th	25th	50th	75th	90th	95th	Max
Birth Characteristics								
Sex	187							
Male	89 (48)							
Female	97 (52)							
Head Circumference (cm)	154	33	34	34	35	36	37	39
Weight (kg)	186	2.6	2.9	3.2	3.5	3.8	3.9	4.2
Height (cm)	181	48	49	50	51	52	54	57
Gestational Age (wks)	185	38	38	39	40	40	41	42
Childhood Psychometric Assessments								
Age at Testing (yrs)	178	7	7	7.5	9	10	10	11
Attention Deficit and Hyperactivity Index	181	42	46	52	59	67	73	90
DSM IV Hyperactivity/Impulsivity	181	45	49	55	63	71	77	89
DSM IV Inattention	181	42	45	51	59	67	72	90
DSM IV Total	181	44	48	53	62	69	75	88
Global Restlessness/Impulsivity Index	181	43	46	52	60	68	74	90
Attention problems	181	37	45	56	62	67	69	76
Hyperactivity	181	39	43	47	55	64	68	83
CPT Conners II (Clinical)	185	30	42	50	69	81	93	100
Hit Reaction Time Block Change	185	38	44	50	58	67	73	100

Table 2.5 Adjusted* multivariable linear regression models for change in psychometric assessment scores associated with medium and high tertiles of maternal third trimester urinary TCPY concentrations compared to the lowest tertile

	N(%) **	All		Males		Females	
Psychometric Assessment		B(95%CI)	p	B(95%CI)	p	B(95%CI)	p
ADHD Index							
Middle TCPY	65 (34)	2.61(-1.54, 6.75)	0.22	2.32(-2.55, 7.20)	0.34	1.63(-5.55, 8.82)	0.65
High TCPY	61(33)	4.00(-0.91, 8.90)	0.11	5.55(-0.19, 11.3)	*0.06*	0.17(-8.28, 8.63)	0.97
p for trend			0.11		*0.06*		0.96
DSM IV Hyperactivity/Impulsivity							
Middle TCPY	65 (34)	-0.56(-5.03, 3.91)	0.81	-0.17(-6.63, 6.29)	0.96	0.33(-6.44, 7.10)	0.92
High TCPY	61(33)	-0.51(-5.80, 4.78)	0.85	1.25(-6.36, 8.87)	0.74	-3.81(-11.8, 4.16)	0.34
p for trend			0.84		0.76		0.35
DSM IV Inattention							
Middle TCPY	65 (34)	2.37(-1.79, 6.53)	0.26	2.33(-2.36, 7.02)	0.32	1.19(-6.09, 8.47)	0.74
High TCPY	61(33)	2.45(-2.47, 7.37)	0.33	2.63(-2.89, 8.16)	0.34	-0.07(-8.64, 8.50)	0.99
p for trend			0.31		0.32		0.99
DSM IV Total							
Middle TCPY	65 (34)	1.23(-2.89, 5.35)	0.56	0.80(-4.48, 6.09)	0.76	1.64(-5.17, 8.45)	0.63
High TCPY	61(33)	1.10(-3.77, 5.98)	0.65	2.06(-4.17, 8.29)	0.51	-1.83(-9.84, 6.19)	0.65
p for trend			0.64		0.51		0.66
Restlessness/Impulsivity Index							
Middle TCPY	65 (34)	-0.15(-4.57, 4.27)	0.95	0.49(-5.71, 6.68)	0.88	-0.48(-7.10, 6.14)	0.89
High TCPY	61(33)	0.38(-4.85, 5.61)	0.89	3.78(-3.52, 11.1)	0.31	-4.90(-12.7, 2.89)	0.21
p for trend			0.89		0.32		0.22
Attention problems							
Middle TCPY	65 (34)	1.79(-2.66, 6.24)	0.43	-0.37(-7.02, 6.27)	0.91	5.81(-0.75, 12.4)	*0.08*
High TCPY	61(33)	3.46(-1.81, 8.73)	0.20	5.59(-2.24, 13.4)	0.16	1.82(-5.91, 9.55)	0.64
p for trend			0.19		0.18		0.62
Hyperactivity							
Middle TCPY	65 (34)	-3.69(-7.88, 0.50)	0.08	-5.00(-12.0, 2.00)	0.16	-0.005(-5.17, 5.16)	1.00
High TCPY	61(33)	-3.35(-8.31, 1.60)	0.18	-3.49(-11.7, 4.73)	0.40	-2.77(-8.84, 3.31)	0.37
p for trend			0.17		0.36		0.37
CPT Conners II (Clinical)							
Middle TCPY	65 (34)	-3.97(-12.5, 4.51)	0.36	-4.29(-15.8, 7.18)	0.46	0.42(-13.2, 14.0)	0.95
High TCPY	61(33)	2.19(-8.11, 12.5)	0.68	0.84(-12.8, 14.5)	0.90	8.55(-7.83, 24.9)	0.30
p for trend			0.73		0.95		0.31
Hit RT Block Change							
Middle TCPY	65 (34)	-4.59(-9.55, 0.36)	0.07	-5.10(-13.1, 2.92)	0.21	-3.79(-10.6, 2.98)	0.27
High TCPY	61(33)	-5.10(-11.1, 0.91)	0.10	-6.86(-16.4, 2.68)	0.16	-2.33(-10.5, 5.82)	0.57
p for trend			0.09		0.14		0.55

*Adjusted by child sex, maternal IQ, maternal education, income, child age at testing, specific gravity, season, breastfeeding, lead, delivery height, delivery head circumference

**N=187 (total)

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Chapter III

Prenatal urinary 3, 5, 6-trichloro-2-pyridinol, paraoxonase I polymorphisms, and attention/hyperactivity in school-age children from Mexico City, Mexico

Abstract

Globally, organophosphate (OP) pesticide usage and exposure is widespread. Studies have found that fetuses and infants are more sensitive than adults to environmental toxicants and that prenatal exposure to low levels of OPs has been associated with Attention Deficit Hyperactivity Disorder-Like Phenotypes (ADHD-LP). Paraoxonase 1 (PON1) is an enzyme involved in detoxifying some OPs and its polymorphisms influence enzyme activity and quantity. The objectives of this study were to: (1) examine whether maternal and child PON1 genotype ($PON1_{R192Q}$ and $PON1_{L55M}$) were associated with ADHD-LP and (2) whether PON1 polymorphisms modified the association of prenatal exposure to OPs (assessed by maternal urinary 3, 5, 6-trichloro-2-pyridinol (TCPY)) and ADHD-LP in a Mexico City, Mexico birth cohort. $PON1_{R192Q}$ and $PON1_{L55M}$ genotypes in mothers ($PON1_{R192Q}$: N=531; $PON1_{L55M}$: N=458) and children ($PON1_{R192Q}$: N=532; $PON1_{L55M}$: N=478) from blood and TCPY concentrations from third trimester urine were determined. We assessed ADHD-LP for children between the ages of 6 and 13 using subscales of Conners' Parental Rating Scales-Revised (CRS-R), Conners' Continuous Performance Test (CPT), and the parental scores for Behavior Assessment System for Children-2 (BASC2). Multivariable linear regression models were used to test

relationships between ADHD-LP and PON1 polymorphisms and assess whether PON1 polymorphisms modified the association between TCPY and ADHD-LP. In main effect models, significant associations were observed with maternal genotypes but not with the children's genotype. An increase in DSM IV Hyperactivity/Impulsivity score (β=3.27 points; 95% CI (0.89, 5.65)) and a 2.17 points increase in child's DSM IV Total (95% CI (0.05, 4.29)) scores were observed for maternal $PON1_{55MM}$ in comparison to $PON1_{55LM+LL}$. A 2.27 point increase was observed for child's attention problems score (95% CI (0.002, 4.53) for maternal $PON1_{192QQ}$. We also found that the relationship between urinary TCPY concentration (a biomarker of exposure to chlorpyrifos, chlorpyrifos-methyl, or TCPY itself) and ADHD-LP was modified by PON1 polymorphisms for child $PON1_{55MM}$ genotypes using secondary subset of the population. PON1 polymorphisms were associated with child ADHD-LP and could be a viable biomarker of susceptibility for ADHD-LP.

Introduction

The use of organophosphate (OP) insecticides, particularly chlorpyrifos (CPF), is of public health concern due to widespread usage and associations with adverse neurodevelopment in fetuses and infants. This is significant because OPs, globally, are still among the most widely used insecticides and exposure is ever-present. CPF, in particular, is the most widely used OP pesticide worldwide (Bradman et al., 2005; Timchalk et al., 2007; Ye et al., 2008). *In utero* pesticide exposure is especially problematic due to the rapid growth, cell differentiation, immaturity of metabolic pathways, and development of vital organ systems of the fetus (Eskenazi et al. 1999; Landrigan et al. 1999). Studies have shown children prenatally exposed to OPs display adverse neurodevelopment including IQ, mental development, psychomotor development, and attention problems (Engel et al., 2011; Eskenazi et al., 2007; Bouchard et al., 2010; Marks et al., 2010). When investigating these associations, it is also important to examine factors that may impart added susceptibility to OP exposure. One such factor related to OP metabolism is Paraoxonase (PON1).

PON1's ability to detoxify OP is considered by various studies to be an important link between environmental exposure to pesticides or other pollutants and disease (Costa et al. 2005; Eaton, 2000; Furlong et al. 2007; Androutsopoulos et al. 2011). PON1 is a liver and serum enzyme involved in a broad range of activities including hydrolyzing or detoxifying organophosphate (OPs) parent compounds and metabolites (Cole et al., 2005). The OP may be detoxified by PON1 before it has the chance to inactivate acetylcholinesterase (AChE) in the peripheral nervous and central nervous systems

(Chen et al., 2003). PON1 has also been found to influence sensitivity to CPF, CPF-oxon and other OP compounds or metabolites due to coding region polymorphisms that alter its activity (Furlong et al., 2005). Higher serum PON1 levels are associated with greater resistance to OP toxicity compared to low circulating levels of PON1. Circulating PON1 is different between and within individuals, and varies genetically and temporally across one's life span (Furlong et al., 2005). Typically, gene variants associated with the amino acid present at position 192 (glutamine (Q) or arginine (R)) are responsible for the variability in circulating PON1 levels or catalytic efficiency of hydrolysis with $PON1_{R192}$ and are considered the protective alloform for paraoxon, CPF and CPF-oxon exposure (Furlong et al., 2005; Li et al., 2000).

In PON1 knockout mice with no detectable PON1 plasma activity, the sensitivity to CPF-oxon toxicity as assessed by measuring brain cholinesterase was modestly increased (Furlong et al., 2005; Li et al., 1995; Li et al., 2000). Exposure to levels of CPF-oxon that produced no symptoms of cholinergic effects and minimal inhibition of brain cholinesterase in knockout mice proved to be lethal (Furlong et al., 2005). The catalytic efficiency for hydrolysis of PON1 polymorphisms are substrate-dependent, as the PON1 Q192 alloform was found to hydrolyzed diazoxon, sarin, and soman more rapidly than PON1 R192 *in vitro* (Costa et al., 2005).

Another coding region single nucleotide polymorphism (SNP), $PON1_{L55M}$, has also been found to be associated with PON1 mRNA, where lower circulating PON1 levels in those with $PON1_{M55}$ compared to $PON1_{55L}$ have been reported using human livers (Leviev et al., 1997; Brophy et al., 2001; Cole et al., 2005). However, recent work on

promoter mutations has suggested that the apparent effect of the L55M polymorphism on enzyme concentration also may be due to linkage disequilibrium (LD) with one of the promoter variants ($PON1_{T\text{-}108C}$) (Brophy et al., 2001; Chen et al. 2003).

Human and experimental studies have shown that the fetus and infant are more sensitive than adults to many environmental toxicants including CPF (Timchalk et al., 2007) and that a mother's exposure is a potential source of fetal exposure (Berkowitz et al., 2004). Newborns have very low PON1 levels (Cole et al., 2005), thus it is reasonable to assume that PON1 levels are even lower in fetuses. This leads to concern about exposure of the fetuses of mothers with low PON1 status or non-protective genotypes to CPF exposure (Furlong et al., 2005). Studies examining *in utero* OP exposure in relation to PON1 levels and/or genotypes in humans have found significant associations with head circumference in offspring (Berkowitz et al., 2004), lower mental development index (MDI) scores for 2 year olds in those with $PON1_{108T}$ (Eskenazi et al., 2010), decreased MDI scores in 12 month olds with mothers with $PON1_{Q192}$ genotype (Engel et al., 2011), and decrements in perceptual reasoning in early childhood (Engel et al., 2011). Trends have also been found between increased OP exposure and decreased neurodevelopment within those with $PON1_{192Q}$ (Engel et al., 2011) and $PON1_{108T}$ (Eskenazi et al., 2010) genotypes.

Attention Deficit and Hyperactivity Disorder (ADHD), a commonly diagnosed and studied neurobehavioral disorder, which is typically characterized by persistent and severe inattention and hyperactivity/impulsivity, has been increasingly diagnosed in recent years (Boyle et al., 2011). Few studies have examined prenatal OP and/or CPF

exposure in relation to ADHD and ADHD-LP such as attention, and even fewer have also considered biomarkers of OP exposure susceptibility.

In this study, we examined the relationship between maternal and child genetic polymorphisms of $PON1_{R192Q}$ and $PON1_{L55M}$ and ADHD-LP in a population of mothers and children from Mexico City, Mexico. In a secondary analysis, we explored the interaction between $PON1_{R192Q}$ and $PON1_{L55M}$ and urinary 3, 5, 6-trichloro-2-pyridinol (TCPY), a metabolite of CPF and CPF-methyl, in relation to psychometric measures in a subsample of the Mexican mother-child pairs.

Methods

Participants were from three sequentially enrolled, prospective birth cohorts conducted in Mexico City, Mexico during: 1994-1997 (cohort 1), 1997-2000 (cohort 2), and 2001-2005 (cohort 3). All three cohorts enrolled homogenous, low-to-moderate income, pregnant women recruited from the National Institute of Perinatology, Hospital General Dr. Manuel Gea Gonzalez, or clinics affiliated with the Mexican Social Security Institute (Braun et al., 2012). Mother-child pairs from the three cohorts (N=827) were re-invited between 2007-2011 to examine childhood and adolescent neurodevelopmental characteristics. Exclusion characteristics for all cohorts included: plans to leave the area within the next 5 years; daily consumption of alcoholic beverages; addiction to illegal drugs; continuous use of prescription drugs; diagnosis of multiple pregnancy, pre-eclampsia, renal or heart disease, gestational diabetes; a history of infertility, diabetes, or psychosis; diagnosis of high risk pregnancy; or suffering

from seizures requiring medical treatment (Hu et al., 2006). Specific objectives of each cohort have been previously published (Hernandez-Avila et al., 2003; Tellez-Rojo et al., 2004; Ettinger et al., 2009). Exposure, outcome, and demographic characteristics were collected from all eligible participants by the same group of investigators and field staff. Participants were informed of the study, associated aims, and uses of biological samples/data and written consent was obtained before enrollment. The Institutional Review Boards of the National Institute of Public Health (Mexico), Harvard School of Public Health, University of Michigan, and participating hospitals approved all study materials and procedures.

Data Collection: Secondary Study

Participants provided second morning void urine samples in the third trimester of pregnancy. In this study, we utilized third trimester urine samples from mother-child pairs that had completed psychometric assessments and 2 or more ml of third trimester urine available for a subset of children (N=187) from cohorts 2 and 3.

We used three psychometric assessments to assess behavioral characteristics of children 6 – 11 years*:* Conners' Parental Rating Scales-Revised (CRS-R), Behavior Assessment System for Children – Parental Rating Scales (BASC-PRS), and Conners' Continuous Performance Test (CPT). Scores from these psychometric instruments indicate ADHD-LP and are not designed as a tool for diagnosis but rather for screening of neurodevelopment. The instructions and prompts were translated into Spanish by a neurodevelopmental examiner in our research group who also trained and supervised

the personnel who administered the assessments. Standardization and quality control checks were conducted by reviews of videotaped evaluations.

Conners' Rating Scales-Revised-Parent

Conners' Parental Rating Scales-Revised (CRS-R) is an assessment tool for parents that are used to determine a child's behavior (Conner, et al. 1998). This 27-question test is used for children and adolescents 3-17 years. Most questions are based on behavioral characteristics that are described in the Diagnostic and Statistical Manual of Mental Disorders-IV (DSM-IV) diagnostic guidelines for ADHD (American Psychiatric Association, 2000; (Deb et al., 2008). In this study, we used the following subscales: ADHD Index, DSM-IV Hyperactivity/Impulsivity, DSM-IV Inattention, DSM-IV Total, and Global Restless/Impulsivity Index. For these scales, the higher the score typically indicates an elevated level of concern with a score of 40-59 considered average and <40 displaying even fewer concerns. The ADHD index identifies children/adolescents that are at risk for ADHD while Global Restless/Impulsivity Index indicates tendencies toward hyperactivity as well as inattention. DSM-IV subscales yield scores between 0-9; scores of 6 and over suggest possible DSM-IV diagnosis (Conner Profile, 2012). DSM-IV Hyperactivity/Impulsivity and DSM-IV Inattention correspond to a diagnostic type of ADHD. DSM-IV Total represents the diagnostic criteria for the combined type of ADHD.

Behavior Assessment System for Children (BASC) – Parental Rating Scales

BASC-PRS is used to measure adaptive and problem behaviors in the community and home setting (Pearson Assessment, 2012). Parental Rating Scales for 6-11 year olds are used to assess attention problems and hyperactivity. Questionnaires include 134-

160 multiple-choice items and are usually completed in 10-20 minutes. For these scales, the higher the score typically indicates an elevated level of concern. Scores of 41-59 were considered average with children displaying typical levels of attention/hyperactivity problems for a child at that age. Scores of <41 indicate an even lower level of concern.

Continuous Performance Test (CPT)

CPT is a 14-minute computer test that measures sustained attention and impulsivity (Wilmshurst et al., 2009) and compares the participant's answers to a reference group and results in a confidence index of clinical and non-clinical profiles. CPT is used to provide a cognitive profile and to test core symptoms of ADHD (i.e. impulsivity and inattention). The clinical index, which measures where the participant will have a higher likelihood of ADHD diagnosis, is presented in this article. CPT has a high sensitivity (83-90%), but is poorer (59-61%) when measured against clinical diagnosis (Linnet et al., 2003).

DNA Extraction and PON1 Genotyping

Blood samples from mother-child pairs who were re-invited for additional studies (N=591) were analyzed for coding PON1 polymorphisms (Q192 and L55M). Promoter PON1 polymorphisms (-909, -169, and -108) were not analyzed due to budgetary constraints.

Blood samples (5ml) were transported to the University of Michigan Sequencing Core for DNA extraction and genotyping. Genomic DNA was extracted from venous blood stored in 8.5 ml Paxgene tubes following the Qiagen Automated purification of

DNA from cell lysate from compromised samples on the Autopure LS® protocol. Steps performed by the Autopure LS® include: protein precipitation, DNA precipitation, DNA washing, and DNA hydration. After removing purified DNA from the instrument, it was incubated at 65 ^{o}C for 1-2 hours to dissolve the DNA into the glycogen solution. Purified DNA was stored at -20^{o}C in until genotyping.

The PON1 genotypes were determined using the Sequenom MassARRAY iPLEX Platform (Bruker Instruments, Billerica, MA). A locus-specific multiplex PCR reaction occurred which was followed by a locus-specific primer extension reaction in which an oligonucleotide primer anneals immediately upstream of the polymorphic site being genotyped (Gabriel et al 2009). Polymerase Chain Reaction (PCR) and extension primers were automatically designed for each SNP and were pooled to allow many SNPs to be analyzed at once in a one-well reaction. Primers were sorted into three groups based on mass to ensure equality in intensity. Uniformity of intensity and accuracy of pooling procedure of low/middle/high mass probes was verified by spotting on SpectroCHIPS and running on detectors (Gabriel et al 2009). DNA samples were amplified via PCR in 384-well PCR plates using the following cycling program (~2.5hr) (Gabriel et al 2009):

1 cycle:	5 min	94^{o}C	(initial denaturation)
45 cycles:	20 sec	94^{o}C	(denaturation)
	30 sec	56 ^{o}C	(annealing)
	1 min	72 ^{o}C	(extension)
1 cycle:	3 min	72 ^{o}C	(final extension)
Final step:	indefinite	4 ^{o}C	(hold).

To remove remaining unincorpated dNTPs, treatment with shrimp alkaline phosphatase (SAP) was performed. In the iPLEX assay, which contained a cocktail of primer, enzyme, buffer, and mass-modified nucleotides, the primer and amplified target DNA were incubated with mass-modified dideoxynucleotide terminators to extend the primer and was performed using the SpectrPREP Multimek robot (Gabriel et al 2009). Products of the primer extension process were spotted on SpectroCHIPS. Then, through the use of matrix-assisted laser desorption ionization-time-of –flight (MALDI-TOF) mass spectrometry, the mass of the extended primer was determined. The primer's mass indicated the sequence and, therefore, the alleles present at the polymorphic site of interest. Sequenom supplied software (SpectroTYPER) that automatically translated the mass of the observed primers into a genotype for each reaction (Gabriel et al 2009). Population sizes for our data analysis involving each SNP were based on the ability to make a successful call for each individual, which was done using Sequenom supplied software SpectroTYPER. The overall call rate was determined by the number of individuals receiving a genotype call divided by the total number of individuals in the population.

TCPY concentrations in urine

Maternal urine samples (2 mL) were transported on dry ice to Emory University for analysis of TCPY in urine. Samples were spiked with stable isotopically labeled TCPY and then subjected to an enzyme hydrolysis. Hydrolysates were extracted using mixed-polarity solid-phase extraction cartridges (CDC, 2006; Olsson et al., 2004). Elutes were concentrated and analyzed using HPLC/tandem MS (Olsson et al., 2004) with both

quantification and confirmation ions monitored. Metabolites were quantified using isotope dilution calibration. The LOD was 0.10 ng/mL; values below the LOD were assigned a value of LOD divided by the square root of two. Urinary specific gravity (SG) was determined using a handheld digital refractometer (ATAGO Company Ltd., Tokyo, Japan). SG-corrected urinary TCPY concentrations were calculated for use in certain statistical analyses.

Statistical Analysis

Covariates

Birth length and head circumference at birth were determined by a nurse at the time of delivery and were used as continuous variables. Maternal IQ was calculated on the basis of a mother's scores on the Information, Comprehension, Similarities, and Block Designs scales of the Spanish Wechsler Adult Intelligence Scale (Téllez-Rojo et al., 2006; Weschler, 1968). A continuous variable was created to capture socioeconomic status and income based on material possessions. Maternal education was the cumulative number of years that the mother attended school and was used continuously. Breastfeeding (yes/no) from a questionnaire administered to the mother during the child's infancy was included in the model and used categorically. Maternal blood lead was measured one-month post-partum and was used continuously.

Statistical Analysis Software (SAS) (version 9.2; SAS Institute Inc., Cary, NC, USA) was used for most analyses. Descriptive statistics for demographic information were calculated for mothers and children included in this study, along with distributions for allele and genotype frequencies for $PON1_{L55M}$ and $PON1_{R192Q}$. Hardy-Weinberg was

calculated for each SNP for each population (and for both mother and child): the "main effects" population (N=591) for whom SNP information was available and the smaller "secondary analysis" subsample (N=187) for whom information on TCPY exposure was also available. Chi-square was used to test whether genotype frequencies were significantly different.

Bivariate analyses between the dependent variables (psychometric assessments-ADHD Index, DSM IV Hyperactivity/Impulsivity, DSM IV Inattention, DSM IV Total, and Global Restless/Impulsivity Index, BASC attention, BASC hyperactivity, and CPT clinical index) and PON1 genotypes, as well as other covariates (maternal IQ, maternal education, socioeconomic status, breastfeeding, child age at testing, child sex, maternal blood lead, birth length, and head circumference at birth) were conducted. ANOVA was used for continuous variables, which were first tested for normality, and Chi-square was used for categorical variables.

Multivariable regression models were created using variables found to be associated with ADHD-LP, TCPY, and/or PON1 genotypes in bivariate analyses ($p<0.05$). Variables were also considered from *a priori* suspicion of being potential confounders of the association between prenatal exposure to CPF, CPF-methyl or TCPY exposure and psychometric outcome. In PON1 main effects models, covariates included: maternal IQ, maternal education, socioeconomic status, breastfeeding, maternal blood lead one month after delivery, child's age at testing, child's sex, birth length, and head circumference at birth. PON1 genotypes were used categorically, comparing the CPF "non-protective" types ($PON1_{55MM}$ and $PON1_{192QQ}$) to $PON1_{55LM+55LL}$ and $PON1_{192QR+192RR,}$

respectively, in separate models. Due to the low number of participants in the $PON1_{55LL}$ group, combined comparison groups, $PON1_{55LM+55LL}$ and $PON1_{192QR+192RR}$, were created for both SNPs for ease in interpretation and comparison between SNPs as opposed to modeling as 3-level categorical variables for each genotype. Models were stratified by maternal or children SNP genotype information. Additionally, in separate analyses, models were stratified by sex of child and by mother/child genotype information.

In exploratory analyses (N=404), maternal and child PON1 genotypes were included in the same model using a population of mother/child pairs who had complete PON1 genotype information ($PON1_{Q192R}$: N=404; $PON1_{L55M}$: N=328). The same PON1 categories described above were used. For these analyses, models were adjusted for mothers and children with the same genotype. For example, models were adjusted for mothers with $PON1_{192QQ}$ and children with $PON1_{192QQ.}$ Potential interaction between mother and child genotypes was assessed. Statistical significance for all models was based on a p-value of 0.05.

Secondary Analysis (N=187)

Descriptive statistics for demographic information were calculated for mothers and children from whom we had data on both PON1 genotypes and urinary TCPY concentrations. Bivariate analyses between the dependent variables (psychometric assessments- ADHD Index, DSM IV Hyperactivity/Impulsivity, DSM IV Inattention, DSM IV Total, and Global Restless/Impulsivity Index, BASC attention, BASC hyperactivity, and CPT clinical index) and TCPY concentrations, as well as other covariates (maternal IQ,

maternal education, socioeconomic status, breastfeeding, child age at testing, child sex, maternal blood lead, birth length, and head circumference at birth), were conducted.

Prenatal TCPY exposure models included the following covariates: natural log-transformed TCPY, mom or child PON1 genotype (with $PON1_{192QR+RR}$ and $PON1_{55LM+LL}$ as reference groups), specific gravity, maternal IQ, maternal education level, age of child at testing, breastfed, and sex of child. Potential interactions between TCPY concentrations and $PON1_{192QQ}$/$PON1_{55MM}$ genotypes for both mothers and children were also explored to assess whether PON1 genotypes modified the relationship between TCPY and ADHD-LP. Statistical significance for all models was based on a p-value of 0.05.

Results

A total of 591 mother/child pairs were genotyped for SNPs of PON1. For $PON1_{Q192R}$, the population for mothers was N=531 while children had N=532 with genotype information. For $PON1_{L55M}$, 458 mothers and 478 children (Table 1) had genotype information. Despite the differences in population sizes, the overall call rate of each SNP population was approximately 80% or greater. The distribution of genotype frequencies for $PON1_{Q192R}$ and $PON1_{L55M}$ were similar for both populations. For mothers, allelic frequencies for $PON1_{Q192R}$ were 41% for A and 59% for G while children's allelic frequencies 40 and 60%, respectively. For $PON1_{L55M,}$ mothers and children both had allelic frequencies of 88 and 12%, for A and T, respectively. Characteristics of mothers and children are presented in Table 2. The median maternal age at delivery was 25 years, with 11 years of schooling and a median IQ of 94. Most mothers were

married (72%) and breastfed their babies (92%). Median blood lead measures (6.2 ug/dl) in moms were above the recommended CDC threshold of acceptable levels in pregnant and lactating women of 5.0 ug/dL (CDC, 2012). The median gestational age for children was 39 weeks with a birth weight of 3.1 kg (6.8 lbs) and length of 50 cm (19.7 in) (Table 2). The median age at testing for children was 9 years and 52% of the population was male.

Results from the PON1 main effects multivariable regression analyses are presented in Table 3. We observed a 2.32-point increase (95% CI (0.26, 4.38)) in DSM IV Hyperactivity/Impulsivity score for children with mothers having $PON1_{55MM}$ in comparison to $PON1_{55LM+LL}$ (Table 3). No other significant associations were found in models stratified by either mother or child genotype information. In models stratified by mother or child genotype and sex of child (Tables 4-5), we did not observe any statistically significant associations with PON1 genotypes in either boys or girls. However, in boys, there were statistically suggestive 2.00, 2.61 and 2.22 point increases for ADHD Index (p-0.09), DSM IV Hyperactivity/Impulsivity (p-0.06), and DSM IV Total (p-0.07), respectively, in relation to mothers' $PON1_{55MM}$. Also, in males who had mothers with $PON1_{192QQ,}$ there was a suggestive 2.81-point (p-0.05) increase for DSM IV Hyperactivity/Impulsivity.

Tables 6-7 show the distribution of genotype frequencies for mother-child populations that only included mother-child pairs who both had genetic information ($PON1_{Q192R}$: N=404; $PON1_{L55M}$: N=328). In an exploratory analysis, models were adjusted for maternal and child genotypes in the same model. In these models (Table

8), we observed a higher increase in points for DSM IV Hyperactivity/Impulsivity score (β=3.27 points; 95% CI (0.89, 5.65)) for mothers' $PON1_{55MM}$ in comparison to models that did not adjust for child genotype. In addition, we found a 2.17-point increase in child's DSM IV Total (95% CI (0.05, 4.29)) scores for children of mother with $PON1_{55MM}$ in comparison to $PON1_{55LM+LL}$. When assessing mothers' $PON1_{192QQ}$ in comparison to $PON1_{192QR+RR}$, a 2.27 point increase was observed for child's attention problems scores (95% CI (0.002, 4.53). None of the interaction terms between maternal and child genotypes were statistically significant.

Secondary Analysis (N=187)

Of the 187 participants with TCPY measurements in maternal urine from the third trimester, $PON1_{Q192R}$ and $PON1_{L55M}$ genotype information was available for N=109 and N=73 mothers, and N=106 and N=86 children, respectively (Table 1). Figure 1 displays the distribution of geometric mean urinary TCPY concentrations by maternal/child PON1 genotype. With regards to TCPY concentrations by PON1 genotype, there was no apparent trend or pattern observed when comparing among or between protective and non-genotypes overall. However, the protective genotype appeared to have slightly lower TCPY concentrations than non-protective genotype for $PON1_{L55M}$.

In multiple variable regression models, we did not observe statistically significant associations between ADHD-LP and lntcpy in models excluding and including maternal/child genotypes (results not shown). We did, however, detect a significant

12.9 point (p=0.003) increase for CPT clinical in relation to child $PON1_{192QQ}$ and a 6.03 point (p-0.02) increase in attention problems in relation to maternal $PON1_{55MM}$ (results not shown) in models for PON1 genotype main effects in this subset of participants. In multivariable models that included the main effects of both lntcpy and PON1 genotypes, similar relationships were observed (Table 9). In models examining whether prenatal exposure to TCPY and mothers/children's genotypes interact to influence ADHD-LP, we observed significant interactions between lnTCPY and PON1 genotype for Global Restlessness/Impulsivity in children with $PON1_{55MM}$ where an ln-unit increase in TCPY was associated with a 1.40 point increase in score (p=0.05) (Table 11). No statistically significant interactions were found using maternal or child genotypes for $PON1_{Q192R}$ (Table 10). Similar results for $PON1_{Q192R}$ and $PON1_{L55M}$were observed in interaction models that adjusted for both maternal and child genotypes in the same model (results not shown).

Discussion

The objectives of this study were to assess whether ADHD-LP behaviors in school-age children were associated with mother and/or child PON1 genotypes. Among a subset of participants in the study, we also explored the interaction of urinary concentrations of TCPY in the third trimester and maternal or child PON1 genotypes in relation to ADHD-LP in school-age children. This is the first study to assess the relationship between PON1 genotypes and ADHD-LP in school-age children, as well as

the first to explore possible interactions between a biomarker of CPF exposure and PON1 genotypes in influencing ADHD-LP in school-age children.

In analyses of PON1 and ADHD-LP, we found that the OP 'non-protective' PON1 genotype, $PON1_{55MM}$, in mothers was associated with poorer DSM-IV Hyperactivity/Impulsivity and DSM IV Total scores. Poorer attention problems scores were also observed in children with mothers that had the non-protective, $PON1_{192QQ}$ genotype. Associations with maternal genotypes were stronger when adjusting for child genotype. No significant associations were found in relation to the child's genotype alone. While there have been no studies examining PON1 genotypes and ADHD-LP, one study has examined the main effects of PON1 genotypes and neurodevelopment using Bayley's Mental Developmental Index (MDI), Psychomotor Developmental Index (PDI), and Pervasive Developmental Disorder (PDD) (Eskenazi et al., 2010). In that study (CHAMACOS) in California (mothers-N=351; children-N=369), no clear patterns of associations with outcomes (MDI, PDI, and PDD) in relation to the mother or child's PON1 192 genotype was observed (Eskenazi et al. 2010). Studies have shown that ADHD and Autism Spectrum Disorder (ASD)/ autism are often co-morbidities (Ceylan et al 2009). One study in Italy found higher odds of autism in Caucasian-American family trios (2 parents and 1 child) (N=107) with $PON1_{192R}$ and $PON1_{55L}$ alleles compared to Italian family trios (N=177) (D'Amelio et al. 2005). Another study in New York City observed small reductions in head circumference in relation to low circulating maternal PON1 activity, but not in relation to PON1 genetic polymorphisms (Q192R, L55M, -909, -162, and -108) in a study of 404 mother/infant pairs (Berkowitz et al 2004). Small head

circumference has been associated with declined cognitive ability in children (Berkowitz et al. 2004).

In the present study, we observed that the association between urinary TCPY and global restlessness/impulsivity was modified among children with $PON1_{55M.}$ As far as we are aware, no studies to date have assessed ADHD-LP in relation to potential TCPY/PON1 genotype interactions. However, one study has examined prenatal exposure to non-specific urinary measures of OP exposure, dialkyl phosphate metabolites (DAPs), among black and Hispanic children and whether or not associations with neurodevelopment at 12 months were modified by the mom's PON1 genotype (Engel et al., 2011). In a study in New York City (NYC) (N=200), maternal $PON1_{192QQ}$ genotype enhanced associations between total DAPs (and total DMP) measured in maternal third trimester urine and decrements in mental development in children at 12 months (Engel et al., 2011). In a follow-up study among children 6-9 years of age (N=169) in the same NYC study, decrements were also found in perceptual reasoning in relation to increasing concentrations of DAPs in children whose mothers possessed the $PON1_{192QQ}$ genotype (Engel et al., 2011). No significant associations were observed with $PON1_{L55M,}$ $PON1_{T-108C,}$ or with circulating PON1 enzyme activity (Engel et al., 2011). Results of that study were similar using child genotypes (Engel et al., 2011). The CHAMACOS study in California found urinary DAP/PON1 genotype interactions in Mexican-American children with $PON1_{192QQ}$ (Eskenazi et al., 2010). The authors reported an inverse association between maternal urinary DAP concentrations measured during pregnancy and PDI scores among 2-year-olds with $PON1_{192QQ}$ (Eskenazi et al., 2010). Similar but weaker

associations between urinary DAP concentrations and neurobehavioral outcomes were observed when stratified by maternal genotype rather than child genotype (Eskenazi et al., 2010). It has been suggested that genotypes may be a more stable, long-term predictor of metabolism potential (Engel et al., 2011) because enzyme levels are highly variable in humans over time (Cole et al., 2003; Costa et al. 2005; Holland et al 2006). For example, measurements of enzyme levels in fetuses and children are much lower than those found in adults (Chen et al. 2003; Furlong et al. 2006; Huen et al. 2010; Eskenazi et al. 2009).

Both fetuses and very young children may be more susceptible to pesticide exposure compared to adults because infants do not approach adult PON1 levels until 6-24 months of age (Chen et al. 2003; Cole et al. 2005). Furthermore, both CPF and CPF-oxon, a neurotoxic metabolite of CPF, can cross the placenta. Thus, maternal exposure may be a proxy for *in utero* pesticide exposure because the fetus is generally less protected regardless of PON1 genotype (D'Amelio et al. 2005). One study suggested that PON1 activity in neonates is affected more than 2-fold in those with $PON1_{55MM,}$ who reportedly have 47 and 80% lesser PON1 activity than 55LM and 55LL, respectively (Chen et al. 2003).

As stated previously, the $PON1_{55M}$ genotype is associated with low PON1 enzyme levels; however, several studies in different populations have suggested that most of this effect is due to its strong linkage disequilibrium with $PON1_{-108T}$, which is the predominant promoter polymorphism contributing to enzymatic activity (Brophy et al. 2001; Chen et al. 2003; Rojas-Garcia et al., 2005), where the two loci are not

independent of another (Costa et al. 2003, Holland et al. 2006). In a CHAMACOS study, effect modification of infant PON1$_{-108TT}$ (susceptible genotype) with maternal urinary DAP concentrations were associated with shorter gestational age (Harley et al. 2011). It was also found that the susceptible PON1$_{-108TT}$ genotype for infants (independent of OP exposure) was associated with shorter gestational age and smaller head circumference (Harley et al. 2011). In another study in New York City, similar associations were found using maternal PON1$_{-108TT}$ (Berkowitz et al. 2004). That study also suggested that *in utero* exposure to CPF has a detrimental effect on fetal neurodevelopment among mothers with low PON1 activity, but did not observe significant interactions between maternal urinary TCPY and PON1 activity level in relation to head circumference (Berkowitz et al. 2004).

The etiology of ADHD is not clear but studies have suggested that oxidative stress may play a role in the etiology of neurobehavioral disorders, particularly ADHD, due to polymorphisms in PON1 leading to altered enzyme activity (Ceylan et al., 2012; Kawatani et al., 2011). Oxidative stress contributes to cell or tissue damage, including the synaptic cell membrane. PON1 activity has been found to have antioxidant properties, which is dependent on the quantity of PON1 activity present. Oxidative stress increases throughout pregnancy with dramatic increases during the 2nd and 3rd trimesters, while PON1 activity decreases (Stefanovic et al., 2012).

In a small study of 35 children diagnosed with ADHD in Turkey, significantly lower levels of PON1 activity were reported in comparison to healthy controls (N=35) (Ceylan et al., 2012). Another study of 103 cardiovascular disease-free adult subjects in

Japan suggested that $PON1_{192RR}$ (in comparison to QR and QQ genotypes) plays a protective role in oxidative stress using the diacron reactive oxygen metabolites test (Kotani et al., 2012). Some studies have found that children diagnosed with ADHD have increased levels of malondialdehyde (MDA), which is associated with a lipid oxidation defect and increased oxidative stress (Essawy et al., 2009; Kawatani et al., 2011). This may be of significance because a study in Egypt of children (N=20) diagnosed with ADHD using DSM-IV criteria in comparison to healthy controls (N=16) had increased levels lipid peroxidation that was significantly correlated with ADHD/Inattentative subtype (Essawy et al., 2009). In our study, we observed statistically significant associations with DSM-IV combined subtypes in main effects models for maternal PON1 genotype.

OPs are capable of generating oxygen free radicals, such as hydrogen peroxide, superoxide, and hydroxyl (Stevenson et al., 1995; Hernandez et al., 2012). Oxidative stress occurs when oxygen free radicals overwhelm the body's ability to regulate them (Hernandez et al., 2012). In an occupational study of 135 adult greenhouse workers who had experience as pesticide applicators in Spain, PON1 polymorphisms ($PON1_{Q192R}$) were found to modify the relationship between pesticide exposure and oxidative stress with $PON1_{192RR}$ being more vulnerable to oxidative stress (Hernandez et al., 2012). That study also found that short-term (as measured by pseudocholinesterase (BChE) activity) and long-term (as measured by AChE levels) pesticide exposures disturbed the redox status in erythryocytes, suggestively by inducing oxidative stress (Hernandez et al., 2012).

This study had several strengths. First, we utilized the largest cohort to date of mother/child pairs to assess the relationship between genotype and ADHD-LP in school-age children, which has not been done previously. We also adjusted for maternal and child genotypes within our models, which allowed us to account for both maternal and child genotype in relation to ADHD-LP. PON1 activity was not measured in this study, which represents a limitation since there is considerable variability in PON1 levels among adults and differences as high as 13-fold have been observed among individuals of the same genotype (Cole et al., 2005; Furlong et al., 2005). However, many studies have reported associations between genotypes and activity level, and this study provides a platform for further analysis. Also, this study did not measure $PON1_{-T108C}$ and studies have suggested that the apparent effect of the L55M polymorphism on enzyme concentration also may be due to linkage disequilibrium (LD) $PON1_{-T108C.}$ Another potential limitation is that our study results may not be generalizable to other race/ethnic groups or geographic regions, since it has been suggested that different populations have different susceptibility to pesticides based on their genetic makeup and mixed ancestry that influences allele frequencies. This study does add to the body of literature on genetic variability in Mexican populations. The interactions explored in our secondary analysis were limited by a modest sample size, but the findings are consistent with previously published literature. Similar to previously published studies, we found that *in utero* exposure to OPs (in our case, TCPY) may be influenced by PON1 polymorphisms and, consequently, impact neurodevelopment in children. Additionally, we measured urinary TCPY at one time point during pregnancy, in the early third

trimester, which may not give an accurate account of CPF, CPF-methyl, and/or TCPY exposure throughout pregnancy. Previous studies of intra- and inter-individual variability in urinary TCPY concentrations between trimesters found fair temporal reliability (intraclass correlation (ICC) of 0.41) ((Landis et al. 1977; Portney et al. 2000; Whyatt et al., 2009; Fortenberry et al., submitted).

In summary, we found evidence for an association between maternal $PON1_{192QQ}$ and $PON1_{55MM}$ and parental reported ADHD-LP in children. These findings have important implications as potential susceptibility factors for ADHD-LP in children, and add to the growing body of evidence that maternal PON1 genotype may affect child neurodevelopment.

This study also cautiously provides additional preliminary exploratory evidence that children with certain non-protective PON1 genotypes may impact the development of ADHD-LP by modifying the exposure to OP pesticides (particularly CPF or CPF-methyl). However, additional studies among larger populations are needed.

Table 3.1 PON1 Genome Variation Estimates

SNP	N	Allele N(%)		Genotype N(%)			HW p-value
$PON1_{Q192R}$		A	G	AA	AG	GG	
Mom	531	433(41)	629(59)	90(17)	253(48)	188(35)	0.65
Child	532	425(40)	639(60)	81(15)	263(49)	188(35)	0.48
Mom*	109	91(42)	127(58)	18(17)	55(51)	36(33)	0.7
Child*	106	85(40)	127(60)	15(14)	54(51)	37(35)	0.51
$PON1_{L55M}$		A	T	AA	TA	TT	
Mom	458	806(88)	110(12)	357(78)	91(20)	10(2)	0.15
Child	478	841(88)	115(12)	372(78)	97(20)	9(2)	0.37
Mom*	73	124(85)	22(15)	53(73)	18(25)	2(3)	0.75
Child*	86	157(91)	15(9)	72(84)	13(15)	1(1)	0.64

*Population with measurements of 3, 5, 6-trichloro-2-pyridinol (TCPY)

HW=Hardy-Weinberg

Table 3.2 Descriptive maternal and child characteristics of ELEMENT cohorts

	Main Effects (N=591)		TCPY Population (N=187)	
Characteristics	N (%)	Median (25th, 75th)	N (%)	Median (25th, 75th)
Maternal Charateristics				
Maternal Age	586	25 (22, 29)	187	26 (22, 30)
Maternal Education	584	11 (9.0, 12)	187	11 (9.0, 11)
Maternal IQ	590	94 (86, 102)	187	96 (88, 103)
Socioeconomic Status	580	8.0 (6.0, 10.5)	178	8.0 (6.0, 10.5)
Blood Lead (ug/dl)	570	6.2(4.2, 8.7)	187	5.4(3.3, 7.8)
Marital Status				
Married	419(72)		139(74)	
Divorced	1(0.2)		1(0.5)	
Separated	2(0.3)		1(0.5)	
Never married	47(8)		15(8)	
Living with partner	117(20)		31(17)	
Breastfed				
Yes	504(92)		167(90)	
No	46(8)		19(10)	
Child Characteristics				
Head circumference (cm)	550	34(33, 35)	154	34(33.5, 35)
Weight (kg)	585	3.1(2.9, 3.4)	186	3.2(2.9, 3.5)
Height (cm)	581	50(49, 51)	181	50(49, 51)
Gestational age (wks)	586	39(38, 40)	185	39(38, 40)
Age at Testing (yrs)	580	9(8.0, 13)	178	7.5(7.0, 9.0)
Sex	586		187	
Male	302 (52)		89 (48)	
Female	284 (48)		97 (52)	

Table 3.3 Adjusted associations of PON1192 or PON155 polymorphisms and ADHD-LP in children between 6-13 years of age with adjustment for maternal and child genotype in separate models

			Mother		Child	
	Mothers	Children	Adjusted Model			
Psychometric Assessment*	N (%)	N (%)	B(95%CI)[a]	p	B(95%CI)[a]	p
ADHD Index						
PON1192 AG+AA	343(65)	344(65)	Ref		Ref	
PON1192 GG	188(35)	188(35)	1.14(-0.74, 3.01)	0.24	0.69(-1.24, 2.61)	0.48
PON155 TA+TT	101(22)	106(22)	Ref		Ref	
PON155 AA	357(78)	372(78)	0.67(-1.15, 2.49)	0.47	-0.48(-2.36, 1.40)	0.62
DSM IV Hyperactivity/Impulsivity						
PON1192 AG+AA	343(65)	344(65)	Ref		Ref	
PON1192 GG	188(35)	188(35)	1.09(-1.05, 3.22)	0.32	0.33(-1.91, 2.57)	0.77
PON155 TA+TT	101(22)	106(22)	Ref		Ref	
PON155 AA	357(78)	372(78)	**2.32(0.26, 4.38)**	**0.03**	0.33(-1.85, 2.52)	0.77
DSM IV Inattention						
PON1192 AG+AA	343(65)	344(65)	Ref		Ref	
PON1192 GG	188(35)	188(35)	1.26(-0.51,3.02)	0.16	0.64(-1.16, 2.44)	0.49
PON155 TA+TT	101(22)	106(22)	Ref		Ref	
PON155 AA	357(78)	372(78)	0.66(-1.05, 2.38)	0.45	-0.36(-2.12, 1.39)	0.68
DSM IV Total						
PON1192 AG+AA	343(65)	344(65)	Ref		Ref	
PON1192 GG	188(35)	188(35)	1.52(-0.38, 3.42)	0.12	0.006(-1.97, 1.95)	0.99
PON155 TA+TT	101(22)	106(22)	Ref		Ref	
PON155 AA	357(78)	372(78)	1.41(-0.43, 3.25)	0.13	-0.37(-2.28, 1.53)	0.7
Global Restlessness/Impulsivity Index						
PON1192 AG+AA	343(65)	344(65)	Ref		Ref	
PON1192 GG	188(35)	188(35)	1.34(-0.71, 3.40)	0.2	-0.43(-2.57, 1.70)	0.69
PON155 TA+TT	101(22)	106(22)	Ref		Ref	
PON155 AA	357(78)	372(78)	1.00(-1.00, 2.99)	0.33	0.14(-1.93, 2.22)	0.89
Attention problems						
PON1192 AG+AA	343(65)	344(65)	Ref		Ref	
PON1192 GG	188(35)	188(35)	1.42(-0.59, 3.43)	0.17	0.07(-1.95, 2.08)	0.95
PON155 TA+TT	101(22)	106(22)	Ref		Ref	
PON155 AA	357(78)	372(78)	0.19(-1.76, 2.13)	0.85	-1.33(-3.30, 0.63)	0.18
Hyperactivity						
PON1192 AG+AA	343(65)	344(65)	Ref		Ref	
PON1192 GG	188(35)	188(35)	0.65(-1.17, 2.47)	0.48	-1.18(-3.01, 0.66)	0.21
PON155 TA+TT	101(22)	106(22)	Ref		Ref	
PON155 AA	357(78)	372(78)	1.34(-0.42, 3.10)	0.13	-0.84(-2.63, 0.95)	0.36
CPT Clinical						
PON1192 AG+AA	343(65)	344(65)	Ref		Ref	
PON1192 GG	188(35)	188(35)	2.78(-0.63, 6.19)	0.11	1.83(-1.60, 5.25)	0.3
PON155 TA+TT	101(22)	106(22)	Ref		Ref	
PON155 AA	357(78)	372(78)	-0.75(-4.06, 2.56)	0.66	-1.74(-5.10, 1.61)	0.31

[a]Adjusted for maternal IQ, maternal education level, age of child at testing, sex of child, breastfed, maternal blood lead, delivery height, delivery head circumference

*PON1 SNPs adjusted in separate models

Table 3.4 Adjusted associations of PON1192 or PON155 polymorphisms and ADHD-LP in **MALE** children between 6-13 years of age with adjustment for maternal and child genotype in separate models

			Mother		Child	
	Mothers	Children	Adjusted Model			
Psychometric Assessment*	N (%)	N (%)	B(95%CI)[a]	p	B(95%CI)[a]	p
ADHD Index						
$PON1_{192}$ AG+AA	207(69)	213(72)	Ref		Ref	
$PON1_{192}$ GG	95(31)	81(28)	1.47(-0.97, 3.91)	0.24	1.16(-1.49, 3.81)	0.39
$PON1_{55}$ TA+TT	124(41)	112(38)	Ref		Ref	
$PON1_{55}$ AA	178(59)	182(62)	2.00(-0.34, 4.34)	0.09	-1.11(-3.58, 1.35)	0.38
DSM IV Hyperactivity/Impulsivity						
$PON1_{192}$ AG+AA	207(69)	213(72)	Ref		Ref	
$PON1_{192}$ GG	95(31)	81(28)	2.81(-0.01, 5.64)	0.05	0.70(-2.38, 3.79)	0.65
$PON1_{55}$ TA+TT	124(41)	112(38)	Ref		Ref	
$PON1_{55}$ AA	178(59)	182(62)	2.61(-0.11, 5.34)	0.06	-1.22(-4.08,1.65)	0.40
DSM IV Inattention						
$PON1_{192}$ AG+AA	207(69)	213(72)	Ref		Ref	
$PON1_{192}$ GG	95(31)	81(28)	0.59(-1.72, 2.90)	0.62	0.99(-1.47, 3.44)	0.43
$PON1_{55}$ TA+TT	124(41)	112(38)	Ref		Ref	
$PON1_{55}$ AA	178(59)	182(62)	1.77(-0.44, 3.99)	0.12	-0.61(-2.89, 1.67)	0.60
DSM IV Total						
$PON1_{192}$ AG+AA	207(69)	213(72)	Ref		Ref	
$PON1_{192}$ GG	95(31)	81(28)	1.84(-0.70, 4.38)	0.15	0.67(-2.09, 3.43)	0.63
$PON1_{55}$ TA+TT	124(41)	112(38)	Ref		Ref	
$PON1_{55}$ AA	178(59)	182(62)	2.22(-0.22, 4.66)	0.07	-0.96(-3.52, 1.61)	0.46
Global Restlessness/Impulsivity Index						
$PON1_{192}$ AG+AA	73(67)	69(65)	Ref		Ref	
$PON1_{192}$ GG	36(33)	37(35)	1.89(-0.83, 4.61)	0.17	-0.07(-3.10, 2.97)	0.97
$PON1_{55}$ TA+TT	20(27)	14(16)	Ref		Ref	
$PON1_{55}$ AA	53(73)	72(84)	1.85(-0.77, 4.48)	0.16	-0.57(-3.39, 2.25)	0.69
Attention problems						
$PON1_{192}$ AG+AA	207(69)	213(72)	Ref		Ref	
$PON1_{192}$ GG	95(31)	81(28)	1.27(-1.48, 4.01)	0.36	-0.21(-3.17, 2.76)	0.89
$PON1_{55}$ TA+TT	124(41)	112(38)	Ref		Ref	
$PON1_{55}$ AA	178(59)	182(62)	1.08(-1.57, 3.73)	0.42	-1.71(-4.46, 1.03)	0.22
Hyperactivity						
$PON1_{192}$ AG+AA	207(69)	213(72)	Ref		Ref	
$PON1_{192}$ GG	95(31)	81(28)	1.31(-1.29, 3.91)	0.32	-1.17(-3.99, 1.65)	0.41
$PON1_{55}$ TA+TT	124(41)	112(38)	Ref		Ref	
$PON1_{55}$ AA	178(59)	182(62)	1.89(-0.83, 4.61)	0.17	-0.22(-2.84, 2.40)	0.87
CPT Clinical						
$PON1_{192}$ AG+AA	207(69)	213(72)	Ref		Ref	
$PON1_{192}$ GG	95(31)	81(28)	2.57(-2.03, 7.16)	0.27	4.06(-1.06, 9.19)	0.12
$PON1_{55}$ TA+TT	124(41)	112(38)	Ref		Ref	
$PON1_{55}$ AA	178(59)	182(62)	1.27(-1.48, 4.01)	0.36	-0.21(-3.17, 2.76)	0.89

[a]Adjusted for maternal IQ, maternal education level, age of child at testing, sex of child, breastfed, maternal blood lead, delivery height, delivery head circumference

*PON1 SNPs adjusted in separate models

Table 3.5 Adjusted associations of PON1192 or PON155 polymorphisms and ADHD-LP in **FEMALE** children between 6-13 years of age with adjustment for maternal and child genotype in separate models

			Mother		Child	
	Mothers	Children	Adjusted Model			
Psychometric Assessment*	N (%)	N (%)	B(95%CI)[a]	p	B(95%CI)[a]	p
ADHD Index						
$PON1_{192}$ AG+AA	192(68)	183(64)	Ref		Ref	
$PON1_{192}$ GG	92(32)	101(36)	0.71(-2.22, 3.64)	0.63	-0.03(-2.89, 2.83)	0.98
$PON1_{55}$ TA+TT	109(38)	102(36)	Ref		Ref	
$PON1_{55}$ AA	175(72)	182(64)	-1.35(-4.23, 1.52)	0.35	0.16(-2.76, 3.07)	0.91
DSM IV Hyperactivity/Impulsivity						
$PON1_{192}$ AG+AA	192(68)	183(64)	Ref		Ref	
$PON1_{192}$ GG	92(32)	101(36)	-0.77(-4.07, 2.54)	0.65	-0.14(-3.51, 3.23)	0.94
$PON1_{55}$ TA+TT	109(38)	102(36)	Ref		Ref	
$PON1_{55}$ AA	175(72)	182(64)	1.68(-1.56, 4.92)	0.31	1.78(-1.65,5.20)	0.31
DSM IV Inattention						
$PON1_{192}$ AG+AA	192(68)	183(64)	Ref		Ref	
$PON1_{192}$ GG	92(32)	101(36)	1.90(-0.86, 4.66)	0.18	0.14(-2.58, 2.85)	0.92
$PON1_{55}$ TA+TT	109(38)	102(36)	Ref		Ref	
$PON1_{55}$ AA	175(72)	182(64)	-0.99(-3.71, 1.73)	0.47	-21(-2.98, 2.56)	0.88
DSM IV Total						
$PON1_{192}$ AG+AA	192(68)	183(64)	Ref		Ref	
$PON1_{192}$ GG	92(32)	101(36)	1.14(-1.78, 4.05)	0.44	-0.88(-3.75, 2.00)	0.55
$PON1_{55}$ TA+TT	109(38)	102(36)	Ref		Ref	
$PON1_{55}$ AA	175(72)	182(64)	0.12(-.75, 2.99)	0.93	0.13(-2.80, 3.06)	0.93
Global Restlessness/Impulsivity Index						
$PON1_{192}$ AG+AA	192(68)	183(64)	Ref		Ref	
$PON1_{192}$ GG	92(32)	101(36)	0.67(-2.52, 3.86)	0.68	-0.91(-4.02, 2.21)	0.57
$PON1_{55}$ TA+TT	109(38)	102(36)	Ref		Ref	
$PON1_{55}$ AA	175(72)	182(64)	0.07(-3.07, 3.21)	0.97	0.05(-3.15, 3.25)	0.97
Attention problems						
$PON1_{192}$ AG+AA	192(68)	183(64)	Ref		Ref	
$PON1_{192}$ GG	92(32)	101(36)	1.76(-1.22, 4.74)	0.25	0.10(-2.75, 2.94)	0.95
$PON1_{55}$ TA+TT	109(38)	102(36)	Ref		Ref	
$PON1_{55}$ AA	175(72)	182(64)	-1.17(-4.11, 1.76)	0.43	-1.08(-3.98, 1.82)	0.47
Hyperactivity						
$PON1_{192}$ AG+AA	192(68)	183(64)	Ref		Ref	
$PON1_{192}$ GG	92(32)	101(36)	0.08(-2.51, 2.68)	0.95	-1.22(-3.71, 1.27)	0.34
$PON1_{55}$ TA+TT	109(38)	102(36)	Ref		Ref	
$PON1_{55}$ AA	175(72)	182(64)	0.67(-2.52, 3.86)	0.68	-1.61(-4.15, 0.93)	0.21
CPT Clinical						
$PON1_{192}$ AG+AA	192(68)	183(64)	Ref		Ref	
$PON1_{192}$ GG	92(32)	101(36)	3.66(-1.56, 8.88)	0.17	-0.52(-5.32, 4.29)	0.83
$PON1_{55}$ TA+TT	109(38)	102(36)	Ref		Ref	
$PON1_{55}$ AA	175(72)	182(64)	1.76(-1.22, 4.74)	0.25	-0.09(-2.75, 2.94)	0.95

[a]Adjusted for maternal IQ, maternal education level, age of child at testing, sex of child, breastfed, maternal blood lead, delivery height, delivery head circumference

*PON1 SNPs adjusted in separate models

Table 3.6 Distribution of $PON1_{Q192R}$ Mother/Child Genotypes

	Child			
Maternal	AA	AG	GG	Total
AA	13	42	16	71
AG	29	100	64	193
GG	24	55	61	140
Total	66	197	141	404

Table 3.7 Distribution of $PON1_{L55M}$ Mother/Child Genotypes

	Child			
Maternal	TT	TA	AA	Total
TT	1	1	5	7
TA	0	21	46	67
AA	6	46	202	254
Total	7	68	253	328

Table 3.8 Adjusted associations of PON1192 or PON155 polymorphisms and ADHD-LP in children between 6-13 years of age with simultaneous adjustment for maternal and child genotype

			Mother		Child	
	Mothers	Children	Mother and Child Adjusted Models			
Psychometric Assessment*	N (%)	N (%)	B(95%CI)[a]	p	B(95%CI)[a]	p
ADHD Index						
PON1$_{192}$ AG+AA	283(64)	284(66)	Ref		Ref	
PON1$_{192}$ GG	159(35)	149(34)	0.57(-1.54, 2.68)	0.60	0.72(-1.40, 2.83)	0.51
PON1$_{55}$ TA+TT	84(22)	86(78)	Ref		Ref	
PON1$_{55}$ AA	295(78)	300(79)	1.30(-0.77, 3.38)	0.22	-1.59(-3.67, 0.49)	0.13
DSM IV Hyperactivity/Impulsivity						
PON1$_{192}$ AG+AA	283(64)	284(66)	Ref		Ref	
PON1$_{192}$ GG	159(35)	149(34)	1.29(1.15, 3.72)	0.30	0.09(-2.35, 2.53)	0.94
PON1$_{55}$ TA+TT	84(22)	86(78)	Ref		Ref	
PON1$_{55}$ AA	295(78)	300(79)	**3.27(0.89, 5.65)**	**0.01**	-1.49(-3.87, 0.89)	0.22
DSM IV Inattention						
PON1$_{192}$ AG+AA	283(64)	284(66)	Ref		Ref	
PON1$_{192}$ GG	159(35)	149(34)	0.73(-1.26, 2.72)	0.47	1.23(-0.73, 3.20)	0.22
PON1$_{55}$ TA+TT	84(22)	86(78)	Ref		Ref	
PON1$_{55}$ AA	295(78)	300(79)	0.96(-1.04, 2.95)	0.35	-1.14(-3.11, 0.82)	0.25
DSM IV Total						
PON1$_{192}$ AG+AA	283(64)	284(66)	Ref		Ref	
PON1$_{192}$ GG	159(35)	149(34)	1.25(-0.92, 3.41)	0.26	0.08(-2.09, 2.25)	0.94
PON1$_{55}$ TA+TT	84(22)	86(78)	Ref		Ref	
PON1$_{55}$ AA	295(78)	300(79)	**2.17(0.047, 4.29)**	**0.05**	-1.67(-3.79, 0.46)	0.12
Global Restlessness/Impulsivity Index						
PON1$_{192}$ AG+AA	283(64)	284(66)	Ref		Ref	
PON1$_{192}$ GG	159(35)	149(34)	0.91(-1.45, 3.27)	0.45	-0.21(-2.57, 2.15)	0.86
PON1$_{55}$ TA+TT	84(22)	86(78)	Ref		Ref	
PON1$_{55}$ AA	295(78)	300(79)	1.16(-1.17, 3.48)	0.33	-0.73(-3.06, 1.60)	0.54
Attention problems						
PON1$_{192}$ AG+AA	283(64)	284(66)	Ref		Ref	
PON1$_{192}$ GG	159(35)	149(34)	**2.27(0.002, 4.53)**	**0.05**	-0.90(-3.17, 1.37)	0.44
PON1$_{55}$ TA+TT	84(22)	86(78)	Ref		Ref	
PON1$_{55}$ AA	295(78)	300(79)	0.70(-1.54, 2.94)	0.54	-1.97(-4.22, 0.27)	0.08
Hyperactivity						
PON1$_{192}$ AG+AA	283(64)	284(66)	Ref		Ref	
PON1$_{192}$ GG	159(35)	149(34)	1.02(-0.99, 3.03)	0.32	-1.35(-3.37, 0.67)	0.19
PON1$_{55}$ TA+TT	84(22)	86(78)	Ref		Ref	
PON1$_{55}$ AA	295(78)	300(79)	1.55(-0.44, 3.53)	0.13	-1.58(-3.57, 0.40)	0.12
CPT Clinical						
PON1$_{192}$ AG+AA	283(64)	284(66)	Ref		Ref	
PON1$_{192}$ GG	159(35)	149(34)	3.01(-0.81, 6.84)	0.12	1.54(-2.31, 5.40)	0.43
PON1$_{55}$ TA+TT	84(22)	86(78)	Ref		Ref	
PON1$_{55}$ AA	295(78)	300(79)	0.39(-3.34, 4.15)	0.84	-3.55(-7.37, 0.26)	0.07

[a]Adjusted for maternal IQ, maternal education level, age of child at testing, sex of child, breastfed, maternal blood lead, delivery height, delivery head circumference

*PON1 SNPs adjusted in separate models

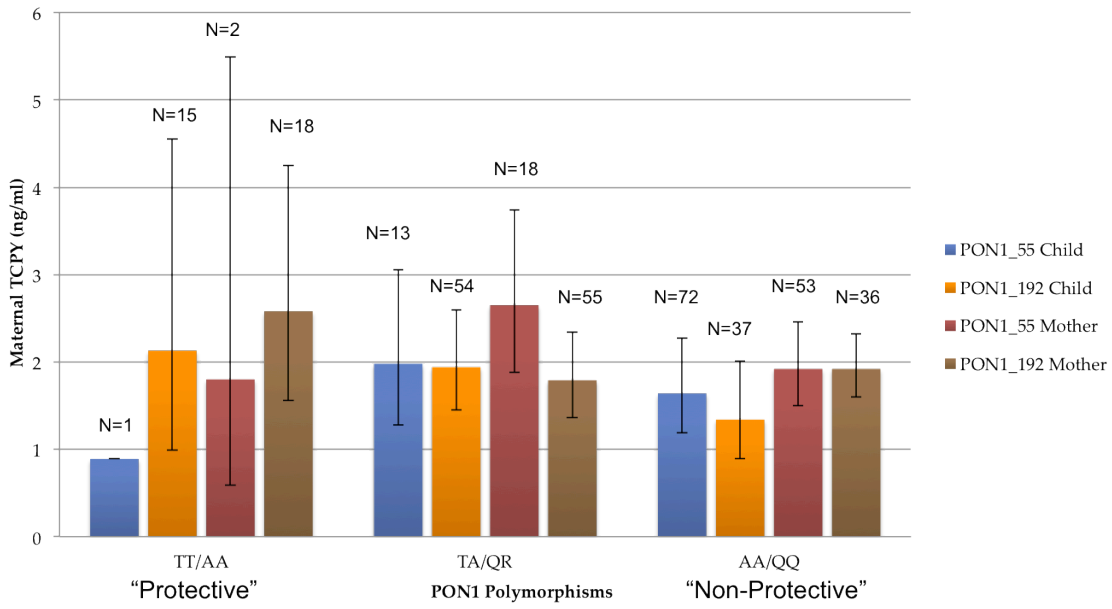

Figure 3.1 Geometric mean and 95% Confidence Intervals of TCPY concentrations by PON1 genotype

Table 3.9 Prenatal LnTCPY-adjusted associations of PON1192 or PON155 polymorphisms and ADHD-LP in children between 6-13 years of age adjusted for maternal or child genotype

			Mother		Child	
	Mothers	Children	Adjusted Model			
Psychometric Assessment*	N (%)	N (%)	B(95%CI)[a]	p	B(95%CI)[a]	p
ADHD Index						
PON1$_{192}$ AG+AA	73(67)	69(65)	Ref		Ref	
PON1$_{192}$ GG	36(33)	37(35)	1.97(0.29, -1.67)	0.76	0.28(0.88, -3.36)	0.88
PON1$_{55}$ TA+TT	20(27)	14(16)	Ref		Ref	
PON1$_{55}$ AA	53(73)	72(84)	1.74(-1.42, 4.90)	0.28	-0.94(-3.99, 2.11)	0.55
DSM IV Hyperactivity/Impulsivity						
PON1$_{192}$ AG+AA	73(67)	69(65)	Ref		Ref	
PON1$_{192}$ GG	36(33)	37(35)	-0.29(-4.19, 3.60)	0.88	-1.30(-5.18, 2.58)	0.51
PON1$_{55}$ TA+TT	20(27)	14(16)	Ref		Ref	
PON1$_{55}$ AA	53(73)	72(84)	1.62(-1.75, 4.99)	0.34	-0.72(-4.96,1.53)	0.30
DSM IV Inattention						
PON1$_{192}$ AG+AA	73(67)	69(65)	Ref		Ref	
PON1$_{192}$ GG	36(33)	37(35)	0.64(-2.90, 4.19)	0.72	0.68(-2.86, 4.22)	0.70
PON1$_{55}$ TA+TT	20(27)	14(16)	Ref		Ref	
PON1$_{55}$ AA	53(73)	72(84)	2.23(-0.83, 5.29)	0.15	-0.88(-3.84, 2.08)	0.56
DSM IV Total						
PON1$_{192}$ AG+AA	73(67)	69(65)	Ref		Ref	
PON1$_{192}$ GG	36(33)	37(35)	0.26(-3.31, 3.83)	0.89	0.14(-3.43, 3.70)	0.94
PON1$_{55}$ TA+TT	20(27)	14(16)	Ref		Ref	
PON1$_{55}$ AA	53(73)	72(84)	2.56(-0.52, 5.63)	0.10	-1.40(-4.38, 1.58)	0.36
Global Restlessness/Impulsivity Index						
PON1$_{192}$ AG+AA	73(67)	69(65)	Ref		Ref	
PON1$_{192}$ GG	36(33)	37(35)	1.42(-2.40, 5.24)	0.46	-0.91(-4.73, 2.91)	0.64
PON1$_{55}$ TA+TT	20(27)	14(16)	Ref		Ref	
PON1$_{55}$ AA	53(73)	72(84)	1.34(-1.98, 4.66)	0.43	0.05(-3.15, 3.25)	0.97
Attention problems						
PON1$_{192}$ AG+AA	73(67)	69(65)	Ref		Ref	
PON1$_{192}$ GG	36(33)	37(35)	2.45(-0.93, 6.49)	0.23	-0.34(-4.39, 3.71)	0.87
PON1$_{55}$ TA+TT	20(27)	14(16)	Ref		Ref	
PON1$_{55}$ AA	53(73)	72(84)	**3.75(0.27, 7.22)**	**0.03**	-1.62(-5.00, 1.77)	0.35
Hyperactivity						
PON1$_{192}$ AG+AA	73(67)	69(65)	Ref		Ref	
PON1$_{192}$ GG	36(33)	37(35)	1.03(-2.63, 4.69)	0.58	0.56(-3.10, 4.22)	0.76
PON1$_{55}$ TA+TT	20(27)	14(16)	Ref		Ref	
PON1$_{55}$ AA	53(73)	72(84)	1.88(-1.29, 5.05)	0.24	-0.92(-3.98, 2.15)	0.56
CPT Clinical						
PON1$_{192}$ AG+AA	73(67)	69(65)	Ref		Ref	
PON1$_{192}$ GG	36(33)	37(35)	0.28(-7.29, 7.85)	0.94	**9.93(2.63, 17.2)**	**0.01**
PON1$_{55}$ TA+TT	20(27)	14(16)	Ref		Ref	
PON1$_{55}$ AA	53(73)	72(84)	-5.53(-12.0, 0.97)	0.09	-0.76(-7.01, 5.49)	0.81

[a]Adjusted for lntcpy, specific gravity, maternal IQ, maternal education level, age of child at testing, breastfed, sex of child
*PON1 SNPs adjusted in separate models

Table 3.10 Adjusted associations of PON1192 genotype-prenatal urinary TCPY interaction and ADHD-LP in children 6-13 years of age adjusted for maternal or child genotype

			Mother		Child	
	Mothers	Children	Interaction Models			
Psychometric Assessment	N (%)	N (%)	B(95%CI)[a]	p	B(95%CI)[b]	p
ADHD Index						
LnTCPY			0.13(-1.43, 1.68)	0.87	0.29(-1.38, 1.96)	0.73
PON1$_{192}$ AG+AA	73(67)	69(65)	Ref		Ref	
PON1$_{192}$ GG	36(33)	37(35)	1.29(-2.97, 5.55)	0.55	0.26(-3.52, 4.04)	0.89
PON1$_{192}$ GG*LnTCPY			1.14(-2.53, 4.80)	0.54	0.08(-2.94, 3.11)	0.96
DSM IV Hyperactivity/Impulsivity						
LnTCPY			-0.51(-2.17, 1.16)	0.55	-0.95(-2.72, 0.83)	0.30
PON1$_{192}$ AG+AA	73(67)	69(65)	Ref		Ref	
PON1$_{192}$ GG	36(33)	37(35)	0.03(-4.53, 4.60)	0.99	-1.77(-5.79, 2.24)	0.38
PON1$_{192}$ GG*LnTCPY			-0.54(-4.47, 3.38)	0.78	1.52(-1.70, 4.73)	0.35
DSM IV Inattention						
LnTCPY			0.07(-1.44, 1.59)	0.92	-0.11(-1.73, 1.52)	0.90
PON1$_{192}$ AG+AA	73(67)	69(65)	Ref		Ref	
PON1$_{192}$ GG	36(33)	37(35)	0.95(-3.20, 5.10)	0.65	0.47(-3.20, 4.13)	0.80
PON1$_{192}$ GG*LnTCPY			-0.51(-4.08, 3.06)	0.78	0.68(-2.25, 3.62)	0.65
DSM IV Total						
LnTCPY			-0.25(-1.77, 1.28)	0.75	-0.50(-2.13, 1.13)	0.55
PON1$_{192}$ AG+AA	73(67)	69(65)	Ref		Ref	
PON1$_{192}$ GG	36(33)	37(35)	0.28(-3.90, 4.47)	0.89	-0.21(-3.90, 3.47)	0.91
PON1$_{192}$ GG*LnTCPY			-0.04(-3.64, 3.55)	0.98	1.12(-1.83, 4.07)	0.46
Global Restlessness/Impulsivity Index						
LnTCPY			-0.59(-2.23, 1.04)	0.47	-1.05(-2.79, 0.70)	0.24
PON1$_{192}$ AG+AA	73(67)	69(65)	Ref		Ref	
PON1$_{192}$ GG	36(33)	37(35)	1.80(-2.68, 6.27)	0.43	-1.48(-5.43, 2.46)	0.46
PON1$_{192}$ GG*LnTCPY			-0.62(-4.48, 3.23)	0.75	1.82(-1.33, 4.98)	0.26
Attention problems						
LnTCPY			0.79(-0.94, 2.51)	0.37	0.69(-1.16, 2.55)	0.46
PON1$_{192}$ AG+AA	73(67)	69(65)	Ref		Ref	
PON1$_{192}$ GG	36(33)	37(35)	2.72(-2.01, 7.45)	0.26	-0.51(-4.71, 3.68)	0.81
PON1$_{192}$ GG*LnTCPY			-0.45(-4.52, 3.62)	0.83	0.54(-2.82, 3.90)	0.75
Hyperactivity						
LnTCPY			-0.57(-2.14, 0.99)	0.47	-0.84(-2.52, 0.84)	0.32
PON1$_{192}$ AG+AA	73(67)	69(65)	Ref		Ref	
PON1$_{192}$ GG	36(33)	37(35)	1.81(-2.47, 6.09)	0.41	0.32(-3.47, 4.11)	0.87
PON1$_{192}$ GG*LnTCPY			-1.30(-4.98, 2.39)	0.49	0.77(-2.26, 3.81)	0.62
CPT Clinical						
LnTCPY			0.53(-2.69, 3.75)	0.75	-0.05(-3.44, 3.35)	0.98
PON1$_{192}$ AG+AA	73(67)	69(65)	Ref		Ref	
PON1$_{192}$ GG	36(33)	37(35)	1.87(-6.94, 10.7)	0.68	9.43(1.84, 17.0)	0.02
PON1$_{192}$ GG*LnTCPY			-2.72(-10.4, 4.96)	0.48	1.52(-4.59, 7.63)	0.62

[a]Adjusted for specific gravity, maternal IQ, maternal education level, age of child at testing, breastfed, sex of child

*Maternal and child genotypes adjusted in separate models

Table 3.11 Adjusted associations of PON155 genotype-prenatal TCPY interaction and ADHD-LP in children 6-13 years of age adjusted for maternal or child

			Mother		Child	
	Mothers	Children	Interaction Models			
Psychometric Assessment	N (%)	N (%)	B(95%CI)[a]	p	B(95%CI)[a]	p
ADHD Index						
LnTCPY			0.33(-1.26, 1.91)	0.68	-0.09(-2.12, 1.94)	0.93
PON1$_{55}$ TA+TT	20(27)	14(16)	Ref		Ref	
PON1$_{55}$ AA	53(73)	72(84)	2.02(-1.74, 5.79)	0.29	-1.27(-4.55, 2.02)	0.45
PON1$_{55}$ AA*LnTCPY			-0.45(-3.71, 2.80)	0.78	0.69(-1.81, 3.18)	0.59
DSM IV Hyperactivity/Impulsivity						
LnTCPY			-0.68(-2.37, 1.02)	0.43	-2.02(-4.16, 0.11)	0.06
PON1$_{55}$ TA+TT	20(27)	14(16)	Ref		Ref	
PON1$_{55}$ AA	53(73)	72(84)	1.35(-2.67, 5.37)	0.51	-2.96(-6.42, 0.50)	0.09
PON1$_{55}$ AA*LnTCPY			0.43(-3.05, 3.90)	0.81	2.59(-0.04, 5.21)	0.05
DSM IV Inattention						
LnTCPY			0.12(-1.41, 1.66)	0.88	-0.82(-2.78, 1.14)	0.41
PON1$_{55}$ TA+TT	20(27)	14(16)	Ref		Ref	
PON1$_{55}$ AA	53(73)	72(84)	2.80(-0.85, 6.44)	0.13	-1.63(-4.81, 1.55)	0.31
PON1$_{55}$ AA*LnTCPY			-0.90-4.05, 2.25)	0.57	1.55(-0.86, 3.96)	0.21
DSM IV Total						
LnTCPY			-0.28(-1.83, 1.26)	0.72	-1.54(-3.50, 0.42)	0.12
PON1$_{55}$ TA+TT	20(27)	14(16)	Ref		Ref	
PON1$_{55}$ AA	53(73)	72(84)	2.66(-1.01, 6.32)	0.15	-2.52(-5.69, 0.65)	0.12
PON1$_{55}$ AA*LnTCPY			-0.16(-3.33, 3.00)	0.92	2.33(-0.08, 4.74)	0.06
Global Restlessness/Impulsivity Index						
LnTCPY			-0.53(-2.20, 1.13)	0.53	-2.02(-4.12, 0.09)	0.06
PON1$_{55}$ TA+TT	20(27)	14(16)	Ref		Ref	
PON1$_{55}$ AA	53(73)	72(84)	1.80(-2.16, 5.75)	0.37	-1.20(-4.61, 2.21)	0.49
PON1$_{55}$ AA*LnTCPY			-0.73(-4.14, 2.68)	0.67	**2.60(0.01, 5.19)**	**0.05**
Attention problems						
LnTCPY			0.59(-1.15, 2.34)	0.50	0.42(-1.83, 2.67)	0.71
PON1$_{55}$ TA+TT	20(27)	14(16)	Ref		Ref	
PON1$_{55}$ AA	53(73)	72(84)	3.17(-0.96, 7.31)	0.13	-1.94(-5.58, 1.71)	0.30
PON1$_{55}$ AA*LnTCPY			0.92(-2.66, 4.49)	0.51	0.67(-2.10, 3.43)	0.64
Hyperactivity						
LnTCPY			-0.72(-2.31, 0.87)	0.37	-1.75(-3.78, 0.27)	0.09
PON1$_{55}$ TA+TT	20(27)	14(16)	Ref		Ref	
PON1$_{55}$ AA	53(73)	72(84)	1.86(-1.92, 5.64)	0.33	-1.86(-5.14, 1.42)	0.26
PON1$_{55}$ AA*LnTCPY			0.03(-3.23, 3.30)	0.98	1.96(-0.52, 4.45)	0.12
CPT Clinical						
LnTCPY			0.31(-2.95, 3.57)	0.85	2.07(-2.12, 6.26)	0.33
PON1$_{55}$ TA+TT	20(27)	14(16)	Ref		Ref	
PON1$_{55}$ AA	53(73)	72(84)	-6.07(-13.8, 1.67)	0.12	0.83(-5.88, 7.54)	0.81
PON1$_{55}$ AA*LnTCPY			0.87(-5.84, 7.58)	0.80	-3.31(-8.45, 1.83)	0.21

[a]Adjusted for specific gravity, maternal IQ, maternal education level, age of child at testing, breastfed, sex of child

*Maternal and child genotypes adjusted in separate models

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Chapter IV

Association between urinary 3, 5, 6-trichloro-2-pyridinol, a metabolite of chlorpyrifos and chlorpyrifos-methyl, and serum T4 and TSH in NHANES 1999-2002

Abstract

Thyroid hormones are vital to a host of human physiological functions in both children and adults. Exposures to chemicals, including chlorpyrifos, have been found to modify thyroid signaling at environmentally relevant levels in animal studies. The aim of this study was to examine circulating T4 and TSH levels in relation to urinary concentrations of 3, 5, 6-trichloro-2-pyridinol (TCPY), a metabolite of the organophosphorus insecticides chlorpyrifos and chlorpyrifos-methyl, using data from individuals 12 years and older from the U.S. National Health and Nutrition Examination Surveys (NHANES). NHANES datasets from 1999-2000 and 2001-2002 were combined, and individuals with thyroid disease, those taking thyroid medications, and pregnant women were excluded (N=3249). Multivariable linear regression models for relationships between log-transformed urinary TCPY and serum total T_4 or log (TSH) were constructed adjusting for important covariates. Models were stratified by sex and a categorical age variable (12-18, 18-40, 40-60, and >60 years). In male participants, an interquartile range (IQR) increase in urinary TCPY was associated with statistically significant increases in serum T_4 of 3.8% (95^{th} CI 0.75 to 7.0) among those 12-18 years of age and 3.5% (95^{th} CI 0.13 to

7.0) in the 18-40 year age group, relative to median T4 levels using unweighted models. An IQR increase in TCPY was also associated with decreases in TSH of 10.7% (-18.7--2.05) among men 18-40 years old and 20.0% (95th CI -28.9 to -9.86) among men >60 years old. Conversely, urinary TCPY was positively associated with TSH in females >60 years of age. Further research to confirm these findings, elucidate mechanisms of action, and explore the clinical and public health significance of such alterations in thyroid hormones is needed. [2]

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Introduction

Globally and domestically, chlorpyrifos is the most widely used non-persistent, organophosphate (OP) pesticide (Ye et al., 2008; Bradman and Whyatt, 2005; Timchalk et al., 2007) with approximately 10 million pounds agriculturally applied annually in the U.S. (EPA, 2011). Residential use of chlorpyrifos in the U.S. was banned in 2000, but widespread exposure remains likely in the U.S. and elsewhere (EPA, 2001). Environmental human exposure is ubiquitous and occurs through inhalation of vapors and aerosols from spray drift (Pang et al., 2002; Whyatt et al., 2009), ingestion of residuals on food and house dust/soil (Pang et al., 2002; Salas et al., 2003), and dermal absorption following skin contact (Panuwet et al., 2008) and is usually excreted within hours or days in urine (Bradman and Whyatt, 2005; Timchalk et al., 2007). Human exposure has been quantified from various media such as indoor air (Pang et al., 2002), carpet dust (Pang et al., 2002), breast milk (Weldon et al., 2011), fruit (Riederer et al., 2009), and water (Carvalho et al., 2002). Human exposure has not only been quantified in the U.S. but in other countries such as The Netherlands (Ye et al., 2008), China (Panuwet et al., 2008), Germany (Koch et al., 2001), and Italy (Aprea et al., 1999).

Environmental exposures to chemicals have been found to modify thyroid hormone signaling (Blount et al., 2006; De Angelis et al., 2009), which are vital to a host of physiological functions in both children and adults (Yen et al., 2001; Thrasher et al., 2002), even at low-levels of exposure. Studies examining thyroid hormones in relation to non-persistent pesticides remain limited but animal and human studies suggest that chlorpyrifos or other organophosphate pesticides may alter thyroid hormone levels

(Lacasaa et al., 2010; Meeker et al., 2006). In animal studies, decreased T4 and cellular changes within the thyroid and pituitary glands in response to chlorpyrifos exposure were observed (Ghisari and Bonefeld-Jorgensen, 2005; De Angelis et al., 2009; Jeong et al., 2006).

In the present study, we assessed the relationship between urinary TCPY, a biomarker of exposure to chlorpyrifos, chlorpyrifos-methyl, or TCPY, and altered serum thyroid hormone levels using data from the U.S. National Health and Nutrition Examination Survey (NHANES).

Methods

Study Population

The National Health and Nutrition Examination Survey (NHANES) is a cross-sectional study designed to be representative of the civilian, non-institutionalized population of the United States. Sample design consists of a stratified, multistage, probability sample of civilian, non-institutionalized adults and children (CDC, 2010). A laboratory subsample (one-third of the total sample) in which both urinary TCPY and thyroid measurements were taken for the 1999-2000 and 2001-2002 NHANES surveys were used for this analysis. This subsample consisted of 3249 individuals after the exclusion of pregnant women (N=194), participants taking thyroid (and antithyroid) medications including levothyroxine sodium (N=103), and those who currently have or have ever had thyroid disease (N=141).

Measurements

Sociodemographic information obtained from NHANES demographic files included sex, race, household income, and age. Body mass index (BMI) (kg/m^2) was obtained from the body measures examination data files. Data on urinary 3, 5, 6-trichloro-2-pyridinol (TCPY), urinary creatinine, serum total thyroxine (T4) and thyrotropin (TSH), and serum cotinine were obtained from the NHANES laboratory data files.

Medications were identified in the prescription drug medication and the analgesics/pain reliever questionnaires and included: estrogens and/or progesterone, other steroid hormones (androgens, adrenal corticosteroids, tamoxifen, raloxifene, and pituitary hormones), non-steroidal anti-inflammatory drugs (NSAIDs), furosemide, beta-blockers, blood glucose regulators, and other medications thought to affect thyroid hormones (amidoarone, carbamazepine,chlorpropamide, carbidopa/levodopa, heparin, interferon, lithium, phenytoin, phenobarbital, and sulfasalazine) (Turyk et al. 2007).

Laboratory Methods

Total thyroxine

Samples collected in 1999-2000 and 2001 were analyzed for serum T_4 using the Hitachi 704 method while in 2002 T_4 levels in serum samples were determined using a paramagnetic particle, chemiluminescent, competitive binding enzyme immunoassay on a Beckman Access2 Immunoassay System(Aoki et al., 2007; CDC, 2006). The analytical range for T_4 was 6.4 to 390 nmol/L (0.5 – 30.3 µg/dL) and the laboratory reference (i.e.,

normal) range was 69.5 to 164.7 nmol/L (5.4 to 12.8 µg/dL) (Aoki et al., 2007; CDC, 2006).

Thyrotropin

1999-2000 and 2001 samples were analyzed for TSH using the IMx ultrasensitive hTSH II microparticle enzyme immunoassay technique while in 2002 a two-site, paramagnetic particle chemiluminescent immunoassay was used. Analytical sensitivity ranged from 0.01 to 100 mIU/L (Aoki et al., 2007); (CDC, 2006). The laboratory reference range for 1999-2000 and 2001 samples were reported as 0.47 to 5.01 mIU/L (Aoki et al., 2007; CDC, 2006). The laboratory reference range for 2002 samples was reported as 0.24 to 5.4 mIU/L (Aoki et al., 2007; CDC, 2006).

TCPY

Urine samples (2 mL) were spiked with stable isotopically labeled TCPy and then subjected to an enzyme hydrolysis to liberate glucuronide- and sulfate-bound TCPy. Hydrolysates were extracted using mixed-polarity solid-phase extraction cartridges (CDC, 2006; Olsson et al., 2004). Elutes (methanol) were concentrated and analyzed using HPLC/tandem MS (Olsson et al., 2004) with both quantification and confirmation ions monitored. TCPY was quantified using isotope dilution calibration. The limit of detection (LOD) of TCPY was 0.40µg/L in urine.

Creatinine

Urine samples were analyzed via the Jaffé rate reaction, in which creatinine reacts with picrate in an alkaline solution to form a red creatinine-picrate complex, using a Beckman CX3 Chemistry analyzer (CDC, 2006; Barr et al 2005).

Statistical Analysis

The Survey Procedures in SAS Version 9.2 was utilized for most analyses. Descriptive statistics for demographic information were calculated along with the distribution of TCPY and thyroid hormones. Values equal to the limit of detection (LOD) (0.40 μg/L) divided by the square root of two were imputed for values of TCPY less than the LOD. Appropriate statistical weights were used to adjust for study design, oversampling and non-response. However, we constructed models both with and without including the sample weights since the weighted method may result in an inefficient analysis due to the large variability in assigned weights, as well as when covariates used in the creation of weights (such as age, sex, and ethnicity) are included in the analysis (Korn and Graubard, 1991).

Because some variables were not normally distributed, we used natural log (ln)-transformations of TSH and TCPY for analysis while serum T4 was modeled untransformed. Bivariate analyses between dependent variables serum T4 and log (TSH) and the independent variable log (TCPY) were conducted as well. Multivariable regression was used to construct separate models for each hormone stratified by gender and a categorical age variable using PROC SURVEYREG. Age was categorized as follows: 12-<18yrs, 18-40yrs, 40-60yrs, and >60yrs. Continuous variables for age, BMI, serum cotinine, and (log) urinary creatinine, and categorical variables for race and income, were included in adjusted models. Race was categorized as: Mexican Americans, Whites, African Americans, and Other. Income was categorized as: less than

$19,999 per year and greater than $20,000 per year. We also considered categories for prescribed thyroid medications: Furosamide, Betablockers, NSAID, Steroids, Otherdrug, estrogen (E2), and Estrogen/Progesterone (E2prog) medications. However, their inclusion did not impact effect estimates and they were not included in final models. To improve interpretability, regression coefficients were presented as a percent change in serum T4 and TSH for an interquartile range (IQR) increase in urinary TCPY levels. Interaction terms for TCPY with sex and age were explored in a secondary analysis. Lastly, using logistic regression models, we explored whether TCPY was associated with being categorized as hypothyroid or hyperthyroid based on laboratory reference ranges.

Results

Descriptive statistics are shown in Table 1. In this population, 16% of TCPY measurements were below the LOD. Table 2 shows results for crude and adjusted regression coefficients for associations between urinary TCPY and serum T4 and TSH, stratified by sex. Results of the crude analyses revealed a positive relationship between urinary TCPY and serum T4 that was significant for males between the ages of 18 – 40 years for the both statistically weighted and unweighted models. In the unweighted multivariable model for males 18 – 40 years of age, an increase of 3.54% (95^{th} CI 0.13 to 6.96) was observed for serum T4 levels in relation to an interquartile range (IQR) increase in TCPY levels. For males 12-<18 years of age, an IQR increase in TCPY was associated with a 3.85% (95^{th} CI 0.75 to 6.95) increase in serum T4.. In males >60 years of age an IQR increase in TCPY was associated with a 14.5% (95^{th} CI -27.6 to -1.11)

decrease in TSH. Conversely, TCPY was positively associated with TSH in women >60 years of age. Effect estimates in the weighted multivariable models were overall similar to those from the unweighted models. An IQR increase in TCPY was associated with 10.7% (95^{th} CI -18.7 to -2.05) 20.0% (95^{th} CI -28.9 to -9.86) decreases in TSH, among males 18 – 40 and >60 years of age, respectively.

In our secondary analysis (results not shown) the interaction term between TCPY and age was statistically significant ($p<0.05$), and the interaction term between TCPY and sex statistically suggestive ($p<0.1$), in relation to serum T4 in multivariable models. Neither of these interaction terms approached statistical significance for TSH. Finally, no relationship was observed between urinary TCPY and hypothyroid or hyperthyroid status.

Discussion

The results of this study suggest that there is a positive relationship between urinary TCPY and serum total T4, and a negative relationship between TCPY and serum TSH, in adolescent males and/or men of reproductive age. There was also evidence of decreased and increased TSH in relation to urinary TCPY among males and females >60 years of age, respectively. These findings add to the existing evidence that exposure to certain organophosphate insecticides or their metabolites may disrupt the hypothalamic-pituitary-thyroid (HPT) axis. However, the exact mechanism of action is not understood, as there are only limited reports to date regarding the relationship between exposure to chlorpyrifos or chlorpyrifos-methyl and thyroid function.

In a human observational study, an inverse association between urinary TCPY and free T4 was reported in 322 adult men of reproductive age recruited through an infertility clinic (Meeker et al., 2006). This was not consistent with results from the present study, where we observed a positive association between urinary TCPY and total T4 in adolescent males (<18 years) and males 18–40 years of age. However, serum levels of free T4 were not available in NHANES 1999-2002, and the study of men from an infertility clinic did not measure total T4. In an occupational study of 136 male floriculture workers that examined the association between thyroid hormones (T3, T4, and TSH) and OP exposure, urinary dialkyl phosphate (DAP) concentrations were associated with increased levels of TSH and total T4 which supports the results of this study; however, urinary DAPs may reflect exposure to numerous OPs and exposure to chlorpyrifos more specifically was not assessed in the study (Lacasana et al., 2010).

Several animal studies investigating thyroid effects related to chlorpyrifos or other OPs may support our results suggesting that TCPY alters thyroid signaling, though the specific findings have not fully consistent between studies. Our observation of sex differences in these relationships is supported by a couple of these studies, whereas experimental support or explanation for the differences we found related to age is lacking. In a study involving male Wistar albino rats subjected to acute organophosphate (methamidophos; dimethyl phosphoramidothioate) exposure, a decrease in serum T4, T3, and TSH levels was observed and resulted in secondary hypothyroidism and sick euthyroidism (Satar et al 2005). A decrease in serum T4 levels was also observed in CD1 mice (both in dams and F1) after developmental exposure to chlorpyrifos at doses low

enough to not elicit inhibition of brain acetylcholinesterase (AchE) (De Angelis et al., 2009). Both sexes of F1 CD1 mice showed reduced serum T4 levels, and, perhaps consistent with the present study, a more significant effect was observed in males compared to females (De Angelis et al., 2009). Jeong et al. (2006) reported that chlorpyrifos-methyl induces hypothyroidism (decreased serumT4 and increased serum TSH) and altered thyroid and pituitary gland weights through sexual maturation and adulthood in rats after long-term *in utero* and postnatal exposure (Jeong et al., 2006). Interestingly, dose-response relationships between exposure and thyroid hormones appeared stronger among the male rates. Finally, the thyroid disrupting potential of chlorpyrifos was also demonstrated *in vitro* by a study of rat pituitary GH3 cells, where chlorpyrifos exposure altered T3-induced cell growth (Ghisari and Bonefeld-Jorgensen, 2005).

Our analysis had several limitations. The analysis was based on a single measure of urinary TCPY and serum thyroid hormone levels. Despite the short half-life of chlorpyrifos (approximately 27 hours in the body) and substantial temporal variability in exposure levels over time (CDC, 2010; Meeker et al. 2005), urinary TCPY is still considered to be the best and most specific biomarker of chlorpyrifos and chlorpyrifos-methyl exposure (Barr et al., 2006). Thyroid hormone levels, especially TSH, may also have significant intraindividual variability over time (Hollowell et al., 2002;Surks et al., 2005). Another limitation is that pregnant women, infants, and young children were not included in this analysis. Human and experimental studies have shown that fetuses and infants were more sensitive than adults to many environmental toxicants, including

chlorpyrifos (Timchalk et al., 2007). Also, there is growing evidence of the adverse impact of exposure to chlorpyrifos on fetal growth and early childhood neurodevelopment (Perera et al., 2005; Eskenazi et al., 2007; Rauh et al., 2006, 2011; Engel et al., 2011). Nevertheless, our findings of a relationship between urinary TCPY and markers of thyroid function may help inform potential mechanisms involved in adverse childhood neurodevelopment associated with OP exposure but should be explored in developmental cohort studies.

Despite the above limitations, this is the largest study to date examining the relationship between thyroid hormones and a biomarker of chlorpyrifos exposure in a human population. This will add to the body of knowledge assessing how environmental exposures impact thyroid signaling, which is vital to numerous human physiologic functions.

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Table 4.1 Descriptive Characteristics of NHANES 1999-2000 and 2001-2002 Population (N=3249)

Variable	12 - <18 years (n=506; n=550)	18-40 years (n=506; n=511)	40-60 years (n=377; n=333)	>60 years (n=200; n=218)
	Median (25th, 75th)			
	Male | Female	Male | Female	Male | Female	Male | Female
Age (yrs)	15.0(13.0, 16.0) | 14.0(13.0, 16.0)	25.0(19.0, 33.0) | 26.0(19.0, 34.0)	49.0(44.0, 53.0) | 49.0(44.0, 53.0)	72.0(66.0, 78.0) | 69.0(63.0, 77.0)
BMI (kg/m2)	21.8(19.1, 26.1) | 21.9(19.5, 26.0)	25.8(22.3, 29.4) | 25.1(22.1, 30.6)	27.6(24.5, 30.5) | 27.9(23.8, 33.0)	27.9(24.6, 31.1) | 27.6(24.6, 31.2)
Creatinine (mg/dL)	168(114, 230) | 139(83.0, 204)	170(116, 232) | 125(76.0, 189)	153.0(106.0, 205) | 90.0(43.0, 141)	115.5(79.5, 162) | 72.0(43.0, 115)
Cotinine (ng/ml)	0.12(0.04, 0.79) | 0.09(0.04, 0.64)	0.92(0.06, 100) | 0.13(0.04, 17.9)	0.27(0.04, 173) | 0.07(0.04, 1.88)	0.041(0.01, 0.89) | 0.04(0.01, 0.15)
T4 (ug/dL)	7.20(6.40, 8.10) | 7.90(6.70, 8.80)	7.55(6.50, 8.50) | 8.10(7.00, 9.30)	7.70(6.60, 8.80) | 8.00(7.00, 9.30)	7.70(6.55, 8.90) | 8.40(7.30, 9.90)
TSH (mIU/L)[b]	1.49(0.99, 1.99) | 1.26(0.88, 1.79)	1.36(0.92, 1.89) | 1.27(0.86, 1.84)	1.34(0.98, 1.94) | 1.47(1.00, 2.14)	1.59(1.12, 1.94) | 1.59(1.15, 2.35)
TCPY (ug/L)[b]	2.99(1.41, 5.61) | 2.47(1.14, 5.37)	2.00(1.00, 4.19) | 1.75(0.74, 3.71)	2.09(0.89, 4.53) | 1.20(0.28, 2.88)	2.72(0.94, 4.53) | 1.34(0.28, 4.28)

NA=Not Applicable;BMI=Body Mass Index; TCPY=3,5,6-trichloro-2-pyridinol

[a]Frequency calculated by age*gender and represents percentage for male population in comparison to female population by age

[b]Geomean and geometric standard deviation provided

Table 4.2 Descriptive Characteristics of NHANES 1999-2000 and 2001-2002 Population (N=3249)

Variable	12 - <18 years (n=506; n=550)	18-40 years (n=506; n=511)	40-60 years (n=377; n=333)	>60 years (n=200; n=218)
	N (%)[a]			
	Male | Female	Male | Female	Male | Female	Male | Female
Mexican American	185(8.3) | 196(9.2)	172(31) | 165(25)	85(12) | 79(10)	32(1.5) | 39(2.2)
Non-Hispanic White	131(5.9) | 145(6.6)	206(21) | 179(19)	189(10) | 152(16)	132(5.1) | 128(6.0)
Non-Hispanic Black	151(8.2) | 164(9.1)	118(23) | 114(24)	76(14) | 70(15)	27(2.2) | 38(4.3)
Other Race	39(8.0) | 45(8.9)	58(26) | 53(24)	27(13) | 32(15)	9(1.7) | 13(3.1)
Menopause:				
No	NA | 547(24)	NA | 459(60)	NA | 101(16)	NA | 0
Yes	NA | 3(0.39)	NA | 18(7.9)	NA | 167(55)	NA | 218(37)
Alcohol Use:	NA			
No alcohol		64(15) | 105(24)	88(18) | 120(24)	74(7.2) | 122(12)
Less than a year		153(25) | 166(29)	111(17) | 131(21)	58(4.0) | 55(4.5)
More than a year		162(33) | 67(15)	163(32) | 63(12)	59(4.4) | 30(3.6)
Income:				
$0-19,999	310(6.3) | 358(7.0)	392(23) | 322(19)	273(19) | 229(16)	133(3.9) | 125(5.6)
$20,000+	138(8.3) | 143(8.7)	97(20) | 132(28)	65(11) | 60(12)	49(4.7) | 63(7.1)

NA=Not Applicable;BMI=Body Mass Index; TCPY=3,5,6-trichloro-2-pyridinol

[a]Frequency calculated by age*gender and represents percentage for male population in comparison to female population by age

[b]Geomean and geometric standard deviation provided

Table 4.3 Percent change[a] in serum T4 (ug/dL) and TSH (mIU/L) levels associated with an IQR increase in urinary TCPY (ug/L) concentration for MALES

		MALES			
		Age group			
		12 - <18 years (n=506)	18 -40 years (n=506)	40 - 60 years (n=377)	>60 years (n=200)
Unweighted	T4	β(95% CI)[c]	β(95% CI)[c]	β(95% CI)[c]	β(95% CI)[c]
	Crude	2.00(-0.58, 4.58)	*2.83(0.17, 5.49)[a]*	-0.51(-3.40, 2.39)	-1.80(-6.04, 2.43)
	Adjusted[b]	*3.85(0.75, 6.95)[a]*	*3.54(0.13, 6.96)[a]*	-1.29(-5.0, 2.42)	-1.62(-7.77, 4.53)
	TSH				
	Crude	0.38(-7.36, 8.78)	-4.34(-12.5, 4.57)	6.81(-2.89, 17.5)	0.75(-13.4, 17.3)
	Adjusted[b]	4.73(-3.91, 14.1)	-6.45(-17.0, 5.48)	9.03(-3.51, 23.2)	*-14.5(-27.6, -1.11)[a]*
Weighted	T4				
	Crude	1.85(-1.55, 5.26)	*2.82(0.14, 5.50)[a]*	-0.83(-4.66, 3.01)	-0.94(-3.82, 1.94)
	Adjusted[b]	3.57(-1.26, 8.41)	3.00(-0.81, 6.82)	-0.74(-5.59, 4.12)	-0.35(-6.28, 5.59)
	TSH				
	Crude	-3.28(-12.6, 7.0)	-4.98(-11.5, 1.98)	7.80(-3.48, 20.4)	-2.91(-19.9, 17.7)
	Adjusted[b]	4.81(-4.12, 14.6)	*-10.7(-18.7, -2.05)[a]*	9.97(-5.98, 28.63)	*-20.0(-28.9, -9.86)[a]*

[a]For T4, percent change relative to population median level.
[b]Adjusted for urinary creatinine, serum cotinine, BMI, age, race, income
[c]$p < 0.05$

Table 4.4 Percent change[a] in serum T4 (ug/dL) and TSH (mIU/L) levels associated with an IQR increase in urinary TCPY (ug/L) concentration for FEMALES

		FEMALES			
		Age group			
		12 - <18 years (n=550)	18 -40 years (n=511)	40 - 60 years (n=333)	>60 years (n=218)
Unweighted	T4	β(95% CI)[c]	β(95% CI)[c]	β(95% CI)[c]	β(95% CI)[c]
	Crude	-0.63(-3.20, 1.94)	0.87(-2.34, 4.09)	-1.66(-5.74, 2.42)	3.57(-1.26, 8.41)
	Adjusted[b]	-0.07(-3.26, 3.11)	-1.65(-5.69, 2.39)	-2.44(-7.09, 2.21)	-2.70(-8.64, 3.25)
	TSH				
	Crude	2.09(-5.71, 10.4)	2.94(-5.63, 12.3)	0.11(-9.46, 10.7)	0.93(-2.19, 22.9)
	Adjusted[b]	3.41(-6.12, 13.9)	6.09(-4.79, 18.4)	6.17(-4.79, 18.4)	*21.5(3.37, 42.8)*[a]
Weighted	T4				
	Crude	0.10(-4.15, 4.35)	-1.15(-5.39, 4.20)	-2.38(-6.99, 3.69)	-1.65(-6.99, 3.69)
	Adjusted[b]	-2.64(-6.44, 1.18)	-5.13(-11.6, 1.36)	-3.01(-8.21, 3.65)	-2.24(-8.13, 3.65)
	TSH				
	Crude	9.19(-2.86, 22.7)	3.18(-5.94, 13.2)	5.90(-4.01, 16.8)	10.7(-2.96, 26.3)
	Adjusted[b]	7.30(-6.65, 23.3)	3.94(-8.06, 17.5)	10.9(-2.27, 25.8)	10.3(-3.57, 26.1)

[a]For T4, percent change relative to population median level.
[b]Adjusted for urinary creatinine, serum cotinine, BMI, age, race, income
[c]$p < 0.05$

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Chapter V

Conclusion

The goal of the research conducted in this dissertation was to quantify a biomarker of exposure, urinary 3, 5, 6-trichloro-2-pyridinol (TCPY; a marker of exposure to chlorpyrifos (CPF), CPF-methyl, and/or TCPY itself), among pregnant, Mexican women and examine it and other risk and susceptibility factors in relation to child Attention Deficit Hyperactivity Disorder (ADHD) and ADHD-Like Phenotypes (LP), specifically measures of attention and hyperactivity. This research has added to the current knowledge on environmental predictors of child neurodevelopment and behavior, and provided additional insights into proposed biological mechanisms, susceptibility factors, and biomarker utility.

Summary of Research

Chapter 2: Distribution, Temporal Variability, and Relationship with Child ADHD and ADHD-Like Phenotypes

The objectives of this study were to define the distribution of TCPY concentrations among a population of pregnant women in Mexico and to assess between- and within-individual variability of urinary TCPY levels over the course of pregnancy. We also explored the relationship between third trimester maternal urinary TCPY concentrations and child characteristics of ADHD and ADHD-LP, particularly

attention and hyperactivity, using subscales from psychometric assessments. We hypothesized that concentrations of urinary TCPY would be measureable in the ELEMENT population and significantly higher than those reported in the U.S. NHANES study. It was also theorized that higher concentrations of urinary TCPY would be associated with adverse ADHD and ADHD-LP.

In this study, we found that urinary TCPY levels were higher in our study population compared to U.S. pregnant women from NHANES investigations in overlapping years. We reported an intraclass correlation (ICC) of 0.41, which indicated a fair level of reliability between urinary TCPY measures across pregnancy (Landis et al. 1977; Portney et al. 2000). Prior studies have also documented larger within-person variability relative to between-person variability in TCPY concentrations measured in repeat spot urine samples from the same individual.

We did not observe any statistically significant associations between tertiles of maternal third trimester urinary TCPY and measures of attention and hyperactivity in children. However, we found suggestive evidence for increases in the ADHD index in relation to urinary TCPY tertiles among boys. This suggests that fetal exposure to increased quantities of CPF, CPF-methyl, or TCPY during the last trimester of pregnancy may influence the display of ADHD characteristics in childhood. We found a suggestive association for increased attention problems when middle TCPY tertile was compared to the lowest tertile in girls.

Chapter 3: Paraoxonase I Polymorphisms and Attention/Hyperactivity in School-age Children

The objectives of this study were to assess whether ADHD-LP in school-age children were associated with mother and/or child PON1 genotypes. Among a subset of participants in the study, we also explored the interaction of urinary concentrations of TCPY in the third trimester and maternal or child PON1 genotypes in relation to ADHD-LP in school-age children. It was hypothesized that those with non-protective genotypes would be associated with ADHD and ADHD-LP. We also postulated that non-protective genotypes would modify the relationship between TCPY and child neurodevelopment.

In analyses of PON1 and ADHD-LP, we found that the OP non-protective PON1 genotype, $PON1_{55MM}$, in mothers was associated with poorer DSM-IV Hyperactivity/Impulsivity and DSM IV Total scores. Poorer attention problems scores were also observed in children with mothers that had the non-protective, $PON1_{192QQ}$ genotype. Associations with maternal genotypes were stronger when adjusting for child genotype. No significant associations were found in relation to the child's genotype alone. We found suggestive associations with the ADHD Index, DSM IV Hyperactivity/Impulsivity and DSM IV Total in boys with mothers who had $PON1_{55MM}$. Additionally, we found suggestive associations with DSM IV Hyperactivity/Impulsivity in boys with mothers who had $PON1_{192QQ}$.

In the exploratory gene-environment interaction study, we observed that the association between urinary TCPY and global restlessness/impulsivity was modified among children with $PON1_{55M.}$

Chapter 4: Association between urinary TCPY and serum T4 and TSH in NHANES 1999-2002

In this study, we assessed the relationship between urinary TCPY concentrations and altered serum thyroid hormone levels, thyroxine (T4) and thyroid-stimulating hormone (TSH), using data from the U.S. National Health and Nutrition Examination Survey (NHANES). The results of this study suggested a positive relationship between urinary TCPY and serum total T4, and a negative relationship between urinary TCPY and serum TSH, in adolescent males (<18 years) and/or men of reproductive age (18-40 years). There was also evidence of decreased and increased TSH in relation to urinary TCPY among males and females >60 years of age, respectively.

Impact/Innovation

These studies have added to the body of knowledge about early life origins of disease in children. There are limited human studies examining the relationship between chlorpyrifos and neurodevelopment and even fewer examining ADHD and ADHD-LP. This was the first study to assess the distribution of urinary TCPY concentrations in a Mexican population, and one of the first to assess temporal variability of TCPY across pregnancy. In addition, this was the first study to explore associations with attention and hyperactivity, using maternal urinary TCPY as a biomarker of exposure to CPF, CPF-methyl or TCPY. Also, this was the first study to assess *in utero* exposure to these chemicals in relation to ADHD and ADHD-LP in school-age children in a Mexico City, Mexico population.

To our knowledge, no other studies have assessed the relationship between PON1 genotypes and ADHD/ADHD-LP. Our findings have added to the literature about

the genetic distribution and variability of PON1 genotypes in a Mexican population. Thus far, this was the largest study to assess the relationship between PON1 genotypes and childhood neurodevelopment. Additionally, this was the first study to explore the effect modification of PON1 genotypes on the influence of chlorpyrifos (or CPF-methyl or TCPY) exposure on ADHD-LP in school-age children.

Lastly, to date, this was the largest study to examine the relationship between thyroid hormones and a biomarker of CPF exposure in a human population. This has added to the body of knowledge assessing how environmental exposures impact thyroid signaling, which is vital to numerous human physiologic functions including neurodevelopment.

In summary, these results are important considering the continued widespread agricultural and possibly residential use of CPF and CPF-methyl in Mexico and the educational implications of cognitive and behavioral deficits. Studies have found that symptoms of ADHD and ADHD diagnosis increase the probability of delinquency and grade repetition, reduce future reading and math scores, and increase the probability of special education (Currie et al., 2006). Early life CPF exposure may not only impact school age children, but could also have implications into adulthood. The concept of developmental programming states that adverse environmental events during sensitive periods of organ development trigger plastic responses that result in long-lasting functional alterations and influence risk for disease later in life (Barker, 2004; Scholtz et al., 2010). It is suggested that inattention persists into adulthood while hyperactivity is not as prevalent (Essawy et al., 2009; Adler et al., 2002), and that for 30% of children

diagnosed with ADHD it will persist into adulthood (Barbaresi et al., 2013).

Research Limitations and Further Research Needs

Although this research does make novel contributions to the literature, there are some limitations and areas that deserve further study. Some imitations include a relatively modest sample size for exploring relationships between urinary TCPY and childhood neurodevelopment and the use of a single urinary measure to estimate exposure in those analyses, from early third trimester, which may not give an accurate account of exposure throughout pregnancy. Despite our modest sample size, our study provides a foundation for a larger study and tentatively supports the limited previously published literature regarding urinary TCPY and childhood neurodevelopment. Data on OP usage, especially non-occupational, in Mexico is limited, thus we are unclear on the contribution of other OPs in the relationship with ADHD-LP.

Future studies should measure pesticide exposure in a larger population of pregnant women during all trimesters, which will also provide insight on potential critical windows of vulnerability to exposure. Additional research on the effect of pesticide mixtures and exposures to other environmental toxicants is important to assess the contributions of a variety of exposures to the development of ADHD characteristics children.

In the gene-environment study, PON1 activity was not measured, which is a limitation since there is considerable variability in PON1 levels among adults and differences as high as 13-fold have been observed among individuals of the same

genotype (Cole et al., 2005; Furlong et al., 2005). However, many studies have reported associations between genotypes and activity level, and this study provides a platform for further analyses that include PON1 status (PON1 activity and genotype). Another potential limitation is that our study results may not be generalizable to other race/ethnic groups or geographic regions, since it has been suggested that different populations have different susceptibility to pesticides based on their genetic makeup and mixed ancestry that influences allele frequencies. In future studies, it would also be interesting to assess oxidative stress in relation to ADHD in this population, and how that relates to environmental exposures as well as PON1 genotype.

Thyroid hormone levels, especially TSH, may also have significant intra-individual variability over time (Hollowell et al., 2002; Surks et al., 2005). Another limitation is that pregnant women, infants, and young children were not included in our NHANES analysis of urinary TCPY in relation to thyroid hormone levels. These populations all have variable thyroid hormone levels in comparison to non-pregnant women and adults. Epidemiological studies have indicated that even a marginally low thyroxine level in a pregnant woman may give rise to reduction of cognitive functions of the offspring (Berbel et al., 2009; Haddow et al., 1999; Pop et al., 2003; Boas et al., 2012). Free T4 (FT4) and Free triiodothyrine (FT3) were not measured in NHANES during 1999-2002, and, consequently, were not assessed in this study. FT4 and FT3 indicate thyroid activity in the human body. Future studies should examine CPF exposure and thyroid function in pregnant women using FT4 and FT3 in addition to total T4 and TSH, because human and experimental studies have shown that fetuses and infants were more sensitive than

adults to many environmental toxicants, including CPF (Timchalk et al., 2007), and altered thyroid function or signaling may represent a viable non-cholinergic mechanism of action.

Moving Forward: Practical Applications

The etiology of ADHD is not clear but exposures to environmental contaminants such as CPF have been implicated as potential risk factors. Based on this research and the research of others, globally, exposure to CPF, CPF-methyl, and/or TCPY during pregnancy should be limited. In general, the use of chemical pesticides should be seen as a last resort and should only be used when other tactics are not available, practical, or effective (Frumkin, 2005). Principles from integrated pest management (IPM), which is an approach that considers multiple control techniques to maintain or manage, pest populations below economically damaging levels while maintaining environmental quality, should be utilized. Specifically, the occurrence of pests can be controlled by various means including: eliminating pest food sources by covering garbage bins, keeping dry food goods in sealed containers, and avoiding the placement of plants close to buildings (Frumkin, 2005). Also, not leaving food out overnight, cleaning under and behind kitchen appliances, and having regular garbage pickups controls the occurrence of pests (Frumkin, 2005). Keeping pests from entering structures by repairing opens or placing screens over attic vents are also effective ways of controlling pest structural entry (Frumkin, 2005).

Other ways to eliminate or reduce pesticide exposure include the selection of

organic food sources (when possible) and washing fruits and vegetables thoroughly before consumption. It has been found that diet could be a large source of exposure to CPF (Pang et al., 2009) and that an organic diet can substantially reduce concentrations of pesticides to undetectable levels (Lu et al., 2006; Sathyanarayana et al., 2012). Also, pregnant and lactating women should avoid areas recently treated with pesticides and not use chemical tick and flea collars. However, if pregnant and lactating women choose to chemically eliminate pests, they should consider using only the services of licensed pesticide applicators and baits and traps instead of sprays, dusts, and bombs (Sathyanarayana et al., 2012).

The diet of pregnant and lactating women may additionally have an impact of the development of ADHD, and pregnant women should strongly consider taking prenatal vitamins. An important factor in fetal neurodevelopment is the micronutrient folate. In a prospective study of 100 mother/infant pairs in the United Kingdom, it was found that lower red blood cell folate and total folate intake in early pregnancy (~14 weeks) was associated with higher childhood hyperactivity (Schlotz et al., 2010). A study of sublethal CPF exposure in fish liver found that vitamin C lessened CPF-induced oxidative damage (Ozkan et al., 2012), which has been implicated in the etiology of ADHD (Ceylan et al., 2012; Kawatani et al., 2011). In Wistar Rats, it was found that vitamin E also decreased oxidative damage after chronic CPF exposure (Ambali et al., 2010). Chronic deficiencies of certain minerals such as zinc, iron, magnesium and iodine and insufficient dietary intake of long-chain polyunsaturated fatty acids may have a significant impact on the development and deepening of the symptoms of ADHD in

children (Ibrahim et al., 2012). Because of the impact of environmental, dietary, and genetic factors on disease susceptibility and occurrence, it is important to simultaneously consider their influence in large prospective studies.

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