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Longitudinal Changes in PON1 Enzymatic Activities in Mexican-American Mothers and Children with Different Genotypes and Haplotypes

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Abstract

The paraoxonase 1 (PON1) enzyme prevents low density lipoprotein oxidation and also detoxifies the oxon derivatives of certain neurotoxic organophosphate (OP) pesticides. PON1 activity in infants is low compared to adults, rendering them with lower metabolic and antioxidant capacities. We made a longitudinal comparison of the role of genetic variability on control of PON1 phenotypes in Mexican-American mothers and their children at the time of delivery (n=388 and 338, respectively) and again seven years later (n=280 and 281, respectively) using generalized estimating equations models. At age seven, children's mean PON1 activities were still lower than those of mothers. This difference was larger in children with genotypes associated with low PON1 activities (*PON1*$_{-108TT}$, *PON1*$_{192QQ}$, *and PON1*$_{-909CC}$). In mothers, PON1 activities were elevated at delivery and during pregnancy compared to seven years later when they were not pregnant ($p<0.001$). In non-pregnant mothers, *PON1* polymorphisms and haplotypes accounted for almost 2-fold more variation of arylesterase (AREase) and chlorpyrifos-oxonase (CPOase) activity than in mothers at delivery. In both mothers and children, the five *PON1* polymorphisms (*192, 55, −108, −909, −162*) explained a noticeably larger proportion of variance of paraoxonase activity (62–78%) than AREase activity (12.3–26.6%). Genetic control of PON1 enzymatic activity varies in children compared to adults and is also affected by pregnancy status. In addition to known *PON1* polymorphisms, unidentified environmental, genetic, or epigenetic factors may also influence variability of PON1 expression and therefore susceptibility to OPs and oxidative stress.

Keywords

paraoxonase; enzymatic assay; genetic control; organophosphate; pesticides; pregnancy; children

Introduction

Paraoxonase 1 (PON1) is a high-density-lipoprotein (HDL)-associated enzyme, which plays a role in both organophosphate (OP) sensitivity and oxidative stress (Azarsiz *et al.*, 2003). PON1 can metabolize the toxic oxon derivatives of several OP pesticides, which are known to

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be acutely neurotoxic (Costa *et al.*, 2005a). There is growing evidence that PON1 may play a role in diseases related to oxidative stress including diabetes and heart disease (Li *et al.*, 2003; Li *et al.*, 2005; Bhattacharyya *et al.*, 2008). *In vitro* and *in vivo* studies have demonstrated that PON1 has antioxidant properties, preventing LDL and HDL oxidation (Aviram and Rosenblat, 2004) and protecting against atherosclerosis (Tward *et al.*, 2002; Rosenblat *et al.*, 2006). Current studies suggest lipophilic lactones are the primary substrate for PON1 (Draganov *et al.*, 2005; Khersonsky and Tawfik, 2005) and it is through this mechanism that PON1 is involved in lipid peroxidation. Although PON1 was named for its esterase activity towards OPs, the endogenous function of this enzyme is more likely its lipolactonase activity (Draganov *et al.*, 2005). In humans, there is a wide variability of PON1 enzymatic activities among adults (Deakin and James, 2004). Individuals with low PON1 activity may be more susceptible to pesticide exposures and oxidative stress since their metabolic capacity and antioxidant defenses are lower compared to those with average or high PON1 activities. Thus, understanding the determinants of PON1 variability, including genetics and age, and how they confer susceptibility to disease or exposures may have broad public health significance.

Several common polymorphisms in the coding and promoter regions of the *PON1* gene influence substrate-specific PON1 enzyme activities (Ferre *et al.*, 2003; Costa *et al.*, 2005b). The single nucleotide polymorphism (SNP) at codon 192 leads to an amino acid substitution from the active-site residue glutamine (Q) to arginine (R) and the catalytic efficiency of the $PON1_{192}$ R alloform towards the oxon derivatives of OP pesticides parathion and chlorpyrifos is greater than that of the $PON1_{192}$ Q alloform in *in vitro* substrate-specific assays. Animal experiments in transgenic mice expressing human $PON1_{192}$ Q and R alloforms have demonstrated that indeed mice expressing the R alloform are more resistant to chlorpyrifos-oxon (CPO) exposure than mice expression the Q alloform (Cole 2005). For paraoxon however, the catalytic efficiency even in the faster R alloform is too slow to provide any protection from *in vivo* exposures (Li et al. 2000). Recent studies found that the $PON1_{192}$ genotype explains a large portion of the variability of *in vitro* PON1 activity towards paraoxon (POase activity); it accounts for 59% of the variability among Caucasian and African-American adults (Bhattacharyya *et al.*, 2008) and 48% of the variability in a Mexican-American population (Rainwater *et al.*, 2009). Several promoter polymorphisms are also known to influence PON1 expression including $PON1_{-108}$, $PON1_{-162}$, and $PON1_{-909}$ (positions are relative to the translation start site). The $PON1_{-108}$ SNP exerts the most noticeable effect on PON1 quantity, as measured by arylesterase activity, accounting for 22.4% of the variability. The $PON1_{-108}$ CC genotype is associated with two-fold higher PON1 levels compared to the $PON1_{-108}$ TT genotype (Brophy *et al.*, 2001; Deakin *et al.*, 2003). The association of the SNPs at positions −162 and −909 with AREase activity likely is due in part to their strong linkage disequilibrium (LD) with the $PON1_{-108}$ SNP (Brophy *et al.*, 2001; Holland *et al.*, 2006). Similarly, the coding SNP $PON1_{L55M}$ is also associated with AREase activity, however most of this effect is attributable to LD with $PON1_{-108}$ (Brophy *et al.*, 2001). While several studies have described the important genetic contribution of these PON1 SNPs on phenotypic variation in multiple populations, few have characterized how the relative influence of genetic control may change through different stages of childhood and by pregnancy status. Furthermore, although genetic polymorphisms account for a large portion of PON1 variability, it is not sufficient in epidemiological studies to consider *PON1* genotypes alone (Richter and Furlong, 1999). PON1 phenotypes range broadly even between individuals with the same *PON1* genotypes because enzyme quantity also varies within these groups (Furlong *et al.*, 2006; Holland *et al.*, 2006). Therefore, studies which measure PON1 activities are more informative than studies that rely solely on *PON1* genotype data.

Children are particularly vulnerable to environmental exposures because they practice behaviors that can lead to increased exposure and often have lower metabolic capacities than adults (Landrigan *et al.*, 2004; Neri *et al.*, 2006; Wigle *et al.*, 2007). For example, children's

susceptibility to the toxic metabolites of OPs and oxidative stress may be heightened as several studies have demonstrated that PON1 activity is lower in newborns compared to adults (Chen *et al.*, 2003; Holland *et al.*, 2006). Early hypotheses suggested that PON1 developmental expression reaches mature levels at or near age two (Cole *et al.*, 2003), however we recently followed a large cohort of Mexican-American children from birth to age seven and found that their PON1 activities continue to increase past age two until at least age seven (Huen *et al.*, 2009a). This age-dependent increase of PON1 enzymatic activity was modified by genetic polymorphisms. For example, children with $PON1_{192}$ R alleles and $PON1_{-108}$ C alleles experienced a steeper rise in activity as they got older compared to children with $PON1_{192}$ Q alleles and $PON1_{-108}$ T alleles. These findings suggest that the window of susceptibility to both oxidative stress and OP exposure may be much longer than previously believed and children with certain genotypes may be particularly vulnerable.

Initially, we reported PON1 activity in a subset of 130 mother-child pairs from the Center for Health Assessment in Children and Mothers of Salinas Valley (CHAMACOS) cohort and determined the effects of genotypes and haplotypes on PON1 phenotype and status (Furlong *et al.*, 2006; Holland *et al.*, 2006). In the present study, we performed a longitudinal comparison of the role of genetic control on PON1 enzymatic activities in the entire CHAMACOS birth cohort of mothers and their children at the time of birth and also seven years later. We also compared PON1 activities between mothers and children at both time points and determined differences in PON1 activities in mothers during pregnancy, at delivery, and seven years later when they were not pregnant.

Materials and Methods

Study subjects

CHAMACOS is a longitudinal birth cohort study of the effects of pesticide and other environmental exposures on neurodevelopment, growth, and respiratory disease in children from primarily Mexican-American families (Eskenazi *et al.*, 2003). The Salinas Valley, which is located in Monterey County, CA, is intensively farmed with approximately 200,000 kg of OPs applied annually (DPR, 2007). Six hundred and one pregnant women were enrolled in 1999–2000 and 531 were followed through the birth of a live infant. Mothers were primarily young (M=25.6 ±5.3 years), married, low-income, Mexican-born, and Spanish-speaking. Many were farm workers themselves (44%) and/or lived with farm workers at the time of enrollment (84%). Ethnicity of children and their mothers was based on mothers' self-report. In this analysis, we included only women and children who were of Hispanic origin, the majority of which were Mexican (>90%), to avoid potential confounding by ethnicity. Study protocols were approved by the University of California, Berkeley Committee for Protection of Human Subjects. Written informed consent was obtained from all mothers and verbal assent was obtained from the children at seven years of age.

Blood collection and processing

Blood specimens were collected from mothers during pregnancy at the time of their glucose tolerance test (approximately 26 weeks gestation) and also at the hospital shortly before or after delivery. They were collected from children at the time of delivery (umbilical cord blood) and when the children were approximately seven years old (mean ± SD:7.05 ± 0.15 yr). Heparinized whole blood was collected in BD vacutainers® (Becton, Dickinson and Company, Franklin Lakes, NJ), centrifuged, divided into plasma, buffy coats and red blood cells, and stored at −80°C. Serum and blood clots were collected in vacutainers containing no anticoagulant. DNA was isolated from clots as described previously (Holland *et al.*, 2006).

Determination of PON1 genotypes

DNA isolated from clots was available for genotyping for 431 mothers and 434 children. The coding polymorphisms, $PON1_{192}$ and $PON1_{55}$, and the promoter polymorphism, $PON1_{-162}$, were genotyped using the Taqman real-time PCR method. Primers for the nucleotide sequence flanking the SNP, and probes specific for the SNPs were custom-designed by Applied Biosystems, Inc. (Foster City, CA). The promoter SNPs, $PON1_{-909}$ and $PON1_{-108}$, were genotyped using a fluorogenic allele-specific genotyping assay (Amplifluor). The $PON1_{-108}$ assay required a two-part nested PCR strategy, where the region surrounding the SNP was pre-amplified using non-allelic flanking primers. The amplicon was then diluted and used as the template for the Amplifluor assay. Quality assurance procedures for genotyping all five *PON1* SNPs included assessment of randomly distributed blank samples in each plate and duplicates of randomly selected samples with independently isolated DNA from the same subjects. Repeated analysis (4% of samples) in several runs showed a high degree (>99%) of concordance. All discrepancies were resolved with additional genotyping.

Determination of PON1 enzymatic activities

PON1 enzyme activity was measured in heparinized plasma samples from 275 pregnant mothers (26 weeks gestation), 388 mothers and 338 newborns at delivery (312 complete mother-child pairs), and from 300 mothers and 281 children at age seven (246 complete mother-child pairs). In this paper, the 26 weeks gestation time point is referred to as pregnancy, measurements made in mothers at the time of delivery are referred to as delivery, and measurements made in umbilical cord blood are referred to as birth or newborns. At the seven-year collection, 21 mothers were pregnant and were excluded from the analysis. PON1 enzyme measurements were obtained from 481 mothers in total; of those, 228 had measurements at two time points, and 127 had measurements at all 3 time points. Enzyme measurements were available from 428 children of whom 191 had measurements at both time points. All samples which were previously assayed for PON1 activity in 130 mother-child pairs (Holland *et al.*, 2006) were completely re-assayed simultaneously with all remaining samples in the CHAMACOS cohort. We found high correlations ($r \sim 0.51–0.79$, $p<0.0005$) with previous measures as reported in Huen *et al.* (2009b) although re-assayed samples had lower activities likely due to storage duration.

We measured PON1 enzyme activities against three different substrates (paraoxon (PO), phenyl acetate (ARE), and chlorpyrifos-oxon (CPO)) in plasma samples using spectrophotometric methods as described previously (Huen *et al.*, 2009b). In this paper, we use these three measurements as markers of PON1 molecular phenotype. The arylesterase (AREase) assay serves as an indirect measure of PON1 enzyme quantity and AREase rates do not vary between $PON1_{192}$ Q and R alloforms as they do for paraoxon hydrolysis (POase). PON1 quantity measured using ELISA and Western blot based methods are highly correlated with AREase activity ($r > 0.85$)(Kujiraoka *et al.*, 2000; Connelly *et al.*, 2008). In contrast, the paraoxonase (POase) and chlorpyrifos-oxonase (CPOase) substrate specific activities reflect both quantity and catalytic efficiency of the enzyme. All assays were performed in triplicate. Quality assurance included assessment of repeat samples (separate aliquots of the same sample run on different days) and internal controls (aliquots of the same sample run on all assay plates). We found a high degree of concordance between repeated samples (3% of samples were repeated). The average coefficient of variation (CV) for repeated samples was 6–9% and the correlation coefficients between repeated runs were between 0.91–0.98 for all three assays. The same internal controls samples were used on every plate for every assay and the inter-assay variability (average CV) for these samples ranged from 7 and 9%.

Storage duration of specimens can affect PON1 activity, particularly in specimens that had been stored for extended periods of time (Brackley *et al.*, 1983; Stenzel *et al.*, 2007; Huen *et*

al., 2009b). For instance, PON1 activity declined on average by 17.1%, 39.4%, and 37.6% for AREase, CPOase, and POase, respectively after five years of storage ($p<0.001$ in all 3 assays). In our study, specimens collected at delivery were generally stored for longer periods of time than specimens collected during the seven year assessment. In order to compare PON1 activity differences between time points (delivery versus seven years later and pregnancy versus seven years later) while taking into account the potential effects of storage duration, we developed a storage duration correction factor using pilot data from a previous study (Huen *et al.*, 2009b). Parallel aliquots of specimens from the same subjects (n=95) were assayed after two and seven years of storage at −80°C. We used these data to construct linear models that predicted the change in PON1 activity (%) as a function of storage duration (years) and storage duration*assay temperature. The models, which included an interaction between storage duration and assay temperature had the lowest Akaike Information Criterion (AIC) and were selected to be used to determine the correction factors. The intercept was constrained to equal zero. Applying the coefficients from these models as correction factors, PON1 activity (AREase, CPOase, POase) was then predicted for a storage time of zero years and these storage duration corrected PON1 activity measures were then used in our generalized estimating equation (GEE) models comparing (1) pregnant mothers versus seven years later and (2) mothers at delivery versus seven years later. The comparison between pregnancy at 26 weeks gestation and at delivery did not require correction for storage duration as the time span was relatively short (~10 weeks).

Statistical analysis

To determine whether there were systematic differences between participants and non-participants in this longitudinal study, we used logistic regression to assess whether missingness was associated with numerous variables related to sociodemographics, exposures, and genotype frequency including: mother's country of birth, length of time in the United States, sex of the child, poverty levels, alcohol and tobacco use, and OP exposure (dialkylphosphate urinary metabolites). These factors were not associated with missingness with the exception of the number of years the mother spent in the United States. At the 7-year collection, the odds that mothers and their children remained in the study were 0.22 and 0.18-fold higher for mothers who had lived in the United States longer ($p=0.01$ and $p=0.03$ for mothers and children respectively).

Previous studies have reported associations of PON1 activity with factors such as nutrition, and alcohol and tobacco consumption (Deakin and James, 2004; Costa *et al.*, 2005b). Therefore, we also explored whether variables such as OP exposure (urinary dialkylphosphate metabolites), alcohol, and tobacco consumption, and some sociodemographic factors were also associated PON1 activity in our CHAMACOS subjects using univariate regression models. The number of years that the mother lived in the United States, which was previously shown to be correlated with differences in nutrition in the CHAMACOS cohort (Harley *et al.*, 2005), was negatively associated with AREase and POase activity in newborns ($p=0.04$ and $p=0.02$, respectively) and maternal smoking during pregnancy was associated with lower POase activity in seven-year olds ($p=0.026$). However, in this paper we focused on the effects of genotypes, age, and pregnancy on PON1 activity; inclusion of maternal smoking during pregnancy and length of time lived in the United States in statistical models did not significantly change the relationship between the main variables of interest and PON1 enzymatic activities (data not shown).

We used a chi-squared goodness of fit test to assess whether allele frequencies for each polymorphism deviate from Hardy-Weinberg equilibrium. Chi-squared tests were also used to determine whether allele and genotype frequencies in mothers and children were significantly different. The Cuzick's non-parametric test for trend was used to assess trends in PON1 activity

(AREase, CPOase, and POase) by PON1 genotypes. Since we performed numerous tests over many time points and three measurements (35 in children, 45 in mothers), we used Bonferonni correction in which a p-value < 0.001 was considered to be significant after adjusting for multiple testing. To infer haplotypes containing all five *PON1* polymorphisms (*PON1*$_{-909}$, *PON1*$_{-162}$, *PON1*$_{-108}$, *PON1*$_{55}$, *PON1*$_{192}$) from genotype data, we used PHASE 2.1 (Stephens *et al.*, 2001; Stephens and Scheet, 2005) software, which utilizes a Bayesian-based approach. To determine the proportion of variance (R^2) explained, separate regression models were constructed for dependent variables AREase, CPOase, and POase. Independent variables included in the model were either the five *PON1* genotypes or the imputed haplotypes. For each of the five polymorphisms, genotype variables were coded 0, 1, or 2 for the number of alleles present. Similarly, for the haplotype regression model, each haplotype with > 1% frequency was coded as a variable in the linear regression model, where the values 0, 1, or 2 denoted the presence of zero, one, or two copies of the haplotype for each subject. Haplotypes with < 1% frequency were pooled into one group for this analysis. Spearman correlation coefficients were calculated to assess correlations of PON1 activities between children at both time points and also between mothers and children at each time point for AREase, CPOase, and POase.

We performed GEE augmentation of linear models to compare AREase, CPOase, and POase activity (1) in mothers during pregnancy and delivery, (2) mothers during pregnancy and seven years later, (3) mothers at delivery and seven years later (4) in mothers at delivery versus children at birth, and (5) in both mothers and children at the seven-year collection. We used an exchangeable correlation structure to account for repeated measures on the same individuals or within the same family. Separate models for each of the three substrate-specific outcomes were generated. To compare PON1 activity in mothers at delivery versus pregnancy, we created an indicator variable where the reference group, mothers at pregnancy, was coded as 0 and mothers at delivery were coded as 1, which served as the independent variable. The other two comparisons, pregnancy versus seven years later and delivery versus seven years later, were treated similarly. To compare measures in mothers versus children at the same time point, we also used an indicator variable (child coded as 1, mother coded as 0) as the independent variable. Since we previously found that assay temperature can affect the PON1 activity measurement (Huen *et al.*, 2009b), assay temperature was included as a covariate in all models. Finally, to determine whether the differences between mothers and children or between mothers at different time points are modified by *PON1* genotypes, we also generated models for each of the five *PON1* SNPs, that included the number of variant alleles (0, 1, or 2) and an interaction term for the indicator variable of the main comparison multiplied by the number of alleles. All analyses were performed in STATA 10.0 (College Station, TX). P-values less than 0.05 were considered significant and p-values less than 0.10 were reported as marginally significant.

Results

PON1 polymorphisms and haplotypes

Genotype distributions did not deviate significantly from Hardy-Weinberg equilibrium. Allelic frequencies in mothers and children for all five SNPs are presented in Table 1. For SNPs *PON1*$_{-909}$, *PON1* $_{-108}$, and *PON1*$_{192}$ allele frequencies were approximately equal in this population. The frequencies of the major allele for *PON1*$_{-162}$ (G) and *PON1* $_{55}$ (L) were 81% and 82%, respectively. Allele and genotype frequencies did not differ significantly between mothers and children (χ^2 test: p-value > 0.05 for all five SNPs) (Table 1, Table 2, and Table 3 and Supplemental Tables 1 and 2). Inferred haplotypes containing all five SNPs were reported in the following order: *PON1*$_{-909}$ *C/G*, *PON1*$_{-162}$ *A/G*, *PON1* $_{-108}$ *C/T*, *PON1* $_{55}$ *L/M*, *PON1*$_{192}$ *Q/R* (Table 4). Haplotype analysis revealed 20 different combinations of alleles. Of these, 11 haplotype combinations accounted for more than 98% of the haplotypes present in

the CHAMACOS population. The most common haplotype (GGCTR) was observed in over 25% of mothers and children.

Effects of genotypes and haplotypes on PON1 activity

The means and ranges of AREase, CPOase, and POase activity by genotype are shown in Table 2 (children) and Table 3 (mothers) for $PON1_{192}$ and $PON1_{-108}$. Supplemental Tables 1 and 2 describe similar data for $PON1_{-909}$, $PON1_{-162}$, and $PON1_{55}$. All three promoter SNPs (*−108, −162, and −909*) were associated with AREase and CPOase activity for mothers and children at all time points (p_{trend} <0.05). The highest mean AREase and CPOase activities were observed in individuals with the $PON1_{-108}$ CC, $PON1_{-162}$ AA, and $PON1_{55}$ LL genotypes. PON_{-108} and $PON1_{-909}$ were also significantly associated with POase activity at all time points (p_{trend} <0.05).

The coding SNP, $PON1_{192}$, was associated with POase activity at all time points and CPOase activity at birth in both mothers and children, with QQ individuals showing the lowest activity levels and RR the highest. Patterns for AREase activity were less consistent; at birth, QQ newborns had the lowest mean PON1 activity levels ($p<0.005$) but QQ mothers had the highest activity levels ($p=0.015$ and $p<0.0005$ for mothers at delivery and during pregnancy, respectively). No significant trends in AREase activity were observed for $PON1_{192}$ at 7 years.

All three substrate-specific measures in both mothers and children were highest in those with the $PON1_{55}$ LL genotype and lowest in those with the $PON1_{55}$ MM genotype (Supplemental Tables 1 and 2). For AREase activity, this trend did not reach statistical significance ($p<0.05$) in mothers at the time of delivery and in seven-year old children, but was significant at all other time points.

The relative genetic contribution of the five *PON1* SNPs (genotypes and haplotypes) on the variation of PON1 phenotypes in mothers and children are shown in Table 5. In some instances, haplotypes can be better predictors of phenotype than genotypes because they incorporate multiple polymorphisms on a single chromosome (Stephens *et al.*, 2001). However, in our study, *PON1* haplotypes did not provide improved assessment of the effects of *PON1* genetic variability on enzyme activities in comparison to *PON1* genotypes. The five *PON1* genotypes explained 26% of the variance of AREase in newborns and seven-year olds. The same genotypes explained less variability in mothers, particularly at delivery (12%). In fact, for all three PON1 measures (POase, AREase, and CPOase), the contribution of these genetic determinants was much lower – almost half for AREase and CPOase – at delivery versus seven years later. At birth, the five *PON1* SNPs explained noticeably less variation of POase both in mothers and children (63% and 49%, respectively) in comparison to the seven-year measures, where POase variability was primarily due to these genetic polymorphisms (almost 80% variation explained), especially $PON1_{192}$.

Correlations

Correlations of enzyme activity between mothers and their children are presented in Supplemental Table 3. In general, children's PON1 activities were weakly correlated with those of their mothers with the exception of newborns and mothers at the time of delivery. As expected since their PON1 activities were closer to those of adults, seven year old children's activities ($r = 0.16–0.51$) were more highly correlated to their mothers than when they were newborns ($r=0.04–0.39$). Correlations of enzyme activity between time points (in the same individuals) are shown in Supplemental Table 4. Correlations of enzyme activities in mothers at birth and seven years later were statistically significant and much higher for POase ($r=0.86$) than for AREase ($r=0.38$) and CPOase and ($r=0.44$). For AREase and CPOase activities in

children at the two time points, correlations were higher than those in mothers (r= 0.47 and 0.58, respectively) but were slightly lower for POase (r=0.80).

PON1 activity in children

AREase, CPOase, and POase activities in children at birth and age seven are described in Table 2. Overall, mean AREase activity increased 3.7-fold and mean CPOase and POase activities both increased 3.5-fold between birth and year seven. We previously reported an age-dependent increase in children's PON1 activities from birth through ages one, two, five and seven (Huen *et al.*, 2009a) and here we focus on the two time points which coincide with maternal sampling (birth and age seven). A graph presenting these data and also PON1 activities in mothers is shown in Figure 1. The graph clearly demonstrates a wide inter-individual variability between study participants at all time points. Among newborns, there was a 38-fold difference between the highest and lowest AREase levels. When we compared the lowest AREase levels in newborns to the highest levels in seven year olds, there was a 64-fold difference. Since cord blood measurements reflect both fetal and placental expression, we explored whether the mother's genotype or phenotype influenced PON1 activity in newborns. Among newborns sharing the same $PON1_{-108}$ genotype, neither maternal AREase activity nor maternal $PON1_{-108}$ genotype affected the AREase activity of their newborns. For instance, among $PON1_{-108}$ CC newborns, their mothers' $PON1_{-108}$ genotype was not significantly associated with the newborns' AREase activity ($p>0.05$). Similar results for POase activity were found such that among newborns sharing the same $PON1_{192}$ genotype, neither $PON1_{192}$ genotype nor POase activity of their mothers affected the newborns' POase activities.

PON1 activity in mothers

AREase, CPOase, and POase activities in mothers during pregnancy, at delivery, and seven years later are described in Table 3. In comparison to pregnancy, PON1 activity at delivery was slightly higher for all three measures and these differences were statistically significant in the GEE models ($p\leq0.001$) (Supplemental Table 5). Before adjusting for storage duration, PON1 activity during pregnancy (26 weeks gestation) appeared slightly lower than seven years later. However after adjustment for storage duration, we found higher AREase, CPOase, and POase activities during pregnancy ($p\leq0.005$, GEE models). Even before applying the storage duration correction factors, means for AREase, CPOase, and POase were elevated in mothers at the time of delivery in comparison to seven years later (7.4, 3.8, and 2.2%, higher respectively). After applying the storage duration correction factors, the difference between delivery and seven years later became even more apparent. Mean AREase and CPOase and median POase activities were 29.0, 49.1, and 20.8% higher at the delivery time point compared to seven years later and this difference was statistically significant in the GEE models ($p<0.001$ for all three measures). Results were similar when we adjusted for each of the five *PON1* SNPs.

PON1 activity in children compared to mothers

Previously we reported that newborns have significantly lower PON1 activities than their mothers and that as children age, their activities increase over time (Holland *et al.*, 2006; Huen *et al.*, 2009a). In this expanded dataset, mean AREase and CPOase and median POase activities at birth were about four-fold lower in newborns than mothers ($p<0.005$ in all three GEE models) (Supplemental Table 5). At age seven, children's PON1 activities were much closer to those in mothers (mean AREase: 4.3%; mean CPOase: 4.1%; median POase: 1.8% lower in children than in mothers). Although children's enzymatic activities at this age were more comparable to mothers, the GEE models indicated the difference in PON1 activity between mothers and children remained statistically significant for AREase ($p=0.05$) and CPOase ($p=0.02$) and marginally significant for POase ($p=0.08$).

Since we previously found that the age-dependent increase of PON1 activity is modified by *PON1* genotype in young children (Huen *et al.*, 2009a), we sought to determine whether the differences in activity between mothers and children are also modified by *PON1* genotype. When we adjusted for *PON1* genotypes and included an interaction term (*PON1* genotype × indicator variable for child) in the GEE models, we found that children's PON1 activities were only significantly lower than those of mothers for specific child genotypes. For instance, among children with the $PON1_{-108}$ CC genotype (the genotype associated with increased AREase activity compared to the TT genotype), children's mean AREase activity was not significantly different than that of mothers (includes mothers of all genotypes). However, among children with the $PON1_{-108}$ CT genotype, children's AREase activities were significantly lower than those of all mothers and this difference was even larger among children with the TT genotype (p-value for interaction term=0.07). Similarly, for CPOase and POase, activities in children with the CC genotype were not significantly different than mothers, however the significant interaction terms (p=0.009 and 0.03, respectively) indicated that activities for CT and TT children were significantly lower than those of mothers (β for interaction term= −458.6 and −100.3 for CPOase and POase, respectively). When we limited the comparison to mothers and children with the same genotype, children's AREase and CPOase activities were lower for all three genotypes however this difference was only statistically significant for the $PON1_{-108}$ TT genotype on AREase activity (p=0.01). For POase activity, children's activities were lower than mothers for CC and CT genotypes but this did not reach statistical significance.

We also identified a significant interaction for the promoter SNP at position −909 when comparing CPOase and POase activity between mothers and children (β for interaction term = − 473.8 and −106.2; p=0.008 and 0.03, respectively). Mean activity for $PON1_{-909}$ CG and CC children was significantly lower than mean activity in all mothers but this difference was not significant in $PON1_{-909}$ GG children (p=0.38 and 0.58 for CPOase and POase, respectively). For the coding SNP at position 192, mean POase activity in QQ children was 289.4 U/L lower than that in mothers (p<0.0005), yet the significant interaction term (β=216.3; p<0.0005) indicated that this difference was much smaller in QR children and the mean POase activity in RR children was actually higher than the overall mean in mothers. However, when we limited comparisons to mothers and children with the same $PON1_{192}$ genotype, children's POase activities were lower in all three genotype groups however, this difference was only statistically significant for the RR genotype (β indicator variable for children =−130.8, p=0.05).

DISCUSSION

In this study, three substrate-specific measures of PON1 quantity and activity were measured in over 400 mothers and children at the time of delivery, when the children were seven years old, and also in mothers during pregnancy. Although children's PON1 activities increased about 3.5-fold between birth and age seven, they still remained 1.8–4.3% lower than levels measured in their non-pregnant mothers. Mothers had significantly higher levels of PON activity during pregnancy than seven years later when they were not pregnant. PON1 activity was particularly high at the time of delivery compared both to 26 weeks gestation (means were 1.9– 8.5% higher at delivery) and seven years later (means were 20.8–49.1% higher at delivery). Using regression analysis, we found that 5 *PON1* genotypes explained a similar proportion of variance compared to inferred haplotypes. The genetic contribution of the 5 *PON1* SNPs on phenotypic variation differed in children between birth and age seven and also between pregnant and non-pregnant mothers suggesting additional factors may affect PON1 expression depending on age and pregnancy status.

In an extended analysis of mothers and newborns, we confirmed our previous data in 130 mother-child pairs that PON1 activities are four-fold lower at birth in comparison to adults (Holland *et al.*, 2006). Therefore, newborns and young infants may have increased

vulnerabilities to OP exposure and oxidative stress. At age seven, children's PON1 activities on average remained significantly lower than in their adult, non-pregnant mothers suggesting that even at school-age, children may remain more susceptible to pesticide exposure and oxidative stress than adults. When adjusting for *PON1* genotypes, we found that PON1 activities in children with $PON1_{192}$ RR and $PON1_{-108}$ CC genotypes (genotypes related to increased catalytic efficiency and enzyme quantity, respectively) were much more similar to adults by age seven, and children with other genotypes (those associated with lower quantity and efficiency of the enzyme) experienced a pronounced lag in developmental expression with significantly lower PON1 activities than mothers at age seven. It is not yet clear if PON1 activities in children with susceptible genotypes will eventually reach average mothers' levels as they continue to age or if their levels will remain lower throughout their lives.

Similar patterns of differential developmental expression have been observed in other metabolic and antioxidant enzymes. For instance, pharmacokinetic studies and *in vitro* data demonstrate that many cytochrome P-450 enzymes (CYPs) at birth are quite low (about 30% that of adults) and then increase quickly until they reach adult levels within six months to a year (Ginsberg *et al.*, 2004). In rat livers, several antioxidant enzymes involved in detoxification of xenobiotics (GPx, GST, and GR) were very low at birth and did not begin to reach adult levels until puberty (Elbarbry 2009). Previously, we reported that whole blood cholinesterase levels in CHAMACOS newborns were 25% lower than in their mothers at delivery, and the difference was even larger (33%) when comparing with maternal levels at 26 weeks of pregnancy (Eskenazi *et al.*, 2004). Data on the ontogeny of different enzymes related to xenobiotic metabolism, included that reported here in PON1, can be used by toxicokinetic models and risk assessment to account for potential susceptibilities during development.

In addition to OPs, variability of PON1 activity may also affect susceptibility to other environmental exposures. As mentioned earlier, it is likely that the endogenous function of PON1 is related to its antioxidant activities involved in lipid peroxidation. Exposure to a wide variety of toxicants including arsenic, polybrominated diphenyl ethers (PBDEs), and bisphenol A (BPA) has been shown to induce oxidative stress in humans and/or human cell lines (Barchowsky *et al.*, 1999; Franco *et al.*, 2009; Gao *et al.*, 2009; Hong *et al.*, 2009; Tagliaferri *et al.*, 2009; Yang *et al.*, 2009). Through this mechanism, variability of PON1 activity and levels may affect vulnerabilities to multiple environmental exposures. For example, two recent studies of arsenic exposure suggest PON1 may modify the effect of arsenic on cardiovascular outcomes (Li *et al.*, 2009; Liao *et al.*, 2009). Therefore young children with low PON1 activity may experience increased susceptibility not only to OP exposures, but to a multitude of environmental hazards due to reduced antioxidant capacity.

In addition to age-related changes observed in children, we also found PON1 activity varied by pregnancy status in mothers; all three substrate-specific activities were elevated during pregnancy, particularly at the time of delivery, in comparison to the seven year time point. Two other studies in pregnant and non-pregnant women corroborate our findings that PON1 activity (AREase and POase) is elevated during pregnancy (Roy *et al.*, 1994; Carpintero *et al.*, 1996). Others studies have reported increased levels of lipid hydroperoxides (markers of oxidative stress), triglycerides, and lipoproteins in late pregnancy accompanied by higher total antioxidant capacity reflected by greater levels of uric acid, and vitamins C and E (Toescu *et al.*, 2002). Increased levels of PON1 during late pregnancy and at delivery suggest yet another mechanism by which the body may maintain oxidant balance during late pregnancy when free radical generation may be accelerated.

We previously determined that 5 *PON1* SNPs account for 23.1% and 8.1% of the variability of AREase activity in newborns and their mothers, respectively (Holland *et al.*, 2006). In this study, we included all Mexican-American mothers and children from the CHAMACOS cohort

(for whom specimens were available) in the analysis and determined the genetic contribution of phenotypic variation both at birth and age seven; results at birth were very similar to those we previously reported in the subset of 130 mother-child pairs. At birth, fifty percent of POase activity was explained by these five genotypes and at age seven, the genetic contribution was even higher (78%). Furthermore, this was primarily due to the $PON1_{192}$ polymorphism (partial correlation coefficient r = 0.58 and 0.82 for newborns and seven-year olds, respectively) which has previously been shown to account for a large proportion of variance in other recent studies (48% and 58.5%, respectively) of POase activity (Bhattacharyya *et al.*, 2008; Rainwater *et al.*, 2009). This finding suggests that the strong genetic control exerted by the $PON1_{192}$ SNP on POase activity is weaker at birth, and other factors may play a significant role at this time.

At the time of delivery, we found that a much lower proportion of PON1 variability (particularly for AREase and CPOase activity) was explained by genotype in mothers. Thus, child birth may represent a time in which environmental and physiological factors or other unidentified genetic factors have a stronger influence on PON1 phenotype. In both mothers and children, $PON1_{192}$ genotype accounted for the majority of POase activity (up to 80%) yet the five *PON1* polymorphisms, including three promoter SNPs only accounted for about 12–26% of AREase activity (PON1 quantity). Therefore, other genetic polymorphisms in the PON1 gene and elsewhere in metabolic networks or other types of unidentified factors like epigenetic modifications and environmental exposures may serve as additional determinants of PON1 expression. Other factors that have been shown to contribute to variability PON1 enzyme activity in adults include environmental and behavioral factors such as alcohol consumption, cigarette smoke, and diet (Li *et al.*, 2003). Physiological conditions such as diabetes, Alzheimer's disease, and cardiovascular disease have also been linked to reduced PON1 activity (Deakin and James, 2004), but it is not yet clear whether depressed PON1 activity leads to these conditions or conversely if these conditions trigger lower activity.

In summary, we examined PON1 genotypes and phenotypes in Mexican-American mothers and their children living in an agricultural community when the children were born and also when the children reached seven years of age. For many children, especially those with genotypes associated with decreased PON1 activities, their PON1 levels remain lower than those of their mothers even at age seven. This finding suggests that young children through school-age, particularly those with the most vulnerable genotypes, $PON1_{-108}$ TT, $PON1_{192}$ QQ, and $PON1_{-909}$ CC, do not have mature levels of the protective PON1 enzyme and may continue to experience increased susceptibility to OP exposure and oxidative stress. Furthermore, this increased vulnerability due to low PON1 activity may also be relevant to additional environmental toxicants like BPA and PBDEs via the oxidative stress pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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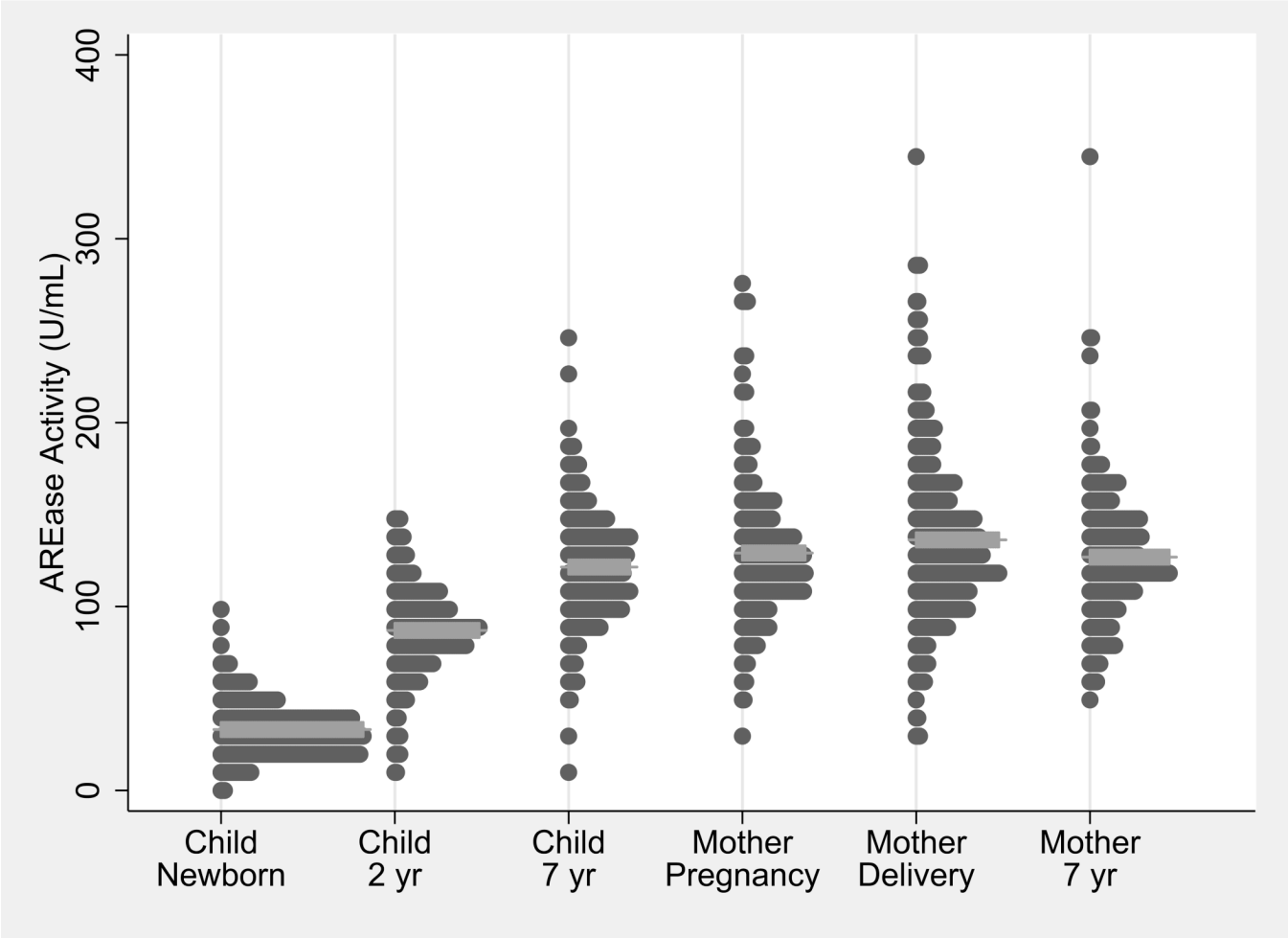

Figure 1.

The distribution of AREase in the CHAMACOS children at birth, age 2, and age seven and in their mothers during pregnancy (26 weeks gestation), at the time of delivery, and when their children were seven years old. Mean AREase activities as indicated by the grey horizontal bars, were 33.1, 89.2, 121.5 U/mL in children at birth, 2, and 7 years of age, respectively. In mothers, mean AREase activities were 129.1, 136.3, and 127.3 U/mL at pregnancy, delivery and seven years later, respectively. PON1 enzymatic activity was very low in newborns and continued to increase over time. At age seven, activities were still significantly lower than that of mothers at the same time point ($p= 0.05$). PON1 activities were elevated in mothers at the time of delivery ($p<0.0005$). This figure contains some of the children's PON1 activity data (children at age two) modified from Huen et al. (2009a).

Table 1

PON1 allelic frequencies (%) in Mexican-American mothers and their children

SNP	Allele	Mothers (n=431)	Children (n=434)	Total Population[a] (n=865)
192	Q	51.1	50.3	50.6
	R	48.9	49.7	49.4
55	L	81.3	82.2	82.0
	M	18.7	17.8	17.0
−108	C	53.1	54.3	53.9
	T	46.9	45.7	46.1
−162	A	19.1	19.2	19.0
	G	80.9	80.8	81.0
−909	C	45.5	45.9	46.5
	G	54.5	54.1	53.5

[a]There were no significant differences observed between PON1 allele frequencies in mothers and children. All distributions did not deviate from Hardy-Weinberg equilibrium.

Table 2

Summary of PON1 Enzyme Activity by *PON1* Polymorphisms in Children at Birth (n=335) and Age 7 (n=268)

PON1		AREase[c] (U/mL)		CPOase[d] (U/L)		POase[e] (U/L)	
	Frequency (%)	Birth Mean(Range)	7 Years Mean(Range)	Birth Mean(Range)	7 Years Mean(Range)	Birth Mean(Range)	7 Years Mean(Range)
192							
QQ	24.4	31.7(3.9–70.9)[a]	129.5(52.9–246.3)	1811.3(175.3–4397.6)[ab]	6885.8(2074.8–11181.3)	110.0(13.1–519.7)[ab]	316.4(119.5–954.7)[ab]
QR	51.6	32.2(3.8–98.0)	119.9(14.5–184.6)	1974.7(153.9–5255.2)	6985.0(904.2–11195.7)	253.7(7.5–682.1)	881.3(133.6–1444.2)
RR	24.0	36.7(9.7–73.6)	117.6(61.1–190.4)	2334.1(569.3–5159.5)	7321.5(3858.3–11576.7)	408.3(62.1–1017.5)	1461.8(782.0–2504.5)
−108							
CC	27.8	42.2(13.9–90.3)[ab]	138.5(73.3–246.3)[ab]	2607.6(823.4–4397.6)[ab]	8286.1(3737.3–11576.7)[ab]	348.8(38.3–742.8)[ab]	1218.2(166.0–2504.5)[ab]
CT	53.2	32.0(3.8–98.0)	119.6(14.5–198.9)	1939.1(153.9–5255.2)	6863.8(904.2–11195.7)	241.9(7.5–1017.5)	844.5(119.5–2005.8)
TT	19.0	23.2(3.9–54.7)	101.7(33.6–159.9)	1389.1(175.3–3409.0)	5734.6(1896.8–7967.2)	157.9(13.1–485.6)	568.2(155.5–1468.2)
All		33.1(3.84–98.0)	121.5(14.5–246.3)	2020.2(4153.9–5255.2)	7035.8(904.2–11576.7)	257.1(7.5–1017.5)	894.2(119.5–2504.5)

In children, the *PON1192* genotype was significantly associated with POase activity at both time points. Children with the RR genotype had the highest mean POase activities compared to QR and QQ children. The promoter polymorphism, *PON1−108*, was associated with all three substrate-specific activities at birth and age 7. On average, children with the TT genotype had lower activities than those with the CT and CC genotypes.

[a] P_{trend} for genotype <0.05, determined by Cuzick's non-parametric test

[b] P_{trend} for genotype significant after Bonferonni correction for multiple testing (n=35 tests, p≤0.001)

[c] AREase = arylesterase activity

[d] CPOase = chlorpyrifos-oxonase activity

[e] POase = paraoxonase activity

Table 3

Enzyme Activity by *PON1* Polymorphisms in Mothers During Pregnancy (270), Delivery (n=382), and 7 Years Later (n=263)

AREase[c] (U/mL)			CPOase[d] (U/L)			POase[e] (U/L)		
Pregnancy Mean(Range)	**Delivery Mean(Range)**	**7 Years Later Mean(Range)**	**Pregnancy Mean(Range)**	**Delivery Mean(Range)**	**7 Years Later Mean(Range)**	**Pregnancy Mean(Range)**	**Delivery Mean(Range)**	**7 Years Later Mean(Range)**
141.1(44.9–263.2)[ab]	143.8(29.9–346.6)[a]	129.3(56.6–247.0)	6635.5(2197.1–10623.3)	7198.0(1766.1–13948.6)[a]	7025.6(2946.1–12302.8)	340.6(93.0–1167.3)[ab]	360.7(75.2–2694.9)[ab]	336.1(144.5–919.9)[ab]
127.7(31.3–272.4)	134.8(40.1–255.9)	130.2(60.4–348.5)	6832.9(1856.0–14810.3)	7576.0(2376.9–13807.1)	7546.8(2937.9–12759.4)	938.7(145.6–1953.7)	989.2(174.8–2856.4)	989.4(243.9–2299.2)
118.7(57.2–263.3)	129.3(27.1–285.9)	120.4(54.5–181.6)	6666.1(3811.5–11735.5)	8067.8(1927.7–14675.8)	7420.9(3368.5–11429.9)	1503.2(699.0–3757.1)	1648.1(294.3–3537.7)	1555.7(666.1–2390.8)
145.8(44.9–272.4)[ab]	151.6(40.1–289.1)[ab]	143.8(54.5–348.5)[ab]	7661.3(2197.1–14810.3)[ab]	8628.8(2376.9–14675.8)[ab]	8497.1(3442.2–12759.4)	1228.8(93.0–3757.1)[ab]	1347.7(148.2–3537.7)[ab]	1324.5(144.5–2390.8)[ab]
127.4(31.3–263.2)	134.5(27.1–265.1)	127.0(63.6–182.9)	6596.2(1856.0–10623.3)	7617.6(1766.1–12931.6)	7411.5(2937.9–12302.8)	861.6(145.6–3256.7)	928.1(75.2–2856.4)	915.6(204.2–2299.2)
114.9(52.6–269.3)	118.3(34.5–346.6)	106.0(56.6–206.4)	6032.9(3191.7–11735.5)	6310.0(2067.7–13948.6)	5891.1(2946.1–8981.8)	730.2(138.2–2387.3)	680.5(134.3–2564.2)	639.3(146.5–1491.3)
129.1(31.3–272.4)	136.3(27.1–346.6)	127.3(54.5–348.5)	6725.4(1856–14810.3)	7613.2(1766.1–14675.8)	7376.0(2937.9–12759.4)	912.1(93.0–3757.1)	987.2(75.2–3537.7)	965.6(144.5–2390.8)

SNP was associated with POase activity at all 3 time points with the highest mean activities in RR mothers and the lowest mean activities in QQ mothers. The coding SNP
d with all three substrate specific activities at all 3 time point with the highest mean acitivities in CC mothers and the lowest mean activities in TT mothers.

5, determined by Cuzick's non-parametric test

ficant after Bonferonni correction for multiple testing (n=45 tests, p≤0.001)

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Table 4

PON1 Haplotype frequencies (%) in Mexican-American Mothers (n=431) and Children (n=434)

Haplotype[a,b]	Mothers	Children
GGCLR	25.1	26.4
CGTMQ	15.2	14.9
CGTLR	15.2	14.7
CGTLQ	14.3	14.3
GACLQ	11.2	10.8
GGCLQ	6.6	6.7
GACLR	5.7	6.4
CGCLR	1.7	1.3
GGCMQ	0.9	1.3
GATLQ	1.4	1.1
GGCMR	1.0	0.6

[a] Polymorphisms in five loci of PON1 gene are listed in the haplotypes using the following order: *−909(C/G), −162(A/G), −108(C/T), 55(L/M),192 (Q/R)*

[b] The eleven haplotypes listed represent 98.4% and 98.5% of the haplotypes present in CHAMACOS mothers and children, respectively. The remaining nine haplotypes (not shown) had frequencies lower than 1%.

[c] *PON1* haplotypes were inferred using PHASE 2.1 software.

Table 5

Proportion of variance explained for PON1 enzyme activity by genotype and haplotype regression models

	Children				Mothers			
		AREase	CPOase	POase		AREase	CPOase	POase
	n	R^2	R^2	R^2	n	R^2	R^2	R^2
Birth	330				395			
5 *PON1* Genotypes		0.266	0.323	0.490		0.123	0.157	0.626
Haplotypes		0.295	0.355	0.510		0.126	0.170	0.619
7 yr	266				253			
5 *PON1* Genotypes		0.260	0.297	0.782		0.201	0.303	0.778
Haplotypes		0.272	0.319	0.775		0.222	0.355	0.780

The proportion of variance (R^2) was determined by running separate regression models for each substrate-specific assay. AREase refers to the arylesterase assay which utilizes a phenyl acetate substrate. CPOase is the chlorpyrifos-oxonase assay, which uses the substrate chlorpyrifos-oxon. POase is the paraoxonase assay using the paraoxon substrate. Models either included a variable for each of 5 *PON1* polymorphisms: *−909(C/G), −162(A/G), −108(C/T), 55(L/M),192(Q/R)* or ordinal variables for haplotypes containing all 5 polymorphisms. These models were performed both in samples collected in mothers and children at time of the child's birth and in samples collected from both mothers and children when children were 7 years old.