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PARAOXONASE-1 AND EARLY LIFE ENVIRONMENTAL EXPOSURES

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Abstract

Background—Acute and chronic exposures to widely used organophosphorus (OP) insecticides are common. Children's detoxification mechanisms are not well developed until several years after birth. The increased cases of neurodevelopmental disorders in children, together with their increased susceptibility to OP neurotoxicity cannot be explained by genetic factors alone but could be related to gene-environment interactions. Paraoxonase-1 (PON1) is an enzyme that can detoxify OPs but its catalytic efficiency for hydrolysis to certain OPs is modulated by the Q192R polymorphism.

Findings—Studies in animals have provided important information on the role of PON1 in protecting against gestational and postnatal toxicity to OPs. The $PON1_{Q192}$ allele is less efficient hydrolyzing certain OPs than the $PON1_{R192}$ allele. Maternal PON1 status (PON1 activity levels, the most important measurement, and functional Q192R phenotype) modulates the detrimental effects of exposure to the OP chlorpyrifos oxon on fetal brain gene expression and biomarkers of exposure. Epidemiological studies suggest that children from mothers with lower PON1 status that were in contact with OPs during pregnancy tend to show smaller head circumference at birth and adverse effects in cognitive function during childhood.

Conclusion—Infants and children are vulnerable to OP toxicity. The detrimental consequences of OPs on neurodevelopment can lead to future generations with permanent cognitive problems and susceptibility to develop neurodegenerative diseases. Improved methods using mass spectrometry to monitor OP-adducted biomarker proteins are needed and will be extremely helpful in early life biomonitoring, while measurement of PON1 status as a biomarker

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of susceptibility will help identify mothers and children highly sensitive to OPs. The use of adductomics instead of enzymatic activity assays for biomonitoring OP exposures have proved to provide several advantages, including the use of dried blood spots, which would facilitate monitoring newborn babies and children.

Keywords

Paraoxonase-1; organophosphates; environmental health; early life exposures; neurodevelopment

INTRODUCTION

While some organophosphorus (OP) insecticides are being phased out in the United States, they are still widely used in developing countries. It is estimated that each year, there are 3 million insecticide poisonings worldwide with 220,000 deaths.[1] About 33 million pounds of OP insecticides were used in 2007 in the United States,[2] mostly for the control of pests in agricultural settings. According to the American Association of Poison Control Centers' National Poison Data System (NPDS), about 32% of the OP insecticide exposures reported in 2013 involved children.[3] Routes of OP exposure include ingestion, inhalation and dermal contact in occupational, residential and non-residential settings. The frequency of neurodevelopmental disorders in children seem to be increasing worldwide. Developmental disabilities include specific learning and intellectual disabilities, autism, and attention deficit hyperactivity disorders.[4] Genetic factors only explain up to 40% of the neurodevelopmental disabilities reported. Growing evidence suggests that environmental exposures in combination with individual genetic susceptibility could trigger these disorders. This review will focus on gene-environment interactions important for modulating exposures to specific OP insecticides in early life.

More than six decades ago, the plasma enzyme paraoxonase-1 (PON1) was shown to hydrolyze toxic metabolites of OP insecticides.[5,6] Early observations noted that species with high plasma paraoxonase levels were more resistant to parathion exposures than species with low plasma paraoxonase levels.[7,8] Thus, for more than 4 decades it was thought that PON1 could protect against exposure to parathion as well as other OP compounds.[9] As noted below, experiments in mice with genetically modified *PON1* genes, showed that human PON1 modulates sensitivity primarily to chlorpyrifos (CPS) and diazinon (DZS), especially for direct exposure to their oxon forms present in most, if not all exposures.[9–12] It is worth noting that all of the safety studies for chlorpyrifos oxon (CPO) have been carried out with highly pure, less toxic parent compound CPS.[13] More valid safety studies should be carried out with exposures containing a percentage of CPO representative to that found in actual exposures.

Several important questions arise from these early observations. 1) Does human plasma PON1 protect against exposure to specific OP compounds? 2) If so, is there genetic and or developmental variability in the plasma levels of PON1 among individuals? 3) Is there developmental regulation of plasma PON1 levels in early life? 4) Is a mother's plasma PON1 protective against OP exposures for her fetus(s)? In this review, we examine these four important questions.

The question of the ability of PON1 to protect against OP exposure was directly tested in 1956 by Main[14] in experiments where he injected partially purified rabbit PON1 into rats increasing their A-esterase activity four to five times and decreasing the toxicity of injected paraoxon (PO). This observation was repeated and extended to the effects of PON1 on CPO toxicity by Costa et al.[15] Many further experiments were carried out in mice showing PON1 protection against CPO and its parent compound CPS. A study in mice injected with rabbit PON1 clearly demonstrated that high levels of PON1 protected against exposure to both CPO and CPS.[16] The effects of low levels of plasma PON1 were examined in *PON1* knockout (*PON1*$^{-/-}$) mice which were shown to be dramatically sensitive to CPO[17] and diazoxon (DZO), with less dramatically increased sensitivity to their respective parent compounds CPS and DZS.[9] Surprisingly, the *PON1*$^{-/-}$ mice showed no increased sensitivity to PO,[9] indicating that mouse PON1 does not have a role in detoxifying PO *in vivo*, even though PON1 had been named for its ability to hydrolyze PO *in vitro*. Resistance to CPO and DZO could be restored by the injection of purified human $PON1_{192}$ alloforms, with both alloforms (Q and R) providing equal protection against DZO and the $PON1_{R192}$ alloform providing better protection against CPO, in agreement with the respective catalytic efficiencies for hydrolysis of these two OPs by each $PON1_{192}$ alloform. Also in agreement with the lack of increased sensitivity of the *PON1*$^{-/-}$ mice to PO, injection of neither human $PON1_{192}$ alloform protected against PO exposure in contrast to rabbit PON1. Injection into *PON1*$^{-/-}$ mice of engineered recombinant human PON1 (rHuPON1) with lysine replacing arginine or glutamine at position 192 provided further evidence for the importance of high catalytic efficiency in PON1 protecting against OP exposure.[18] The $rHuPON1_{K192}$ when injected into *PON1*$^{-/-}$ mice protected against 2 or 3 LD_{50} levels of exposures of the *PON1*$^{-/-}$ mice to DZO. The $rHuPON1_{K192}$ has higher catalytic efficiency for the hydrolysis of DZO, CPO and PO than the native $PON1_{192}$ Q or R alloforms and was designed based on the high catalytic efficiency of rabbit PON1 for hydrolysis of CPO.

These experiments clearly demonstrate the importance of PON1 in protecting against CPS and DZS exposures, especially to the oxon forms present in these exposures.[9] They also point out the potential for treating acute, life-threatening OP exposures with injections of rHuPON1 or engineered rHuPON1, which will be necessary for treating exposures to parathion/PO.[9,16,18]

Since PON1 plays an important role in resistance to exposure by at least two commonly used OP insecticides, the role of genetic and developmental variability become important questions related to early life exposures. PON1 is a polymorphic enzyme. Early studies in the 1960s and 1970s used PO as the substrate to measure PON1 activity in human populations. Most of them reported a high variability of PON1 activity between individuals, showing either a bimodal or trimodal distribution of serum PON1 activity levels[19] leading to the suggestion that the paraoxonase activity of PON1 exhibited a genetic polymorphism.[20] Several assays were developed to examine this polymorphic distribution of PON1 activity, all used PO as a substrate under varying conditions of pH, salt concentration, calcium, buffer composition and EDTA. Interestingly, this polymorphic distribution was not observed when using phenyl acetate as a substrate. Taking advantage of these substrate differences, an improved PON1 phenotype resolution was achieved by measuring PON1 activity with the two different substrates PO and phenyl acetate. This two-substrate assay/analysis, pioneered

in La Du's laboratory[21] clearly resolved individuals with low PON1 activities by plotting rates of PO (with high salt in the assay) *vs.* phenyl acetate, however, the intermediate and high metabolizers were not resolved by this two-dimensional analysis. Cloning and sequencing of the human PON1 gene revealed two major coding polymorphisms, L55M and Q192R.[22] The basis of the high *vs.* low rates of PO hydrolysis was subsequently shown to be determined by the Q192R polymorphism with the $PON1_{R192}$ alloform hydrolyzing PO much more efficiently than the PON_{Q192} alloform.[23,24] Further improvement of this two-substrate analysis, referred to as PON1 status,[25] involved plotting rates of DZO hydrolysis *vs.* PO hydrolysis.[26,27] This assay clearly resolved all three $PON1_{192}$ phenotypes (Q/Q, Q/R and R/R). Results obtained from PON1 status analyses show inter- and intra-assay reproducibility, although samples should be analyzed within 2 years from collection.[28] A version of the PON1 status analysis that makes use of non-highly-toxic substrates has recently been developed to reduce exposure of laboratory personnel to the highly toxic oxons of PO and DZO.[29]

A typical PON1 status plot (for 704 individuals) is shown in Figure 1.[30] This plot also shows the importance of the functional analysis of the two-dimensional PON1 status plot. The activity level for each individual is revealed by the plot which also identified 4 individuals where the functional analysis was discrepant from the DNA single nucleotide polymorhipsm (SNP) analysis. Sequencing the PON1 gene from these individuals revealed the defect in one allele in each, where the individuals genotyping as $PON1_{192}$ heterozygotes functionally showed only one active PON1 alloform. It is important to note that for epidemiological studies, a SNP analysis provides only information about the PON1 SNPs present in the individual's genome and no information about the PON1 activity levels, the most important factor in determining risk of exposure or disease. One could characterize all ≈200 SNPs (Fig. 2) and not be able to predict PON1 activity level. We emphasize this point since many dozens of epidemiological studies have been carried out using only an analysis of SNPs, ignoring the most important factor affecting risk, which is PON1 activity level.[31]

The question of developmental regulation of PON1 activity levels has been examined in at least two recent studies. Cole et al. reported that PON1 levels of newborns are approximately one third to one fourth the levels of adults. It takes between 6 months and two years for the PON1 levels in babies to reach adult activity levels.[32] On the other hand, plasma PON1 activity reaches adult levels in mice at three weeks of age.[33] When the human $PON1_{Q192}$ and $PON1_{R192}$ transgenes, including their respective 5' regulatory regions, are expressed in $PON1^{-/-}$ mice, the time course is the same as observed in wild type mice with activity peaking at ≈3 weeks of age, indicating a strong conservation of PON1 regulatory elements between humans and mice.[32] A study carried out in collaboration with the University of California Children's Health Study Program showed a large variability of PON1 activity levels among both mothers and newborns (Fig. 3).[34] The average PON1 activity levels in the newborns were comparable with the PON1 levels in the *PON1* transgenic mice allowing for the prediction of the relative sensitivity to CPO and DZO. The predicted range of sensitivity for CPO exposure between the newborn with the lowest PON1 activity level and the mother with the highest PON1 activity level was 131- to 164-fold and for DZO ≈65-fold. A major concern would be for the mothers with low PON1 activity levels to protect their fetuses from exposure to these OP compounds. The data supporting these concerns are discussed below.

ROLE OF PON1 IN PRE- AND POSTNATAL OP EXPOSURES IN ANIMAL STUDIES

OP insecticides are developmental neurotoxicants that can lead to permanent brain damage. Concern for prenatal and postnatal exposure to OP insecticides such as CPS and their potential effect in neurodevelopment and neurotoxicity has prompted many studies on OP exposures in dams, fetuses and pups. Several *in vitro* studies have shown potential neurodevelopmental effects of CPO at levels that do not inhibit acetylcholinesterase (AChE).[35] This is of special interest, as chronic low-level environmental OP exposures are common.

PON1, one of the main CPO detoxifying enzymes, is modulated by age. As mentioned above, studies of PON1 expression during development in transgenic mice expressing either human $PON1_{Q192R}$ alloforms ($tgHuPON1_{Q192}$ and $tgHuPON1_{R192}$) indicated that PON1 activity is low at birth and it plateaus at 3 to 4 weeks of age.[32] This finding was in agreement with previous studies in wild type mice and rats,[33,36] suggesting that the developing brain may be more susceptible to the toxic effects of certain OPs. Creation of the $PON1^{-/-}$ mouse was key to demonstrate the role of PON1 in detoxification of certain OPs such as CPO and DZO, and their parent compounds.[9,17] The “humanized” tgHuPON1 mice generated on the $PON1^{-/-}$ background provided a better understanding of the effect of PON1 Q192R polymorphism in OP exposures *in vivo*. As observed in studies injecting the two PON1 alloforms in $PON1^{-/-}$ mice,[9] adult transgenic mice carrying only the $PON1_{Q192}$ allele were more sensitive to CPS and CPO exposure than $tgHuPON1_{R192}$ mice.[37]

There are very few studies on pre- and postnatal effects of OP exposures in relation to PON1. In a study of oral gestational exposure to CPS in rats from gestational day (GD) 14 to GD18, the authors concluded that fetal brain total cholinesterase [AChE and butyrylcholinesterase (BChE)] was able to recover faster between dosages than maternal brain total cholinesterase.[38] As expected, PON1 activity in fetal tissues (liver and placenta) was quite low compared to the maternal tissues, providing little potential for CPS detoxification in rat fetuses by PON1. A similar study using C57BL/6 dams chronically exposed to CPS via subcutaneous injection from GD6 to GD17 also showed fetal resistance to CPS toxicity, with fetal brain AChE less inhibited than maternal brain AChE.[39] In order to study the neurotoxic effects in fetuses and the protective effect of maternal PON1 Q192R polymorphism in repeated CPO exposures, a more recent gestational study has been published using wild type, $PON1^{-/-}$, $tgHuPON1_{Q192}$ and $tgHuPON1_{R192}$ mice.[40] Pregnant female mice were dermally exposed from GD6 to GD17 to doses of CPO that caused minor inhibition of maternal brain AChE in wild type mice. Maternal biomarkers of OP exposure (plasma BChE, carboxylesterase, red blood cell acylpeptide hydrolase and brain AChE) showed significantly decreased activity, independently of the mouse strain tested (except for brain AChE activity in wild type dams). However, only fetal plasma BChE activity seemed to be affected by CPO toxicity, despite reported fast recovery of cholinesterases in fetuses.[38] These effects seemed to be modulated by maternal PON1 activity levels and dependent on the maternal PON1 Q192R polymorphism, as fetuses had extremely low plasma PON1 activity. A much more rapid CPO detoxification in maternal plasma could prevent CPO from

reaching fetal brain and inhibiting AChE. Conversely, studies reporting some fetal brain AChE inhibition used CPS,[38,39] which is first metabolized by cytochrome P450s to the reactive CPO in maternal and fetal tissues (CPS can cross the placenta) and then detoxified.

As predicted from other studies,[9,37] fetuses of *PON1*$^{-/-}$ dams were the most susceptible to chronic CPO exposure, followed by fetuses from *tgHuPON1*$_{Q192}$ dams. These findings were supported by gene expression data obtained in this same study. Following CPO exposure, brain from fetuses of *tgHuPON1*$_{Q192}$ dams, compared to fetal brain from *tgHuPON1*$_{R192}$ dams, were enriched in gene sets related to the mitochondrial respiratory chain, lipid metabolism, cell cycle, vesicle membranes, and neurotransmission by glutamate receptors, among many others.[40] Therefore, this study demonstrated that maternal PON1 status could modulate the effects of gestational exposure to CPO on fetal brain gene expression and biomarkers of exposure. An earlier study conducted in C57BL/6 mice reported similar gene sets affected in fetal brain resulting from CPS-exposure to dams.[39]

Studies on neonatal OP exposures have been very important in demonstrating the role of PON1 status in determining susceptibility to developmental neurotoxicity. Repeated daily exposure of wild type, *PON1*$^{-/-}$, *tgHuPON1*$_{Q192}$ and *tgHuPON1*$_{R192}$ mice to CPO from postnatal day (PND) 4 to PND21 resulted in changes in cerebellar gene expression, body weight and neurobehavioral deficits.[41,42] In humans, PND4 to PND21 would parallel the neurodevelopmental period comprised between late gestation and the first two years of life. A 2.5-fold difference in resistance to CPO between wild type and *PON1*$^{-/-}$ mice was already observable in 4-day-old mice.[41] Although the doses of CPO used in that study where low and not lethal to *PON1*$^{-/-}$ mice, chronic exposure to CPO reduced brain AChE activity more significantly in the *PON1*$^{-/-}$ and *tgHuPON1*$_{Q192}$ mice, strains that have lower capacity of CPO detoxification. These two strains of mice also showed the same cerebellar pathways affected by exposure, mostly related to mitochondrial dysfunction and oxidative phosphorylation,[41] consistent with other studies that have associated oxidative stress with OP exposures.[43] Extensive neurobehavioral testing of the CPO-exposed *PON1*$^{-/-}$ mice only showed changes in startle latency and dose-related transient hyperkinesis.[42] Perhaps the effects of CPO exposure in mice are age-dependent, or alternative neurobehavioral tests assessing other brain neurotransmitter systems and social interactions should have been tested. Thus, PON1 protection against CPO toxicity during postnatal development is determined by the level of PON1 expression, with juvenile mice with low PON1 status (*tgHuPON1*$_{Q192}$) being more susceptible to CPO toxicity than *tgHuPON1*$_{R192}$.

PON1 AS A BIOMARKER OF SUSCEPTIBILITY TO NEURODEVELOPMENTAL EFFECTS

Children are particularly susceptible to environmental toxicants. OP insecticides such as CPS, the most used OP insecticide in the USA, can reach fetal tissues by crossing the placenta, with exposure continuing after birth through breast milk.[4] Of note is that the blood-brain barrier is not completely developed until around the first year of life and, therefore, does not protect against chemicals crossing into the brain. There have been numerous studies on the effects of CPS in pregnant women and children, most of them

accurately reviewed by Eaton and colleagues.[35] As this review focuses on PON1, in this section we will limit the studies discussed to those that included assessment of both PON1 genotype and phenotype, as genotype alone is uninformative.[31,34]

Given the role of PON1 in protecting against OP exposures and the lower PON1 levels at birth, the study of the role of PON1 in OP exposures and related outcomes during pregnancy and childhood continues to be the focus of intense research. The lower PON1 levels in children were first reported in 1963.[44] However, since we now know that the serum arylesterase activity is catalyzed by PON1,[18,24] the relationship of PON1 levels with neurochemical and neurobehavioral changes following OP exposures was not evidenced until recently.

Several reports on a multiethnic birth cohort study at the Mount Sinai Medical Center (New York City) have documented effects on fetal growth and neurodevelopment from *in utero* exposures to CPS in relationship with maternal PON1 activity and genotype. In their first study, smaller head circumferences were reported in babies from mothers with low PON1 activity, compared to the offspring from mothers with higher PON1 arylesterase activity or non-exposed mothers.[45] A later study by the same investigators on prenatal exposures to OPs and other pesticides in another cohort in the same geographical area confirmed their previous finding of lower PON1 arylesterase activity leading to decreased head circumferences in babies.[46] In addition, they also found statistically significant relationships between certain urinary dialkylphosphate metabolites (DAPs), low PON1 status (low PON1 arylesterase activity and $PON1_{QQ192}$ genotype), and decreased birth weight and birth length. They also assessed abnormalities in neonatal behavior and primitive reflexes with the Brazelton Neonatal Behavioral Assessment Scale, an index to assess newborn's abilities. There was an increased number of abnormal primitive reflexes in neonates from mothers with low PON1 arylesterase activity and high DAP metabolite levels during pregnancy.[47] In a follow-up study of the cohort at ages 1–2 and 6–9 years old, the authors evaluated the impact of early life exposures in development.[48] In this case they used the Bayley Scales of Infant Development, which provides age-standardized norms of mental and psychomotor development. The results of years 1–2 were somewhat contradictory in terms of maternal $PON1_{192}$ genotype, probably due to sample size and heterogeneous race/ethnicity. However, their findings suggested that prenatal exposure to OPs (estimated by maternal urinary DAPs) had a detrimental effect on perceptual reasoning (cognitive development) in 1–2 year old infants. This evidence was stronger in 6–9 year old children of mothers homozygous for the $PON1_{Q192}$ polymorphism. No effect of PON1 arylesterase activity from maternal blood or cord blood on the neurodevelopmental assessment performed was noted. This was the first study to examine a possible association between *PON1* Q192R genotype and neurobehavioral end points in children.

The Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) study is a longitudinal birth cohort study from an agricultural Mexican-American community from the Salinas Valley (CA). Farmworker mothers had been exposed to relatively high levels of OP pesticides during pregnancy, as assessed by urinary DAP metabolites. When measuring PON1 status in mothers and their babies, at birth, children's PON1 levels were one-fourth the level of the mothers, with a predicted sensitivity to DZO

exposure of 26-fold vs.14-fold among the mothers.[34] The calculated range of sensitivity to CPO exposure was of 131–164-fold between the *PON1*$_{QQ192}$ newborn with lowest PON1 status and the *PON1*$_{RR192}$ mother with highest PON1 status. That was the first study to assess PON1 status in a cohort of mothers and babies exposed to OPs. Prenatal maternal urinary DAPs were associated with detrimental pervasive and mental development in children at age of 2, as measured with the Bayley Scales of Infant Development.[49] Decreased mental development scores appeared to be related to the child's promoter *PON1*$_{-108T}$ allele, while psychomotor developmental scores in *PON1*$_{QQ192}$ children were inversely associated with urinary DAP levels.[50] At ages 5 to 7, full-scale IQ scores tended to be lower in *PON1*$_{-108TT}$ children and in children from mothers with higher DAP levels and lower PON1 arylesterase activity during pregnancy, with the latter also showing lower verbal comprehension.[51] Altogether, these results suggest that PON1 genotype and low activity may be related to adverse cognitive functioning in vulnerable children, i.e. children from mothers with low PON1 activity exposed to OPs during pregnancy.

BIOMONITORING OF ORGANOPHOSPHORUS EXPOSURES

Primary routes of OP exposure in the United States are through food consumption and spray drift in agricultural communities.[10,52] While the states of Washington and California have ongoing biomonitoring programs of their agricultural workers, who can potentially suffer acute occupational exposures to the insecticides sprayed, there are a lack of methods sensitive enough to quantify non-occupational low level chronic OP exposures in the general population. Biomonitoring of acute and chronic environmental exposures is especially important for pregnant women and children, which are more vulnerable to the effect of neurotoxicants. Therefore, improved assessment of children's exposures is highly needed.[53] Upon entering the human body, the parent OP organophosphorothioates are either detoxified by specific cytochrome P450s (and eliminated in the urine) or are converted to their highly toxic oxon forms (Fig. 4). The highly toxic oxons interact with serine active-site enzymes (e.g. AChE and BChE) creating a covalent bond with their active site serine that ages to an irreversible inhibitor of the enzyme. The adducted protein is stable and will remain in circulation until degradation, which depends on the protein's half-life in circulation (11 days for plasma BChE and 33 days for red blood cell AChE). The oxons can also undergo spontaneous or enzymatic (i.e. PON1) hydrolysis resulting in the formation of DAP metabolites and an OP-specific leaving group. These metabolites do not show inhibitory properties and are eliminated in the urine.

Urinary DAP metabolites and cholinesterase enzymatic inhibition assays are the most common methods used for biomonitoring OP exposures.[54] The two approaches offer advantages and disadvantages. Measurement of six DAP metabolites has been used to assess exposure of at least 28 OPs and is not an invasive method since it uses urine samples.[55] However, OP metabolites have short half-lives, ranging between 24 and 48 h, the OP or mixture of OPs involved in the exposure may form the same metabolites during hydrolysis and, therefore, the OP of exposure cannot be distinguished, and the metabolites cannot be discriminated from direct environmental exposure to non-toxic OP degradation products, which would result in overestimation of the exposure.[54] As OPs target cholinesterases, among other serine hydrolases, inhibition of their activity in blood has been used for many

years as a surrogate to estimate the level of brain AChE inhibition. Inhibition of plasma BChE is one of the early biomarkers of OP exposure commonly used. The Ellman colorimetric enzymatic assays,[56] which use red blood cells to determine AChE activity (with acetylthiocholine as substrate) or plasma for BChE activity (with butyrylthiocholine as substrate), are quite accurate, reliable and inexpensive. Although used for monitoring agricultural workers, the drawbacks of these assays are significant. The requirement of a pre-exposure sample to be used as the individual's baseline activity, the high intra- and inter-individual variability and the inability to assess chronic low-level exposures that usually cause no cholinesterase inhibition are some of the disadvantages of the Ellman assays.[54] A more accurate method for biomonitoring OP exposures is needed. An ideal biomonitoring protocol should accurately quantify exposures and provide some information about the OP compound, should be sensitive enough to monitor low level exposures, should be automatable and reproducible, and should require samples obtained through the least invasive route possible. In recent years, a focus on mass spectrometry (MS) as a tool for biomonitoring OP exposures has emerged.[57] MS provides high sensitivity methods for targeting posttranslational modifications in proteins, including OP adducts. A myriad of biological processes result in adducted proteins and DNA, so in the recent years, the general study of adducts on biological molecules has been termed adductomics. Mass spectrometric analyses provide significant improvements since they can target any biomarker of OP exposure, not just the cholinesterases. Furthermore, proteins are very abundant, their half-lives are much longer than those of urinary metabolites, and baseline activity measurements are not necessary.[57] Although blood collection adductomics protocols is invasive and requires the sample to be processed and stored properly, the high sensitivity provided by MS has been demonstrated in preliminary experiments to provide adequate sensitivity for quantifying OP exposures from dried blood spots (Marsillach et al. manuscript in preparation). Dried blood spot samples are much easier to collect from finger sticks as well as easier to ship and archive. They should also allow for analysis of OP exposures from archived dried blood spots from newborn heel sticks.

CONCLUSIONS AND FUTURE DIRECTIONS

Early life exposures have important effects on brain development that can lead to permanent damage, affecting the children's future development in society and increasing their susceptibility to develop neurodegenerative diseases. Genetic variability of the PON1 gene and the resulting effects on protein structure and levels have provided an early, well-characterized example of gene-environment interaction. All of the early research examined the role that PON1 played in modulating exposures to OP insecticides. Cloning and characterizing the PON1 cDNA along with purifying the two Q192R alloforms of PON1 allowed for the determination of the effects of this coding region polymorphism on the catalytic efficiency of OP hydrolysis. The development of the PON1 genetically variable mice has provided an invaluable asset for understanding the physiological function of PON1.[17,37] For many years, it was thought that high metabolizers of PO would be resistant to exposures to parathion/PO, however, experiments from our laboratory showed this not to be the case and demonstrated the importance of high catalytic efficiency for determining whether PON1 could protect against specific exposures.[9] These experiments showed that the

genetic variability in PON1 governed primarily sensitivity to the oxons of CPS and DZS with some protection afforded to exposures to the respective parent organophosphorothioates. These experiments clearly pointed out that hydrolysis of OPs with *in vitro* assays was not a good indicator of the ability of PON1 to protect against exposure. PO and the nerve agents soman and sarin provide good examples of this point. The development of a protocol for generating native recombinant human PON1 (rHuPON1) from an *E. coli* expression system allowed for the characterization of the protective capacity of PON1 under physiological conditions and at the same time demonstrated the potential to make use of rHuPON1 as a therapeutic for treating OP exposures.

Another physiological function of PON1 in preventing cardiovascular disease came to light with the discovery by Mackness and colleagues that PON1 on the high-density lipoprotein particles was responsible for preventing the oxidation of low-density lipoprotein particles.[58] This observation initiated many studies on the relationship of PON1 genetic variability to various oxidative stress-related diseases. This subject is not covered in this review, but is worth mentioning for readers who would like to follow up other gene-environment interactions involving genetic variability of PON1. These studies also emphasize the importance in epidemiological studies of examining PON1 status, which reveals the activity of PON1 as well as the Q192R phenotype of individuals. Studies that include PON1 status have found that low PON1 activity levels are a risk factor not only for OP exposure but also for cardiovascular disease.[59–61] Studies that examine only SNPs are missing the most important measure of risk, the activity levels of PON1, and have often led to inconclusive results.[31] The assays for determining PON1 status are high throughput and, therefore, convenient for use in most laboratories.

Another important point to note in summarizing PON1 gene-environment interactions in early life exposures is the developmental variability of PON1 where newborns have much lower PON1 levels (also variable among infants) than adults and are thereby more sensitive to exposures for which PON1 modulates risk.

Epidemiological studies with appropriate measurement of PON1 status as a biomarker of susceptibility coupled with accurate measures of exposures are still needed. The focus on MS analysis of OP-adducted biomarker proteins has several advantages over the Ellman assays or the urinary DAP metabolite analyses. No additional blood draw is required to determine subjects' baseline activity levels, which would eliminate thousands of blood draws from agricultural workers before the spray season starts, and avoid the difficulty of obtaining multiple blood draws. The MS analyses simply provide an accurate determination of the percentage adduction of the active-site serine in biomarker proteins. In addition, adducted proteins have a much longer half-life than DAP metabolites, increasing the window of time for exposure detection. Additionally, the high sensitivity of MS also provides some information about the OP to which the individual was exposed. The use of finger stick/dried blood spot analyses significantly simplifies sample collection, shipping and archiving. MS is still an emerging field, and implementation of this technology in clinical laboratories is still underway. However, thanks to the significant accomplishments obtained in recent years, the future of MS as a tool to quantify children's exposures at any life stage is very promising.

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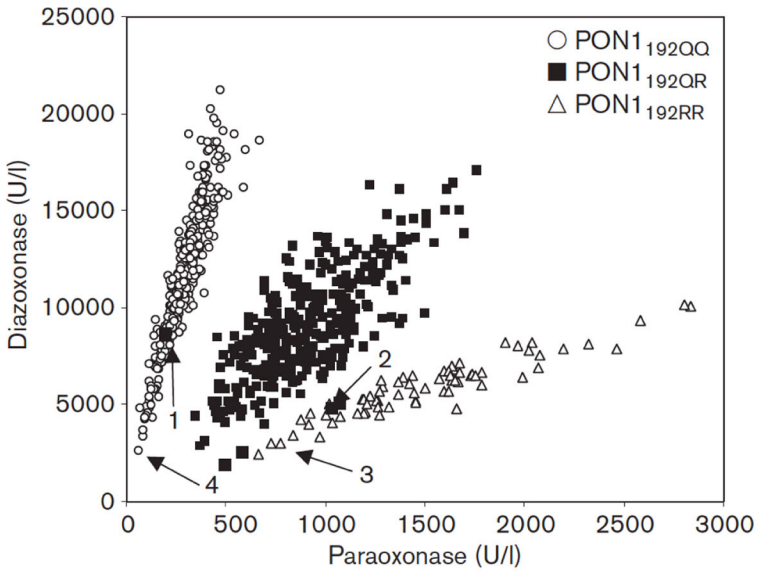

Figure 1.

PON1 status plot of 704 subjects identifying 4 individuals (arrows) discordant with respect to DNA SNP analysis and those with coding region changes. The 3 *PON1*$_{192}$ phenotypes are clearly resolved. Arrow 1: *PON1*$_{Trp194stop\ allele}$; arrow 2: suspected of having a partial deletion of the coding region; arrow 3: coding region change *PON1*$_{Asp124missplice}$; arrow 4: coding region change *PON1*$_{Pro90Leu}$. Reproduced with permission from Jarvik et al.[30]

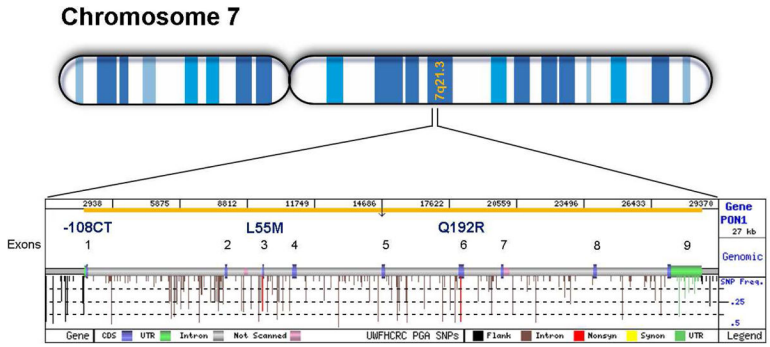

Figure 2.

Gene structure of the human *PON1* gene showing the 9 coding exons and the frequencies of SNPs in the 5′ and 3′ non-coding regions, in introns and exons. Note that there are approximately 200 SNPs identified with different frequencies. Modified from Seattle SNPs (http://pga.gs.washington.edu/data/pon1/welcome.html).

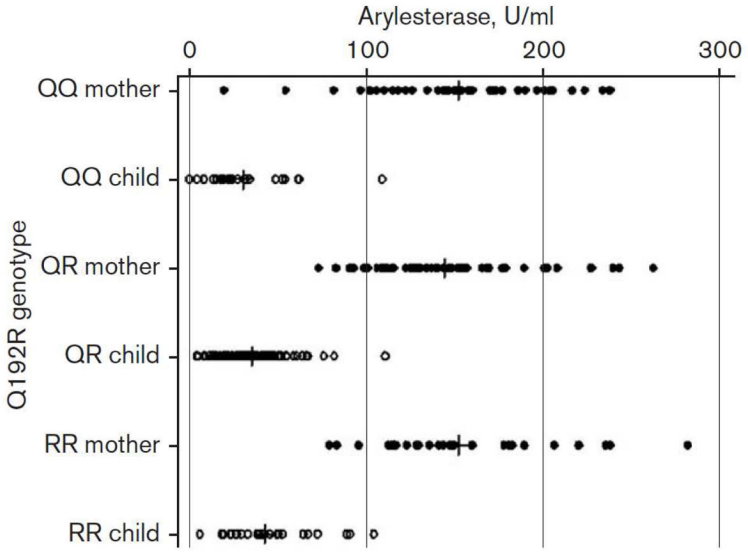

Figure 3.

Individual data points for arylesterase activities in mothers (solid circles) and newborns (open circles) for each *PON1*$_{192}$ genotype as indicated. Means are indicated by the crossbars. Reproduced with permission from Furlong et al.[34]

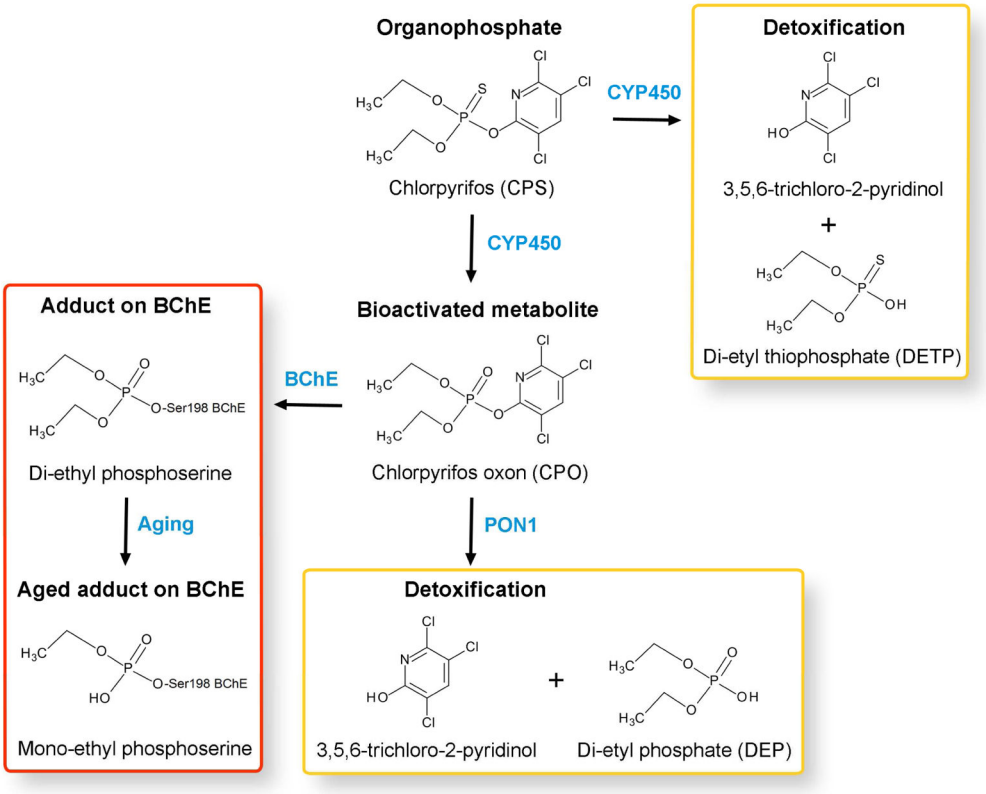

Figure 4.

Biotransformation of the organophosphate insecticide chlorpyrifos. The chemical structures in yellow boxes are dialkylphosphate metabolites. The red box contains organophosphate-adducted butyrylcholinesterase (BChE).