Recent progress in the genetics and epigenetics of paraoxonase: why it is relevant to children’s environmental health

Nina Holland, Daneida Lizarraga, and Karen Huen

INTRODUCTION

Paraoxonase 1 (PON1) is an enzyme involved in oxidant defense by hydrolyzing oxidized lipids [1] and also plays a key role in the detoxification of some organophosphate pesticides [2]. Thus, individuals with low PON1 levels and activities may be more susceptible to organophosphate exposures and oxidative stress, which occurs when there is an excess of damaging reactive oxygen species. PON1 genetic variants and lower enzyme levels have been linked to adverse health outcomes including oxidative stress-related conditions such as cardiovascular disease and obesity [3–7]. Therefore, it is of considerable clinical interest to characterize the protective role of endogenous antioxidant enzymes against the development of obesity and metabolic syndrome (MetS) in children. Previous reviews of PON1 research have shown that age and genetics are key factors associated with PON1 variability and, thus, susceptibility. Here, we highlight novel developments in PON1 research including epigenetic mechanisms such as DNA methylation and non-coding RNAs, as well as the effects of genetic admixture, especially important for studies of minority populations, and the relationship of genetic and epigenetic markers with obesity and metabolic disease.

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KEY POINTS

- The broad variability of PON1 levels and activities in humans can confer differential susceptibility because this multifunctional enzyme can detoxify organophosphate pesticides and has antioxidant properties.
- Genetics and age are strong determinants of PON1 variability and, therefore, susceptibility to oxidative stress and organophosphate exposure.
- Epigenetic modifications such as DNA methylation and miRNAs may provide additional insights into the mechanisms of PON1 variability.
- In addition to commonly used measurements for PON1 status, as well as AREase and POase activity, future studies of PON1 function may be enhanced by incorporation of lactonase activity as a reliable indicator of PON1 phenotype.
- In order to use PON1 as a model to predict risk of obesity and other health outcomes, it should integrate enzyme activities, genetic and epigenetic factors, and take into account genetic ancestry as a potential confounding factor.

PARAOXONASE SUBSTRATE-SPECIFIC ACTIVITIES, SINGLE NUCLEOTIDE POLYMORPHISMS, AND STATUS

Human PON1 enzyme is a 43kDa protein composed of 354 amino acids. It is referred to as a multifunctional enzyme because it can metabolize a number of substrates including some oxon forms of organophosphate pesticides (this is where the name paraoxonase originates from) and aryl esters, among others. Spectrophotometric methods have been established for the most commonly used substrates for the measurement of PON1 molecular phenotype. These include phenyl acetate and paraoxon, which measure arylesterase (AREase) and paraoxonase (POase) activity, respectively. AREase activity is considered an indirect measure of PON1 protein level and has been highly correlated with western blot and ELISA experiments [8,9]. POase activity reflects both quantity and catalytic efficiency of the enzyme.

More recently, several studies have demonstrated that the native substrates for PON1 are likely lactones [10] and researchers are now beginning to incorporate the use of lactonase substrates such as dihydrocoumarin [11*] and 5-thiobutyl butyrolactone (TBBL) in PON1 experiments [11*,12*,13]. For instance, Ferre et al. [12**] found that TBBLase, but neither POase nor PON1 concentrations, determined by ELISA, was significantly associated with obesity status in prepubertal and adolescent children (age 9–15 years) as well as several obesity markers such as BMI, body fat percentage, and triglyceride levels (Table 1). Obese children with MetS had even lower levels of TBBLase than obese children who did not have MetS [12**]. As lactones are the endogenous target of PON1, lactonase activity may be an important and relevant marker of PON1 molecular phenotype particularly for studies related to oxidative stress rather than organophosphate pesticide exposure.

The polymorphic PON1 gene is a member of the PON family cluster of genes including PON1, PON2, and PON3, which all reside adjacent to each other on chromosome 7. PON1 specifically has been mapped along the chromosome 7q21.3–22.1 region [17,18] and contains nine exons. Over 200 gene variants have been identified in the PON1 gene; however, only a few promoter single nucleotide polymorphisms (SNPs) significantly influence protein levels as measured by AREase activity [19–23]. In particular, rs705379 (PON1_108), is the strongest predictor of AREase activity. Furthermore, the PON1_108CC genotype is associated with two-fold higher PON1 enzyme levels compared with the PON1_108TT genotype [24,25]. The SNP at the position 192 (rs662) in the coding region impacts the catalytic efficiency of the enzyme in detoxifying organophosphate pesticides with the PON1_192QQ genotype coding for the enzyme with the lowest efficiency [26]. A number of studies have examined the association of numerous PON1 SNPs with PON1 molecular phenotype but the majority of SNPs do not explain a substantial amount of additional variation of enzyme quantity or activity in comparison to the commonly studied PON1_108 and PON1_192 SNPs [22,27]. One recent study identified a few rare PON1 variants and some transacting variants located in the FTO and SERPINA12 genes that displayed a modest association with AREase activity independent of common PON1 SNPs [28]. However, these associations were not as strong as those with previously identified PON1 SNPs.

Although most studies of PON1 focus on just a small set of the most common PON1 SNPs, one recent study characterized 16 PON1 genetic variants and their relationship with several different substrate-specific activities, including lactonase (using dihydrocoumarin substrate), POase, AREase, and diazoxonase (DZOase) activities in prepubertal children (ages 4–16 years) [11**]. The relationships of these SNPs with the different substrate-specific PON1 activities were highly dependent on their location in the gene. For instance, promoter region SNPs were highly associated with AREase, lactonase, and DZOase, whereas 3’ untranslated SNPs were more strongly associated with POase and less
Table 1. Summary of studies related to paraoxonase, obesity and metabolic syndrome in children

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AREase, arylesterase; DZOase, diazoxonase; MetS, metabolic syndrome; NS, no significant relationship; POase, paraoxonase SNPs, single nucleotide polymorphisms. Negative or positive association with obesity and other health outcomes is indicated by (−) or (+) symbols.

Table 1. Summary of studies related to paraoxonase, obesity and metabolic syndrome in children

- Andersen et al. [14] studied 141 children, including 88 pesticide exposed and 53 unexposed, aged 6–11. They found no significant association between PON1 and obesity.
- Huen et al. [15] examined 373 children aged 2 and 5, finding a significant association between the PON1 rs662 SNP and obesity.
- Ferre et al. [12] investigated 110 obese children and adolescents aged 9–15, measuring PON1 activities and finding no significant association with obesity.
- Krzyteek-Karpacka et al. [16] assessed 156 children and adolescents aged 14 ± 2, where they observed a significant association between PON1 AREase and waist circumference.

**OTHER GENETIC EFFECTS: ADMIXTURE AND ANCESTRY INFORMATIVE MARKERS**

In genetic association studies of admixed populations, heterogeneity of genetic background can lead to spurious associations if ancestry is related to both a candidate gene and the disease outcome of interest (Fig. 1a); this is also referred to as genetic confounding due to population stratification [31]. Structured association methods enable us to adjust for potential genetic confounding. This is done by genotyping a number of ancestry informative markers, genetic variants known to vary widely between different ethnic groups, and using the known frequencies among reference populations to estimate proportion of ancestry in individuals. The estimated parameters can then be included in statistical models as covariates. Figure 1b and c show examples of proportional ancestry distribution estimated by the genotyping of over 100 ancestry informative markers for two different studies of Latino populations, Center for Health Assessment of Mothers and Children of Salinas (CHAMACOS) [15] (Mexicans) and Genes-environments and Admixture in Latino Americans (GALAI) [32]. Ancestral distributions range quite broadly within populations in both studies and between populations for GALAI.

Both functional PON1 genetic variants PON1<sub>192Q</sub> and PON1<sub>192Q</sub> among others, vary substantially among ethnic groups. For instance, the frequency of the Q allele for the PON1<sub>192</sub> SNP is 0.73 for Caucasians [33], 0.37 for African–Americans [33], and 0.48 for Mexicans [21,34]. Furthermore, many of the oxidative stress-related health outcomes ranging from birth weight to obesity and cardiovascular disease differ in prevalence by ethnic groups, making genetic ancestry an important factor to consider in PON1 studies, especially in admixed populations. We recently examined associations of PON1, obesity, and genetic ancestry in young Mexican–American children [15]. Although a trend of higher African ancestry with higher BMI z-scores and odds of obesity was observed, these relationships did not reach statistical significance after adjusting for multiple testing. We also identified a strong increased odds of obesity in children with the PON1<sub>192QQ</sub> genotype at ages 2 and 5 and found...
similar trends in relation to waist circumference at age 5. After controlling for genetic ancestry, this relationship with $PON1_{192}$ genotype remained, although beta coefficients changed moderately (9–15%), demonstrating suggestive evidence of genetic confounding by population stratification. Presence of genetic confounding may explain some of the inconsistencies between reported studies as it can introduce bias, yet to our knowledge, few other studies have attempted to adjust for population stratification in $PON1$ genetic association studies [11**,35].

**PARAOXONASE EPIGENETICS**

$PON1$ genetics does not completely explain the broad variability of $PON1$ molecular phenotype and other factors should be considered. There is a growing recognition that epigenetics may play an important role in key biological processes and mechanisms of disease development [36–38]. Epigenetic mechanisms regulate gene expression without changes in DNA sequence and include DNA methylation, histone modifications, and noncoding RNAs [39–41]. Here, we focus on DNA methylation and microRNA (miRNA) because, in contrast to chromatin modification assays that are mainly qualitative and require large sample volumes, the state-of-the-art methodologies available for these marks are more amenable to human population studies. Furthermore, a 2011 review of $PON1$ research describing potential regulators of $PON1$ expression highlighted epigenetics as an unexplored field [42].

DNA methylation is the most extensively investigated epigenetic mechanism and refers to the potential of a cytosine (C) base to be methylated at its fifth carbon if followed by a guanine (G) base in the DNA code, called a CpG site. The human genome contains about 30 million CpG sites. CpG sites are distributed throughout several regions of the genes referred to as CpG islands, shores, shelves, and gene bodies. CpG islands are stretches of DNA with a high frequency of CpG dinucleotides that often occur in proximity to gene promoter regions [43]. It was previously believed that the majority of functional changes occurred in CpG islands, but new research has shown that DNA methylation changes along CpG shores (regions within 2 kb of

FIGURE 1. (a) shows that if differences in ancestry are associated both with genotype and the outcome of interest, genetic ancestry can act as a source of genetic confounding. Bar plot of genetic ancestry estimates are generated by STRUCTURE software (Stanford University, Stanford, CA). Estimates were expressed as proportion of European, African and Native American ancestry in (b) Mexican–American Center for Health Assessment of Mothers and Children of Salinas (CHAMACOS) children ($n=375$) and (c) GALAII participants of Hispanic origin ($n=6021$). Each vertical bar represents the ancestral distribution in one participant. For each participant, the proportions of Native American (red), African (blue), and European (tan) ancestry are displayed. Figure 1b is reproduced (with minor modifications) with permission from [15**] and Figure 1c is reproduced with permission from [32]. $PON1$, paraoxonase 1; SNPs, single nucleotide polymorphisms.
islands) and within the gene body may also have functional effects on gene expression [44,45]. The amount and patterns of DNA methylation are established during the prenatal period and may vary by tissue and cell type [46,47]. Gain or loss of DNA methylation, referred to as hypermethylation and hypomethylation can lead to gene silencing or overexpression, respectively [48].

The PON1 promoter has one CpG island comprising 19 CpG sites with a second island located near exon 7 (eight CpG sites). There are a total of 287 CpG sites in PON1 including 66, 48, and 146 CpG sites within shores, shelves, and other regions referred to as the open seas, respectively (Fig. 2). Data on PON1 methylation are scarce. Only one study has examined associations between PON1 methylation at several CpG sites with molecular phenotype [49**]. De la Iglesia et al. [49**] reported an inverse association between methylation levels of several promoter region CpG sites and AREase activity in 47 adults with MetS in an energy-restricted dietary weight-loss intervention. This relationship was strongest for CpG sites that were closest in proximity to the PON1 transcription start site. Additionally, a parallel decline in AREase activity was seen with decreases in several obesity-related parameters such as BMI, fat mass, blood pressure, and triglyceride levels. Another small study of 24 adults also reported an association between methylation at two PON1 promoter CpG sites with body weight and waist circumference, providing further evidence that PON1 DNA methylation may influence obesity risk [50].

Another class of epigenetic marks includes miRNAs, which are small (~23 nucleotide) noncoding RNAs that regulate gene expression by pairing with protein-coding mRNAs and directing their posttranscriptional repression. To date, approximately 2500 miRNAs have been identified in humans; however, the majority of their target-binding sites are not yet known [51]. Putative-binding sites within coding sequences can be identified by sequence complementarity to candidate miRNAs [52]. MiRNAs regulate protein expression by the cleavage of homologous mRNA or by specific inhibition of translation. It is estimated that 10–30% of human protein-coding genes are targets of miRNA binding [53], and aberrant expression of miRNA has been implicated in numerous diseases [54,55]. MiRNAs are an excellent epigenetic biomarker to study for the following reasons: they are ubiquitously expressed in tissues and body fluids including blood, urine, and saliva; as miRNAs are released into the bloodstream from target tissues (i.e., brain, liver) circulating miRNAs may reflect profiles of target tissue [56]; and they are highly stable and resistant to RNase activity as well as effects of pH and temperature in stored specimens over time [55].

In-silico analyses using miRanda software (Memorial Sloan Kettering Cancer Center, New York, NY), and considering conservation and good support vector regression (SVR) scores, have yielded 25 putative miRNA binding sites in the PON1 3’ untranslated region (Fig. 2). One recent study showed that a PON1 SNP located in a different miRNA binding site (miR-616) was associated both with changes in PON1 expression and increased risk of ischemic stroke and carotid atherosclerosis [57**]. Unlike the other putative miRNAs that may target PON1, this is the only miRNA that has been functionally validated and shown by reporter assay to bind PON1. Furthermore, Liu et al. [57**] were able to show that the PON1 SNP, rs3735590, affected the binding affinity of miR-616 to PON1 and resulted in differences in PON1 expression. These data demonstrate the molecular mechanisms through which the interplay of genetics and epigenetics influence PON1 expression and demonstrate further the clinical significance of PON1 variability. Although the complex interactions between DNA methylation, miRNAs, and genetics are not yet

**FIGURE 2.** Map of PON1 CpG sites putative miRNA binding sites. PON1 contains two CpG islands, one in the promoter region and one in exon 7. It has 278 CpG sites spread across the CpG islands, shores, shelves, and open seas. In-silico analyses using the most recent version of miRbase (version 21) has identified 25 putative miRNA binding sites in PON1. However, thus far, none of these sites has been functionally validated with PON1. miRNA, microRNA; PON1, paraoxonase 1.
well understood, recent data suggest that miRNA gene expression can also be regulated by aberrant DNA methylation of miRNA genes [58–61].

PARAOXONASE, OBESITY, AND METABOLIC SYNDROME

Few studies have examined the associations of PON1 substrate-specific activities or genotypes with obesity or MetS in children; however, this field of research has grown substantially over the past 2 years (Table 1). AREase activity, a marker of PON1 enzyme quantity, was inversely associated with obesity status [62] and waist circumference [16*] in two studies of children and adolescents. Yet one study of older children in Spain, which measured PON1 concentration by ELISA [12**], and our study in 2-year-old Mexican–American children [15**] found positive associations of PON1 quantity with obesity. Interestingly, at age 5, we found that AREase activity was inversely associated with obesity status in PON1<sub>192QQ</sub> but not PON1<sub>192RR</sub> children, indicating the associations with obesity can change with age and by genotype [15**]. Lactonase (TBBLase) and PON1-specific activity (POase divided by PON1 concentration) were also inversely associated with several obesity parameters (BMI, % body fat) in children [12**]. The only other study to examine lactonase activity using dihydrocoumarin did not observe associations of lactonase or other substrate-specific activities with obesity in prepubertal children, but did report a relationship of lactonase activity with lipid profiles [11**].

Two studies, including ours, found higher BMI z-scores, waist circumference, and odds of obesity with the PON1<sub>192Q</sub> allele in young children [15**] as well as school-age children [14]. However, some studies did not observe the same relationship [11**,12**]. Furthermore, Ruperez et al. [11**] identified a different intronic PON1 SNP that was significantly associated with several substrate-specific PON1 activities (DZOase, AREase, and lactonase) as well as obesity. Ruperez et al. [11**] and Ferre et al. [12**] also examined associations with haplotypes, which incorporate combinations of multiple SNPs on a single chromosome [63]. Nevertheless, based on our study [22] and recent publications that also examined PON1 haplotypes [11**,12**], there is no apparent advantage in using haplotypes in the analysis of PON1 effects.

CONCLUSION

PON1 serves an important example of a susceptibility factor, whose characterization can help us to understand the cause of multiple diseases. Here we have highlighted several methodologies that may broaden the scope of PON1 research and help to explain some of the molecular mechanisms linking PON1 to disease. More generally, many of these concepts can be applied to other susceptibility markers involved in metabolism, inflammation, or other important biological pathways. Identifying the factors that modulate gene expression, accounting for differences in study populations, and trying to assess the complex interplay of different molecular markers (genetics, epigenetics, enzyme levels, etc.) may increase our understanding of these susceptibility markers. This is essential for elucidation of the cause of complex diseases in children and reliable interpretation of data used for personalized medicine.
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Conflicts of interest
None.

REFERENCES AND RECOMMENDED READING
Papers of particular interest, published within the annual period of review, have been highlighted as:
• of special interest
• of outstanding interest

• 11. This study reported a decrease in PON1 AREAse activity associated with central rather than general obesity in overweight and obese children and adolescents.


This is the first study that reported methylation levels of some CpG sites of the PON1 gene and their association with AREase activity.


This is the first study that validated a human microRNA that modulates PON1 gene expression.