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Pharmacogenetics of paraoxonase activity: elucidating the role of high-density lipoprotein in disease

Daniel Seung Kim[1], **Judit Marsillach**[1], **Clement E Furlong**[1], and **Gail P Jarvik**[1,*]

[1]Departments of Genome Sciences & Medicine (Division of Medical Genetics), University of Washington School of Medicine, Box 357720, University of Washington, Seattle, WA 98195-7720, USA

Abstract

PON1 is a key component of high-density lipoproteins (HDLs) and is at least partially responsible for HDL's antioxidant/atheroprotective properties. PON1 is also associated with numerous human diseases, including cardiovascular disease, Parkinson's disease and cancer. In addition, PON1 metabolizes a broad variety of substrates, including toxic organophosphorous compounds, statin adducts, glucocorticoids, the likely atherogenic L-homocysteine thiolactone and the quorum-sensing factor of *Pseudomonas aeruginosa*. Numerous cardiovascular and antidiabetic pharmacologic agents, dietary macronutrients, lifestyle factors and antioxidant supplements affect PON1 expression and enzyme activity levels. Owing to the importance of PON1 to HDL function and its individual association with diverse human diseases, pharmacogenomic interactions between PON1 and the various factors that alter its expression and activity may represent an important therapeutic target for future investigation.

Keywords

antioxidants; cardiovascular disease; drug interactions; gene-by-environment interactions; oxidative stress; paraoxonase; pharmacogenetics; pharmacogenomics; PON1; statins

High-density lipoprotein (HDL) has long been viewed as cardioprotective. However, recent failed HDL Mendelian randomization studies [1] and randomized clinical trials [2] have raised new doubt about the merits of using HDL-cholesterol (HDL-C) levels alone in cardiovascular risk-stratification. Focus has instead shifted to the numerous individual components of HDL that are not captured by HDL-C measurements, including PON1, a liver-produced, HDL-associated glycoprotein enzyme that is itself protective against numerous human diseases.

PON1 is one of the three paraoxonase gene family members, aligned next to each other on chromosome 7q21.3-22.1, with *PON2* being the oldest evolutionary member and *PON1* the most recent [3]. As lactones are commonly found in plants and as natural food flavors, the paraoxonases may have evolved for their native lactonase activity [3,4]. Moreover, this lactonase activity also allows PON1 [5], PON2 [6] and PON3 [5] to hydrolyze the quorum-sensing factor of *pseudomonas aeruginosa* and protect against infection and lethality from

*Author for correspondence: pair@u.washington.edu.

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this bacteria [5,6]. However, the paraoxonases, and in particular PON1, are perhaps best known for esterase activity and ability to hydrolyze organo phosphorus (OP) insecticide metabolites and nerve agents [7]. PON1 has been discovered to be antioxidant and athero protective [8], which has spurred many investigations that revealed associations between the paraoxonases and numerous human diseases, including cardiovascular disease, diabetes and cancer; the paraoxonases may also serve as biomarkers for Parkinson's disease (PD) [9].

Owing to the extremely broad substrate specificity of the paraoxonase enzymes and their numerous functions in human physiology, a crucial area of investigation is in the pharmacogenomic interactions between the paraoxonases and drugs or dietary supplements [10]. Within this review, we will summarize the current known pharmacologic and organic targets of the paraoxonases. In addition, we will describe the numerous determinants of PON1 activity, which given its extremely broad substrate specificity and disease associations, may prove to be a valuable method of modulating PON1 levels and activity for therapeutic purposes.

PON1 function

Basic biochemical and physiological principles dictate that it is the activity of a given enzyme that is important with respect to its function. This section will review what is known about measuring PON1 function, especially with respect to epidemiological studies. Moreover, this section will highlight the importance of using ‘PON1 status’, or the combination of both PON1 enzyme activity measurements and *PON1* genetic variants, for characterization of PON1 variation in human epidemiological studies investigating risk of disease or exposure. It is important to note that studies that do not include proper measurements of PON1 function will likely fall short of being useful.

History of PON1 status

The earliest studies on the PON1 activity variation included important observations that are often ignored in current studies. Despite not knowing the basis for the variation in PON1 activity, it was clear to the early workers that serum/plasma paraoxonase was polymorphically distributed in human populations; that simple histograms could distinguish low metabolizers of paraoxon (a toxic OP compound found to be hydrolyzed by PON1) from high metabolizers; that the paraoxonase activity levels varied significantly among both the low and high metabolizers and the frequency of low metabolizers exhibited a large variability among populations of different ethnic and geographical distributions. The large variability in activity within each PON1 phenotypic group was further validated with a unique two-substrate assay protocol developed in the laboratory of the late Bert La Du [11]. A plot of rates of paraoxon hydrolysis (POase) versus phenyl acetate hydrolysis (AREase) plus analysis of ratios of rates of POase versus AREase allowed them to assign genotypes as *ESA*A* (low metabolizers), *ESA*AB* (heterozygotes) and *ESA*B* (high metabolizers; reviewed in [12]).

Purification and cloning of human PON1 revealed the two common coding region SNPs that have since been extensively studied, L55M and Q192R [13]. Further efforts reported that the Q192R SNP was responsible for determining high versus low POase activity with the $PON1_{R192}$ alloform hydrolyzing paraoxon approximately eight-times more efficiently than the $PON1_{Q192}$ alloform [14,15]. The term PON1 status was introduced to incorporate both an individual's $PON1_{192}$ functional genotype as well as the level of PON1 activity in their plasma [16]. Extension of the two-substrate activity/analysis plots revealed that measurement of rates of diazoxon versus paraoxon hydrolysis clearly resolved all three functional *PON1* genotypes (Q/Q, Q/R and R/R) and at the same time determined the activity level of an individual's plasma PON1 [17]. The two-substrate analyses also

demonstrated that the nerve agents, soman and sarin, were hydrolyzed more rapidly by the $PON1_{Q192}$ alloform than by the $PON1_{R192}$ allo-form. As noted below in the 'PON1 protection from OP compound exposure' section, different rates of *in vitro* substrate hydrolysis do not necessarily reflect a differential sensitivity to that substrate.

The PON1 status analysis protocol was used to demonstrate that for epidemiological studies examining the relationship between *PON1* genetic variability, it is most informative to analyze PON1 levels within each $PON1_{192}$ genotype separately. The rationale for these stratified analyses stems from the different properties of the $PON1_{192}$ alloforms, especially different rates of paraoxon hydrolysis and that the allele frequency varies significantly among different ethnic groups. Application of this approach to the study of carotid artery disease (CAAD) revealed that Q/Q and Q/R individuals with low PON1 levels had a higher risk of CAAD than individuals with higher levels of PON1 [18]. In that study, the frequency of the $PON1_{R192}$ allele in the population made up primarily of individuals of northern European origin was too low to observe a relationship between PON1 activity levels and risk of CAAD.

Since the initial protocol for determining PON1 status made use of two highly toxic substrates, a novel protocol to provide PON1 status with nontoxic substrates was developed [19]. Tables for interconverting rates of hydrolysis of different substrates were also published [20].

PON1 genetic variation does not accurately predict PON1 levels or activity

The human *PON1* gene is encoded by nine exons [21], and is the most evolutionarily recent of the three paraoxonase gene family members found on chromosome 7 [3]. Five *PON1* promoter SNPs have been characterized by three different research groups [22–24]. The C(−108)T SNP [23] marks a binding site for Sp1, a transcription factor, and has the most influence on levels of expression of PON1 [25], accounting for up to 30% of the observed variation in plasma PON1 activity levels, with the $PON1_{C\text{-}108}$ allele expressing more PON1 than the $PON1_{T\text{-}108}$ allele [9]. The initial resequencing efforts of the human *PON1* gene from 47 individuals revealed eight new promoter region SNPs, 162 intronic SNPs and nine additional 3′-UTR SNPs that have yet to be characterized [9].

Further studies that characterized both *PON1* genotype by DNA analysis and PON1 status by functional activity assays revealed a number of individuals whose genotype information disagreed with the enzymatic assay assignment of functional genotype. While the DNA analysis indicated that they were heterozygous for $PON1_{192}$, the PON1 status assays indicated that they were homozygous for $PON1_{Q192}$ or $PON1_{R192}$. DNA sequencing of the *PON1* gene from these individuals identified rare inactivating mutations that resulted in expression of only one active PON1 protein [26].

However, characterizing all of the known *PON1* SNPs will not allow for an accurate prediction of plasma PON1 levels or activity, which are the most important predictor of risk of disease or exposure [27]. For example, a recent study in a cohort of 1328 European subjects found that *PON* gene cluster (*PON1–3*) genetic variation only accounts for 19.02% of PON1 activity [27]. Moreover, while no genetic variant was associated with CAAD, PON1 AREase activity was significantly inversely correlated with CAAD risk [27]. Thus, it makes no sense to characterize only *PON1* DNA polymorphisms in looking for associations between PON1 variability and risk of disease or exposure. High-throughput functional assays provide the most useful data for examining the association of PON1 with risk of disease or exposure.

PON1 activity versus protein levels

One additional important consideration has recently come to light. Besler *et al.* demonstrated that healthy controls had high levels of plasma PON1 activity, but low levels of PON1 protein, whereas the reverse was the case for individuals with vascular disease (see [28]). Its is also noteworthy that POase activity is more depressed than AREase activity [28]. Similar observations have been seen in chronic kidney [29] and liver disease [30] as well as HIV infection [31]. More investigation is necessary to uncover the physiologic interpretation of these findings.

Importance of PON1 status

While it is tempting to take advantage of the high-throughput analysis of *PON1* SNPs for epidemiological studies, they will be un informative when used alone as many studies have now shown [32]. It is more important to make use of the high-throughput functional assays since it is, after all, the activity of PON1 that is important in determining risk of disease or exposure [12,33,34]. The PON1 status assays also provide the activity levels for each individual and functional assignment of the $PON1_{192}$ genotype. Comparing the functional PON1 status assays with analysis of the PON_{192} SNP will however reveal rare mutations where either the PON1 activity is exceptionally low or where there is a discrepancy between $PON1_{192}$ genotyping and the functional determination of PON1 status [26].

PON1 protection from OP compound exposure

This section will review the history and recent research regarding PON1's hydrolysis of toxic oxon metabolites of OP compounds (chlorpyrifos and diazoxon), which are frequently found at significant levels in insecticides. In addition, bioengineered PON1 will be introduced owing to potential usage as a therapeutic for OP compound exposure.

Historical experiments of PON1 & OP compounds

In Aldridge's early studies of enzymes that inactivated toxic OP compounds, he found that some proteins catalytically inactivated OP compounds while others only inactivated a single OP compound before themselves becoming irreversibly inactivated. He designated the catalytic esterases ‘A esterases’ and the stoichiometric esterases ‘B esterases’ [35]. PON1 was designated an A esterase. Following the demonstration that PON1 could catalytically inactivate paraoxon, Aldridge measured PON1 activity in different tissues from different organisms [36]. He found that rabbit plasma had very high levels of paraoxonase activity.

Early studies by several research groups correlated the activity of plasma esterases from different species with their sensitivity or resistance to OP insecticides (reviewed in [12]). The first direct test of the ability of PON1 to protect against OP exposure was carried out by Main in 1956 when he injected partially purified rabbit PON1 into rats and found protection against paraoxon exposure [37]. This observation was confirmed and extended by Costa *et al.* who demonstrated protection against paraoxon and chlorpyrifos oxon exposure by injecting purified rabbit PON1 into rats [38]. Li *et al.* followed this work with a series of experiments with wild-type mice demonstrating that injection of purified rabbit PON1 into mice provided some protection against paraoxon exposure, but much better protection against chlorpyrifos oxon [16] and its parent compound chlorpyrifos [39]. As mentioned previously, the term PON1 status was introduced in this study as a term that included both $PON1_{192}$ genotype and plasma activity level.

Genetically modified mouse models & OP sensitivity

The most important contributions to understanding the role of PON1 in protecting against OP exposure resulted from the generation of *PON1* genetically modified mice by Shih *et al.*

and Cole *et al.* at UCLA [40,41]. PON1 knockout mice (*PON1*$^{-/-}$) were dramatically more sensitive to dermal chlorpyrifos oxon exposure than wild-type mice. They also showed increased sensitivity to the parent insecticide chlorpyrifos. The *PON1*$^{-/-}$ mice were more susceptible to atherosclerosis when fed a high-fat, high-cholesterol diet [40]. Both their HDLs and low-density lipoproteins (LDLs) were more susceptible to oxidation by cocultured cells than the lipoproteins from wild-type littermates.

Further studies by Cole *et al.* and Li *et al.* expanded our understanding of the role of PON1 in modulating OP exposure [41,42]. *PON1*$^{-/-}$ mice exhibited very high sensitivity to diazoxon inhibition of brain cholinesterase compared with wild-type mice. Sensitivity of brain acetyl cholinesterase in the *PON1*$^{-/-}$ mice to the parent insecticide diazinon was less dramatic. The most surprising observation in these studies was that *PON1*$^{-/-}$ and *PON1*$^{+/-}$ mice showed no increase in sensitivity to paraoxon exposure. Additional experiments where purified human $PON1_{Q192}$ or $PON1_{R192}$ were injected into the *PON1*$^{-/-}$ mice demonstrated that both alloforms protected equally well against diazoxon exposure while $PON1_{R192}$ provided better protection against chlorpyrifos oxon exposure. Neither human alloform was able to protect against paraoxon exposure. The explanation for these observations was provided by determining the catalytic efficiency of the purified $PON1_{192}$ alloforms for the hydrolysis of these substrates. The catalytic efficiencies of the two $PON1_{192}$ alloforms were equivalent for diazoxon hydrolysis with $PON1_{R192}$ having a higher catalytic efficiency for hydrolyzing chlorpyrifos oxon than $PON1_{Q192}$. Despite the large difference in the rates of the two alloforms for hydrolyzing paraoxon, the catalytic efficiency for the most efficient alloform $PON1_{R192}$ was too low to provide protection against paraoxon exposure. These observations were further confirmed in characterizing PON1 humanized mice (mice expressing either human $PON1_{Q192}$ or $PON1_{R192}$ on the *PON1*$^{-/-}$ background). The *tgHuPON1*$_{R192}$ mice exhibited increased resistance to chlorpyrifos oxon exposure compared with the *tgHuPON1*$_{Q192}$ mice while both PON1 humanized strains were more resistant to chlorpyrifos oxon exposure than *PON1*$^{-/-}$ mice, except for high dose exposure, where the *tgHuPON1*$_{Q192}$ mice were as sensitive as the *PON1*$^{-/-}$ mice. These and the earlier experiments demonstrated the potential for the use of purified or recombinant PON1s for therapeutic applications.

Modulation of mixed exposures by PON1: PON1 as a bioengineered therapeutic

Another area of interest to toxicologists has been the role of PON1 in modulating mixed OP exposures. One set of experiments has directly addressed this issue. Carboxylesterases (CESs) are important in detoxifying malathion/malaoxon and pyrethroids. OP compounds are potent inhibitors of CESs. It would be expected that PON1 would play a role in protecting CESs from inhibition if PON1 efficiently hydrolyzed an OP compound in a given mixed exposure. This was found to be the case. Using the PON1 humanized strains of mice, Jansen *et al.* showed that PON1 played an important role in protecting against malaoxon exposure by detoxifying diazoxon and chlorpyrifos but not paraoxon as expected [43].

These observations were key for understanding the *in vivo* role of PON1 for modulating OP exposures. The catalytic efficiency of nerve agent hydrolysis by native PON1 is too low for use as a therapeutic for treating agent exposure. Engineering of PON1 for higher catalytic efficiency will be necessary if PON1 is to be used for treating agent exposure. Two efforts are underway to provide engineered PON1 for treating OP exposures where higher catalytic efficiency is required, for example, paraoxon or agent exposures. One effort is making use of a recombinant PON1 (rEngPON1) engineered by Tawfik's group in Israel. In efforts to make a more soluble PON1 that could be expressed in bacteria, they made use of domains from human, rabbit, rat and mouse PON1s. They achieved a rEngPON1 that could be purified as an active protein in reasonable yields from an *Escherichia coli* expression

system. Furthermore, they were able to crystallize the purified protein and generate a crystal structure for the recombinant PON1. This structure has been important for understanding the structure–function relationships of PON1 and for the rational design of variants with increased catalytic efficiency of agent hydrolysis [44]. Separately, the University of Washington team was able to purify active native PON1 (rHuPON1) in an *E. coli* expression system and demonstrate increased catalytic efficiency of OP insecticide hydrolysis by a single amino acid substitution at position 192 ($PON1_{K192}$) [7]. These two approaches are informing each other. Efforts are underway to make the rEngPON1 more human-like with increased catalytic efficiency for agent hydrolysis and to minimize immunological responses to injected rEngPON1 [45] and separately, to engineer the rHuPON1 for higher catalytic efficiency with as few changes as possible from the native human sequence.

PON1 & OP compound hydrolysis summary

These observations point out that hydrolysis of a given OP compound with an *in vitro* assay does not indicate that PON1 will protect against an exposure to that OP compound. It is important to determine the catalytic efficiency of hydrolysis and to test wild-type and $PON1^{-/-}$ mice for differential sensitivity to the OP compound in question. The experiments described above provide useful guidelines for answering the question of the role of PON1 in modulating exposure to a given OP compound.

Paraoxonases & human disease

The paraoxonases, and in particular PON1, are associated with numerous human diseases. This section will present the history of PON1 with regard to the investigation of its lactonase ability. In addition, we will review the link of PON1 to cardiovascular disease while emphasizing the importance of PON1 status in clinical studies. In addition, recent evidence of the paraoxonases as proteins that can be delivered to specific tissues to combat oxidative stress will be presented, a function that may underlie the wide variety of associations that PON1 has with human diseases.

PON1 lactonase activity

Although PON1 is historically known for its esterase activity that allows it to hydrolyze OP compounds, the usage of insecticides over the past century is too short of a time for OP compounds to likely be the native substrate of PON1. It was instead postulated that the ability of PON1 to hydrolyze L-homocysteine thiolactone, a human biomarker associated with atherosclerotic disease, was PON1's native substrate for which it had been evolved [46]. To provide evidence for this potential hypothesis, Draganov *et al.* [47], and Khersonsky and Tawfik [4] separately used biochemical enzyme kinetic experiments of PON1 (and PON2 and PON3 for Draganov) and various classes of substrates. Both groups' results suggested that lactonase activity was PON1's native enzyme function [4,47].

This knowledge of PON1 lactonase activity was furthered by the efforts of Ozer *et al.*, who reported that both human and murine PON1 were able to hydrolyze the quorum-sensing factor of *Pseudomonas aeruginosa* [5]. This finding, in conjunction with the report that the oldest paraoxonase family member, PON2, was also protective against *P. aeruginosa* infections [6], provided another potential evolutionary focus for the paraoxonases: lactonase activity that protected against potentially lethal bacterial infections.

Cardiovascular disease

Although the PON1-mediated inactivation of toxic OP compounds was well known, it was not until the 1990s that PON1 was linked to chronic human disease. Mackness *et al.* are generally credited with sparking the interest of the medical community by demonstrating

that both HDLs or pure PON1 were able to prevent the oxidation of LDLs [48]. Through interpretation of these findings, the authors suggested for the first time that a possible biological function of PON1 might be hydrolysis of lipid peroxides formed during oxidation of LDLs, thereby making PON1 an atheroprotective enzyme. Previous publications had already related low PON1 activity levels with myocardial infarction [49], and diabetes mellitus or familial hyper cholesterolemia [50]. Although other HDL-associated proteins have been shown to possess this antioxidant activity, PON1 is the major contributor [51]. A few years later, Mackness *et al.* extended these observations, confirming their previous findings and suggesting that PON1 enzymatic activity could explain the antioxidant properties of HDL [8]. Following these publications, Aviram *et al.* reported that PON1 also protected HDL from oxidation [52] and that the esterase active site of the enzyme is not involved in this activity [53]. Watson and colleagues also suggested that LDL-oxidized phospholipids could be a substrate for PON1 [54]. Altogether, these observations generated an increasing medical interest in the study of PON1 and its role in protecting against atherosclerotic disease.

The creation of the *PON1*$^{-/-}$ mouse by Shih and colleagues provided a platform for examining the physiological role of PON1 [40]. They demonstrated that these mice are extremely sensitive to chlorpyrifos oxon exposure (as mentioned earlier) and that they are more susceptible to lipid peroxidation and development of atherosclerosis when fed a high-fat, high-cholesterol diet, compared with wild-type mice. The atherosclerotic susceptibility and LDL oxidation were dramatically enhanced when the double knockout mouse, that combined the *PON1*$^{-/-}$ and the apolipoprotein E (*apoE*$^{-/-}$) mouse (a mouse model for the study of atherosclerosis), was generated [55]. On the other hand, the human transgenic *PON1* mouse (*HuTgPON1*), which showed a two- to four-fold increase in PON1 activity levels in plasma, was more effective at protecting against LDL oxidation and its higher levels of PON1 protected them against development or progression of atherosclerosis [56].

These and other functional studies describing the atheroprotective nature of PON1 spurred numerous clinical studies investigating the link between the known *PON1* polymorphisms (usually the two coding mutations: *PON1*$_{M55L}$ and *PON1*$_{Q192R}$) and cardiovascular phenotypes. However, as these studies did not take into account PON1 status, or the combination of the genotypes and PON1 enzymatic activities, results were mixed. A recent meta-analysis did find a statistically significant, though weak, association between the *PON1*$_{192R}$ variant and coronary artery disease [57]. However, upon removal of studies with less than 500 subjects that may have been prone to publication bias, there was no significant effect of the *PON1*$_{192R}$ polymorphism [57]. These results are similar to a previous meta-analysis that showed a significant association for *PON1*$_{192R}$ only when considering larger studies [58]. By contrast, numerous studies have shown a strong association between PON1 enzyme activity and cardiovascular phenotypes [18,27,59], again highlighting the importance of taking into account PON1 status in epidemiological studies.

Association of PON1 with HDL

To help determine the specific components of HDL that PON1 was associated with, Mackness *et al.* examined PON1 enzyme activity from the plasma of patients with congenital HDL deficiencies. In 1987 and 1989, Mackness *et al.* reported that PON1 activity was dramatically reduced in two genetic disorders associated with low HDLC, Fish-Eye disease [60] and Tangier disease [61]; with a stronger association with apolipoprotein AI (apoAI) than apoAII. More recently, an analysis of 1402 subjects has found that apoAI, rather than HDL-C levels, is the strongest predictor of PON1 AREase activity [25].

In addition to its strong relationship with apoAI, the *in vitro* antioxidant effect of PON1 has been reported to be strongest in the small, dense subfractions of HDL (e.g., HDL3 > HDL2),

although PON1 activity alone did not account for all LDL antioxidant effects in this study [62]. Similarly, a recent epidemiologic study of 104 healthy volunteers reported a negative association between HDL particle size and PON1 POase activity, and a negative correlation between HDL2:HDL3 ratio and PON1 POase activity [63]. Taken together, these results suggest that PON1 is strongly associated with apoAI, and that they are found more frequently associated with the small, dense subfractions of HDL.

Antioxidant delivery of PON1 by HDL

Recent *in vitro* functional studies of PON1 have yielded important findings regarding the role of PON1 and HDL in antioxidant pathways. As previously described, PON1 is primarily synthesized in the liver and found in circulation, mainly associated with HDLs. Deakin and colleagues described how hepatic PON1 is transferred from the external membrane of the cell to nascent HDL [64]. According to the authors, both apolipoproteins and phospholipids are necessary not only for the release of PON1 from cells to HDLs, but also for preserving its enzymatic activity. When using protein-free phospholipid vesicles, the authors also observed a transfer of PON1 from membrane-to-vesicles. This observation was confirmed in a publication by Sorenson *et al.* [65]. Following the observation of the transfer of PON1 from membrane to vesicles, Deakin *et al.* examined the possibility of the reverse transfer of, PON1 from HDL to cell membranes [66]. They demonstrated that PON1 can be incorporated from HDL-like membranes into the membranes of cells devoid of PON1 synthesis. Interestingly, PON1 immunolocalization had been reported in many mouse tissues, which included cells that do not synthesize PON1 [67]. Altogether, these studies suggest that HDL can also function as a delivery vehicle, transporting antioxidant proteins such as PON1 to other tissues of an organism in need of antioxidant activity. At the same time, these data suggest a possible implication of PON1 in other diseases where inflammation and oxidative stress are involved [67], broadening the medical interest in this multifaceted protein.

Association of the paraoxonases with human diseases related to oxidative stress

PD & neurotoxicity from oxidative stress—Epidemiologic studies have not consistently linked *PON1* genetic variants with PD. While numerous small studies [68,69] and a meta-analysis [70] have linked the $PON1_{L55M}$ coding polymorphism with PD [68,69], null results have also been reported [71]. A recent meta-analysis found no association for a link between the $PON1_{L55M}$ variant and PD [72].

In contrast to these conflicting epidemiologic studies that did not take into account PON1 status, more recent evidence from human functional studies do support a protective role of PON2 and possibly PON1 in oxidative stress related neurologic diseases, such as PD. Giordano *et al.* reported that male mice – who had lower levels of PON2 protein, expression and lactonase activity – had three- to four-fold higher sensitivity to oxidative stress induced toxicity compared with females [73]. This sex-dependent susceptibility to neural oxidative stress was abrogated in $PON2^{-/-}$ mice [73]. Similarly, estradiol treatment increased astrocyte PON2 mRNA in both males and females, and protected wild-type mice from oxidative stress induced neurotoxicity [73]. Efforts to demonstrate this neuroprotective effect with PON1 are currently underway.

Cancer—Cellular oxidative stress leading to DNA damage could contribute to neoplastic growth. Given the broad antioxidant effects of PON1, numerous studies have investigated the association of the functional *PON1* polymorphisms with cancer risk. As expected from studies not taking into account PON1 status, conflicting results regarding the effects of *PON1* genetic variants and cancer risk were obtained. However, a recent meta-analysis of approximately 7000 cancer cases and 9000 controls from 25 studies has found strong

evidence of a link between the $PON1_{L55M}$ mutation, which decreases PON1 enzyme activity, and overall cancer risk [74]. Additionally, in analyses stratified by cancer type, an increased risk of breast and prostate cancer was found for carriers of $PON1_{M55}$ [74]. Moreover, this meta-analysis found a decreased risk of cancer for individuals of Asian ancestry carrying the $PON1_{R192}$ variant, which drastically increases PON1 POase activity [74]. In addition to this meta-analysis, a separate, meta-analysis of breast cancer has found similar results, with $PON1_{M55}$ being a risk allele, while the $PON1_{R192}$ allele was protective against the risk of breast cancer [75].

Other diseases—PON1 has been associated with numerous human diseases, including diabetes and mental illnesses, which are beyond the scope of this review. For more information, Camps *et al.* have previously reviewed the various human diseases associated with PON1 [76].

Targets of paraoxonase hydrolysis

Owing to their broad lactonase and esterase activities, the paraoxonases metabolize a broad variety of organic and pharmacologic substrates. The relevant data are summarized in **Table 1**.

Pharmacologic targets

Owing to its ability to hydrolyze lactones, cyclic carbonates and esters, PON1 can metabolize numerous pharmacologic drugs. As an example, PON1 has been reported to bioactivate the fluoro quinolone antibiotic, prulifloxacin, by Tougou *et al.* [77]. Moreover, these authors found evidence that the rate of this bioactivation was related to the $PON1_{Q192R}$ genotype, which produces a more active enzyme [77]. In addition, PON1 has been used to limit the systemic effects of glucocorticoids through a mechanism where serum PON1 inactivates a glucocorticoid once it reaches the circulation, thereby limiting the glucocorticoid effects to the site of application [78].

PON3, which has similar tissue expression patterns as PON1, has been found to metabolize bulkier pharmacologic drugs. Notably, Draganov *et al.* found that recombinant purified PON3 was able to hydrolyze lovastatin, spironolactone and spironolactone's active metabolite, canrenone, while PON1 and PON2 did not have detectable activity for any of the aforementioned drug substrates [47].

A more recent study by Riedmaier *et al.* found that both PON1 and PON3 hydrolyzed atorvastatin δ-lactone, an inactive metabolite of atorvastatin that is associated with statin toxicity [79]. Through genotyping of 40 polymorphisms within the PON gene family locus, Riedmaier and colleagues also found that *PON1* and *PON3* promoter polymorphisms (including $PON1_{-108T/C}$) were associated with alterations in both *PON1* expression and atorvastatin δ-lactone hydrolysis [79].

Clopidogrel (Plavix®) controversy

Clopidogrel is a prodrug that requires two steps of bioactivation to generate an active thiol metabolite (H4) that inhibits ADP binding to a sulfhydryl group on the ADP receptor P2Y12 on the surface of platelets resulting in the inhibition of ADP-mediated activation of the glycoprotein BPIIb–IIIa complex. A paper by Bouman *et al.* first reported in December 2010 that PON1 was a major determinant of clopidogrel efficacy. They proposed that the two-step bioactivation of clopidogrel involved the introduction of a keto group on the thiophene ring to produce 2-oxo-clopidogrel by CYP450 followed by the hydrolysis of the resulting thioester by PON1 to form the pharmacologically active thiol metabolite [80]. A

response to this publication by many experts in PON1 research also appeared in *Nature Medicine* [81], which expressed concerns about the methodology used in the Bouman *et al.* report, particularly the use of EDTA preserved serum for PON1 measurements and the assessment of the kinetics of the purified PON1. In the same issue of Nature Medicine, Dansette *et al.* provided an alternative mechanism of clopidogrel bioactivation that did not include the enzymatic activity PON1 [82], with a detailed report following a year later [83]. The first step in generating 2-oxo-clopidogrel is carried out by the CYP450s 2C19, 1A2 and 2B6. The second step in generating the physiologically active metabolite involves another CYP450 catalyzed step in the presence of glutathione to generate the active metabolite. The ability to clarify the metabolism was dependent on sophisticated liquid chromatography/mass spectrometric analyses including derivatization of the microsomal generated metabolites [83,84]. The metabolite referred to as H4 was shown to be the pharmacologically relevant metabolite with the H2 metabolite having approximately half the biological activity measured *in vitro* [84]. Hydrolysis of a metabolic intermediate with an 'endo' double bond by PON1 leads to an inactive metabolite present at lower levels than the active metabolite H4 [83]. These observations have been confirmed in a series of follow-up studies ([85] and references cited within).

It is important to ask the question as to how the Bouman team could have carried out such a highly detailed study and reached an incorrect conclusion. It appears that they were unfamiliar with both the fundamental PON1 biochemical findings and protocols for analyzing the effects of *PON1* genetic variability on risk of disease, exposure or drug metabolism as well as the detailed liquid chromatography/mass spectroscopy protocols required for a detailed understanding of clopidogrel metabolism. It is one aim of this review to present sufficient guidance for epidemiologists to follow in carrying out meaningful studies on the role of genetic variability in PON1 related to disease, exposure or xenobiotic metabolism. It is not possible to overemphasize the importance of including PON1 status in epidemiological studies and avoid relying on genotyping alone [18]. One could characterize approximately all 200 *PON1* polymorphisms and not be able to predict the activity levels of an individual's PON1, the most important factor in rates of metabolism of a toxic metabolite (oxidized lipid) or xenobiotic (drug or insecticide).

Lifestyle determinants of PON1 activity

The effects of nonmodifiable (e.g., age and sex) and modifiable (e.g., alcohol, tobacco use and dietary lipid intake) risk factors have been studied with regard to PON1 activity. These data are summarized in this section and also in **Table 2**, with emphasis provided for human studies (both cohort and cell based).

Nonmodifiable factors

Age/development—PON1 activity in both the serum and liver are extremely low during human fetal development, as evidenced by a 24% decrease in activity in preterm babies (33–36 weeks gestation) compared with full-term neonates [86]. PON1 activity remains low at birth and increases to a plateau between 6–15 months of age for humans [87]. PON1 activity then remains relatively constant over time into adulthood, until a progressive decline in PON1 activity occurs in the geriatric stages of human life [88].

Gender—A significantly higher mean value of PON1 activity has been found for females, as compared with males in human epidemiologic studies [25]. Mouse models using inbred laboratory strains have also demonstrated a 14–26% higher PON1 activity and liver expression in females versus males [89]. However, follow-up studies examining the effects of female sex hormone supplementation have yielded mixed results. In post menopausal women, intranasal estradiol was found to have no effect on serum PON1 activity [90], while

hormone replacement therapy (estrogen and progesterone) was found to increase serum PON1 activity in diabetic, postmenopausal women [91].

Interestingly, gonadectomy of the male mice resulted in an increase in PON1 mRNA, which was not observed after comparable treatment of the female mice [92]. This finding of male gonadectomy increasing *PON1* expression may be due to the effect of male-pattern growth hormone, which has been reported *in vivo* to decrease PON1 liver mRNA in mice [93]. Moreover, male/female sex hormones and female-pattern growth hormone did not affect *PON1* expression in mice liver [93]. This finding is in concordance with an *in vitro* study of human HuH7 liver cells, which reported that estradiol increased PON1 activity, but did not affect gene expression [94].

Together, these findings suggest that the higher levels of PON1 activity and liver PON1mRNA expression in females compared with males may be driven by two factors: the suppression of *PON1* expression by male-pattern growth hormone [93] and the direct stabilization and/or regulation of the PON1 protein by estrogen [94].

Modifiable lifestyle factors

Alcohol use—In concordance with the notion of mild alcohol use being cardioprotective, the majority of research has found moderate alcohol consumption (~≤40 g/day) increases PON1 activity in humans and animal models. Three initial, small human observation studies found conflicting results: in a randomized cross-over study of 11 healthy middle-aged men, PON1 activity was found to significantly increase after 3 weeks of moderate alcohol consumption [95]. Similarly, a study of ten men and nine postmenopausal women undergoing a randomized cross-over study reported an increase in PON1 activity after 15 days of moderate alcohol consumption [96]. By contrast, a study of 14 healthy men comparing PON1 activity before and after 3 weeks of alcohol consumption (approximately 26 g/day) found no statistical difference in PON1 activity [97]. Recently, a retrospective analysis of 1402 subjects from a CAAD case–control cohort using dietary intake data from food-frequency surveys has reported a significant increase in PON1 activity with alcohol consumption [25]. Two studies using mouse [98] and rat [99] models also support the findings of moderate alcohol consumption leading to an increase in PON1 activity, with both studies demonstrating an increase in both serum PON1 activity (+64% for mice, +20–25% for rats) and hepatic PON1 mRNA expression (+31% for mice, +59% for rats) [98,99].

In contrast to moderate alcohol consumption, heavy alcohol drinking leads to a significant decrease in PON1 activity. A human cohort study of 328 chronic alcoholics and 368 healthy individuals reported that PON1 activity was significantly decreased in chronic alcoholics (−53%) and even further depressed in alcoholics with liver cirrhosis (−72%) [30]. A human and rat comparative study by Rao *et al.* found that human heavy drinkers had a 45% lower serum PON1 activity compared with nondrinkers, while rats given heavy ethanol exposure had a 25% decrease in serum and liver PON1 activities and a 51% decrease in liver PON1 mRNA expression [99].

One hypothesis linking alcohol and PON1 mRNA activity/expression levels [99] postulates that alcohol-induced increased membrane fluidity releases PKC, which activates the transcription factor Sp1 [100]. Sp1 is known to bind to the upstream promoter region of *PON1* and increase transcription [100]. Moreover, overexpression of PKC has been found to decrease *PON1* promoter activity despite its role in activating Sp1 [100]. This proposed mechanism provides an elegant, yet unproven, model for how moderate alcohol consumption increases PON1, while heavy alcohol consumption decreases PON1 levels and activity.

Note that quercetin and resveratrol, two antioxidant components of alcohol and specifically, red wine, are discussed below for their individual effects on PON1 levels and activity.

Smoking—Extract from cigarette smoke has been found to inhibit PON1 activity in experiments on plasma from healthy human subjects, possibly through modification of PON1's free thiol group, which is required for antioxidant effects on LDL peroxidation [101]. Accordingly, the inhibitory effect of cigarette smoke extract in human plasma was reversible through addition of antioxidant compounds such as glutathione or *N*-acetyl cysteine [101]. Follow-up studies in human cohorts confirmed this finding, demonstrating a strong and statistically significant inverse relationship between current cigarette smoking and PON1 activity [25,102]. Moreover, the decrease in PON1 activity was only found to be significant in current smokers (as compared with controls and former smokers), indicating that the effects of cigarette smoke on PON1 are short-term [102].

Lipid-rich diets

Cholesterol—Dietary cholesterol intake was first reported to be positively associated with PON1 activity in baboons by Rainwater *et al.* [103]. Follow-up research in a human cohort of 1402 subjects confirmed the positive association between dietary cholesterol intake and PON1 activity [25]. This finding is paradoxical, as PON1 activity is athero protective, while dietary cholesterol intake is atherogenic. Rainwater *et al.* hypothesized that dietary cholesterol served as a surrogate for HDL levels, where PON1 is localized [103]. However, Kim *et al.* found that dietary cholesterol remained significant in regression models adjusting for the significant effects of apoA1 in humans [25]. Moreover, Kim *et al.* reported that dietary cholesterol was positively and significantly associated with the ratio of PON1 AREase hydrolysis and HDL-C, thereby indicating that the increase in PON1 activity exceeds any increases in HDL-C [25].

Fatty acids—Phospholipid intake may differentially influence PON1 activity dependent on the specific fatty acid composition of the diet. For example, rats had significantly increased PON1 activity (+46%) when fed triolein; however, when the rats were fed fish oil, their PON1 activity significantly decreased (−39%) [104]. Similarly, human studies examining the relationship between dietary fat intake and PON1 activity have had contrasting results dependent on the fatty acid composition. A further study of 12 healthy males found that postprandial serum PON1 activity decreased by 27% after eating a meal rich in cooking fat [105]. Similarly, usage of trans fat in lieu of saturated fats resulted in a decrease in serum PON1 activity in both healthy men and women [106]. However, thermally stressed olive oil consumption has been reported to increase postprandial serum PON1 activity in a study of 14 diabetic patients, with the effect greater in females than males [107]. In addition, dietary oleic acid from olive oil, as determined from a 12-h recall survey, was significantly associated with increased serum PON1 activity, although the effect was only observed in subjects with a homozygous RR genotype at $PON1_{192}$ [108].

The discrepancies between these studies may, in part, be explained by results from *in vitro* experiments investigating the relationship between specific lipids and PON1 activity. Polyenoic acids (fatty acids having more than one double bond; e.g., linoleic acid) were found to decrease PON1 activity *in vitro*. Interestingly, monoenic acids (e.g., oleic acid) were found to increase PON1 activity [109], and moreover, were found to stabilize PON1 *in vitro* [109].

Sugar intake

PON1 enzymatic activity is consistently lower in subjects with diabetes compared with healthy controls [110]. However, Ikeda *et al.* have also reported that hyperglycemic

conditions (specifically, increased D-glucose concentration) in cultured human hepatocytes leads to enhanced *PON1* promoter activity and increased *PON1* expression [111]. Thus, Ikeda and colleagues have speculated that their findings represent a compensatory mechanism to increase antioxidant capacity under physiologic conditions that favor oxidative stress [111].

Iron & other metal intake

Recently, Kim *et al.* reported that dietary iron intake was negatively correlated with PON1 activity [25] from a cohort of 1402 human subjects. This was the first reported association between dietary iron intake in normal subjects (e.g., nonanemic) and PON1 activity. This finding is consistent with prior *in vitro* studies reporting an inhibitory relationship between iron and PON1 activity [112,113]. Moreover, other divalent cations, such as lead, have also been found to inhibit PON1 activity *in vivo* in a cross-sectional analysis of workers exposed to lead [114]. The exact mechanism for PON1 activity inhibition via metal intake is yet unknown. It has been postulated that metal inhibition of PON1 occurs through binding of the free sulhydryl group at residue 284 [44,115], a key amino acid required for PON1-mediated protection from LDL oxidation [53]. This proposed mechanism is supported by *in vitro* evidence that PON1 enzyme inhibition by metal cannot be reversed through increased concentration of its substrate [112].

Pharmacologic determinants of PON1 levels and/or activity

Numerous pharmacological agents have been tested for their effects on PON1 activity, with a particular emphasis on those used for cardiovascular or diabetic conditions. This section summarizes research on pharmaceutical drugs and PON1 activity (see **Table 3**), with emphasis provided for human studies (both cohort and cell based).

Cardiovascular drugs

Statins—Statins have been implicated in altering serum *PON1* expression levels and/or activity; however, the direction of effect has been disputed. Numerous studies in humans have demonstrated a small increase in PON1 activity (~5–23%) in response to treatment with atorvastatin [116–118] or simvastatin [116,119]. By contrast, a recent study found no effect for simvastatin pharmaco-therapy affecting PON1 activity, although the sample size was limited to 14 Type 2 diabetic subjects [120].

Studies using human cell lines to investigate the effects of statins on PON1 activity have drawn similarly conflicting results, with one study using HuH7 human hepatoma cells reporting a decrease in PON1 activity in response to statins [121]. However, the majority of other human cell line studies have implicated statins as increasing PON1 activity. To begin, Aviram *et al.* first reported an increase in PON1 activity in isolated HDL in the presence of atorvastatin metabolites (but not the parent compound) [122]. Follow-up research by other investigators in HepG2 [119,123] human hepatoma and HEK293 [123] human embryonic kidney cells confirmed the effects of atorvastatin on PON1 activity, and also discovered similar effects for pivastatin and simvastatin [123].

The effects of statins on PON1 activity appear to be driven by an increase in transcription of the *PON1* gene. Arii *et al.* have reported in HuH7 cells that pivastatin acts through the MAPKs p44/p42 to activate the transcription factors, Sp1 and SREBP-2, which in turn increase *PON1* promoter activity and gene expression [124]. Similar results have been reported for simvastatin, with Deakin *et al.* demonstrating the role of SREBP-2 and Sp1 in binding the *PON1* promoter and increasing gene expression in response to simvastatin stimulation in HepG2 cells [125]. In addition, Deakin *et al.* found that the increase in *PON1*

expression with simvastatin treatment only occurred in subjects with the $PON1_{C\text{-}108}$ allele, which modulates PON1 levels in the absence of statin pharmacotherapy [125]. However, Sardo *et al.* have also reported conflicting results, indicating that the atorvastatin-mediated increase in PON1 activity is independent of the $PON1_{C\text{-}108T}$ variant) [126].

Other lipid-lowering medications—Fibrates have also been investigated for their effects on PON1 activity since Aviram *et al.* reported a metabolite of gemfibrozil increased PON1 activity in isolated HDL [122]. Experiments in HuH7 cells revealed a 70% increase in PON1 activity and mRNA levels after treatment with gemfibrozil [121]. However, the results from human cohort studies have been conflicting: one study of 51 patients with metabolic syndrome found a statistically significant increase in PON1 activity with ciprofibrate pharmacotherapy [127], while another study of 29 patients with type IIb hyperlipoproteinemia found no increase in PON1 activity after treatment of either gemfibrozil or bezafibrate [128].

In brief, several other lipid-lowering medications have been reported to affect PON1 activity. Probucol, which increases LDL catabolism, was found to increase PON1 mRNA levels and activity levels in rabbits fed a high-cholesterol diet [129]. Ezetimibe, which decreases dietary cholesterol absorption from the small intestines, was similarly found to increase PON1 in a cohort of hyperlipidemic patients [130]. Finally, berberine, a botanical alkaloid frequently used in Chinese medicine recently found to have hypotensive and lipid-lowering effects, was reported by Cheng *et al.* to increase PON1 in both HuH7 and HepG2 cell lines [131]. More over, Cheng *et al.* were the first to demonstrate the possible role of JNK and c-Jun signal transduction leading to the binding of both (but particularly c-Jun) to the AP1 promoter site upstream of *PON1*.

Other cardiovascular drugs—Acetylsalicylic acid (aspirin) was first reported to increase both PON1 activity and concentration by Blatter-Garin *et al.*, who studied a prospective cohort of approximately 800 subjects collected for a coronary artery disease study [132]. Follow-up investigation by Jaichander *et al.* using both live mice and rat and human cell lines replicated the results of Blatter-Garin, with mice having a twofold increase in PON1 activity and induction of *PON1* gene expression in the liver [133]. Similarly, aspirin pharmacotherapy significantly increased PON1 activity and expression in both rat hepatocyte and human HepG2 cell lines. Finally, Jaichander *et al.* reported the necessity of the arylhydrocarbon receptor (AhR) for the *PON1* expression and activity response to aspirin. It should be noted, however, that in a study of 30 healthy volunteers, aspirin use did not significantly increase PON1 concentration or activity (although the raw values did trend positively with aspirin use) [134].

Other cardiovascular drugs have also been reported to affect PON1 activity. Eplerenone, an aldosterone antagonist frequently used in the treatment of congestive heart failure, has been found to increase PON1 activity in both diabetic and control mice [135]. In 49 patients with chronic kidney disease, supplementation of both erythropoietin-β and iron resulted in a significant increase in overall PON1 activity [29].

Antidiabetic drugs

Rosiglitazone & sulfonylureas—Rosiglitazone, a PPAR-γ agonist used in the treatment of Type 2 diabetes, has been reported in several small studies to increase PON1 activity. First, in a study of 19 human subjects with Type 2 diabetes, rosiglitazone was found to increase fasting and postprandial PON1 activity [136]. Additional studies in rabbits [137] and rats [138] demonstrated similar results as those found in humans: the rabbits had a 21% increase in serum PON1 activity after 6 weeks of rosiglitazone therapy [137], while the rats

exhibited a 68% increase in hepatic PON1 activity after 2 weeks of rosiglitazone therapy immediately following 6 weeks of a high-fructose diet used to mimic metabolic syndrome [138].

In addition to rosiglitazone, the sulfonylureas glimepiride and glibenclamide, which block K_{ATP} channels in the pancreas and increase insulin secretion, have recently been investigated for their effects on PON1 activity. Using diabetic and control rats, Wojcicka *et al.* found that sulfonylurea treatment increased PON1 activity in hepatic tissues only, with serum PON1 activity being unaffected by pharmacotherapy [139].

Other drugs

Numerous other drugs, including oral contraceptives, selective serotonin reuptake inhibitors and certain antibiotics have been associated with PON1 activity. An excellent, detailed review of these pharmacologic determinants of PON1 activity has been previously provided by Costa *et al.* [10].

Antioxidant determinants of PON1 activity

Since the discovery that antioxidants can preserve PON1 from inactivation by oxidized LDL [140], research into PON1 has focused on the role of dietary and supplemental antioxidants. A summary of research within this field is presented in this section and in **Table 4**.

Vitamins C & E

Vitamins C and E were first reported to be positively associated with PON1 activity through an analysis of food frequency data from 189 male European-ancestry subjects [141]. A recent follow-up analysis confirmed the positive effects of vitamin C on PON1 activity in a much larger, and partially overlapping, cohort [25]. These effects of vitamin C on PON1 activity remained significant with the removal of overlapping subjects from prior analyses by Kim *et al.* and Jarvik *et al.* [25,141]. Moreover, vitamin E was significantly and positively associated with PON1 activity if vitamin C was first removed from the regression model; thus, vitamin E was likely dropped from the final model owing to high correlation with vitamin C [25]. Other analyses of vitamins C and E in human models have demonstrated a protective effect of these antioxidants on PON1 activity. For example, a study in healthy college-aged males found that supplementation of vitamin E prevented exercise-induced PON1 activity loss [142]. Similarly, vitamin C was found to block hypochlorite-induced loss of PON1 activity in human plasma [143]. However some null results have also been reported: usage of vitamin E-coated dialysis membranes for 3 months in patients with end-stage renal disease did not affect serum PON1 activity. Moreover, a study of 95 healthy Finnish subjects found a negative correlation between vegetable intake, potentially rich in vitamins C and E, and PON1 activity [144].

Quercetin & resveratrol

The red wine polyphenols, quercetin and resveratrol, have been well studied for their effects on PON1 activity. While the only known human experiment on 35 healthy subjects did not demonstrate a significant difference in serum PON1 activity after administration of quercetin [145], there remains considerable human cell line and animal model evidence for a positive association between quercetin/resveratrol and PON1 activity. To begin, human cell line experiments in HuH7 cells have indicated that both PON1 activity and mRNA levels are increased after quercetin supplementation [146]. This effect appears to be mediated through the aforementioned AhR, and also requires a xenobiotic-response element-like sequence within the *PON1* promoter [146]. Resveratrol has been reported to also act through the AhR to increase *PON1* expression in HuH7 cells [147]. Interestingly, a recent report suggests that

quercetin may also act through the also aforementioned SREBP2 transcription factor to exert its effects on *PON1* expression [148]. In addition to these cellular results, there are numerous animal experiments that support quercetin increasing PON1 protein levels and activity (please see [10] and references included within).

Polyphenols in pomegranate juice

Pomegranate juice, and in particular its polyphenol components, are positively associated with serum PON1 activity (26–43% increase in mice [149]; 20% in humans [150]) and are thought to be cardioprotective. Follow-up research in 30 subjects with Type 2 diabetes replicated these findings, with subjects having a 34–45% increase in PON1 activity compared with baseline after pomegranate juice supplementation [151]. Moreover, it was noted that PON1 enzyme binding to HDL was increased by 30% and that the PON1 enzyme itself became more stable *in vitro* following pomegranate juice consumption [151]. Finally, experiments in HuH7 cells have reported that pomegranate juice and its major polyphenols increase PON1 mRNA expression through activation of PKA and the PPAR-γ pathway [152].

Conclusion & future perspective

Future research outside of increasing PON1 activity should focus on further understanding the genetic control of *PON1* expression. A recent report of 1052 European subjects has found that both common and rare genetic variation within the genes *FTO*, *ITGAL* and *SERPINA12* are highly associated with PON1 activity [153]. Larger studies should assess whether other genetic loci are also influencing PON1 expression and/or activity. Moreover, with the recent publication of the ENCyclopedia Of DNA Elements (ENCODE) project [154], it may now be possible to unite the numerous reported factors affecting *PON1* expression (as reviewed, the AhR, p44/42 mitogen cascade, PKA, PKC, XRE, SREBP2 and Sp1) and unify them into a mechanistic pathway that influences *PON1* transcription.

Additionally, given the recent failures of HDL to deter adverse cardiovascular events, research should again focus on the role of the paraoxonases and atherosclerosis. For example, PON1 activity is highly protective against atherosclerotic phenotypes [27]; however, common genetic variation that predicts PON1 activity is not significantly associated with atherosclerotic end-organ damage phenotypes [27]. Areas for research in this field include developing more sophisticated animal models to determine the exact effects of varying levels of PON1 activity (e.g., not only knockout models). Additionally, rare genetic variation likely underlies a large portion of trait heritability [153].

The resources are available for addressing many of these questions. Protocols using nontoxic substrates are available for determining PON1 status, which provides $PON1_{192}$ functional genotype and the activity level, which in most cases is more important than *PON1* genotype [19,20]. Procedures are described for purifying each of the $PON1_{192}$ alloforms for any kinetic studies [155], or recombinant native PON1 for other studies [7]. *PON1* genetically modified mice ($PON1^{-/-}$, $tgHuPON1_{Q192}$ and $tgHuPON1_{R192}$) are also available for determining physiological relevance of hypotheses. Commercial antibodies are available for quantitative western blots or ELISA assays. These resources should be utilized to validate proposed involvement of PON1 in physiological processes.

In summary, the paraoxonases, and PON1 in particular, affect numerous human disease processes through the metabolism of pharmacologic agents, neutralization of toxic OP compounds, prevention of *Pseudomonas aeruginosa* infection, and protection against the oxidative stress involved in atherosclerosis, diabetes and potentially PD and cancer through modulation of oxidative stress. Future strategies to increase PON1 expression and activity

through the exogenous compounds and drugs outlined in this and other review articles may likely yield broad and measurable benefits.

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Executive summary

PON1 function

- PON1 status includes both PON1 enzyme activity functional assays and genotype
  - – A high proportion of PON1 activity variation is not captured by genotype alone.
  - – Use of genotypes alone (e.g., in clinical studies) will likely not find associations.
- The $PON1_{Q192R}$ polymorphism affects organophosphate substrate hydrolysis
  - – Extension of two-substrate activity plots developed by La Du resolves three $PON1_{Q192R}$ genotype classes (Q/Q, Q/R and R/R).
  - – Comparison of PON1 status assays with $PON1_{Q192R}$ genotype can reveal rare inactivating mutations.
  - – It is essential to determine PON1 activity levels within each $PON1_{Q192R}$ genotype class separately owing to different rates of allele frequencies across ethnic groups and differential properties of PON_{192} alloforms.
- PON1 activity versus protein levels
  - – Healthy controls tend to have high PON1 activity but lower PON1 protein levels.
  - – Exact meaning of this finding is yet unknown.

PON1 protection from organophosphate exposure

- Aldridge's early studies
  - – ‘A esterases’ catalytically inactivated organophosphorous (OP) compounds.
  - – ‘B esterases’ stoichiometrically inactivated OP compounds.
- Animal models
  - – First performed by Main in 1956; rabbit PON1 protects rats from paraoxon.
  - – Follow-up by Costa and Li also demonstrated protection against chlorpyrifos.
  - – Mouse knockout models were created by Shih, Lusis and Tward:
  - – $PON1^{-/-}$ mice are much more sensitive to chlorpyrifos and atherosclerosis.
  - – $PON1^{-/-}$ mice are much more sensitive to diazoxon.
  - – No change in sensitivity to paraoxon in $PON1^{-/-}$ mice.
- Bioengineered therapeutics
  - – PON1 protects against diazinon/diazoxon and chlorpyrifos/chlorpyrifos oxon.
  - – Two groups engineering PON1 with altered catalytic efficiency for OP compounds.
  - – Tawfik's group in Israel used hybrid protein, which included domains from human, rabbit and mouse PON1s.

– Furlong's group at the University of Washington expressed altered human PON1 in an *Escherichia coli* system with higher catalytic efficiency for OP compounds.

Paraoxonases & human disease

■ Native paraoxonase enzyme activity is probably a lactonase

– PON1 hydrolyzes *Pseudomonas aeruginosa* quorum-sensing factor.

■ Mackness *et al.* first reported that high-density lipoprotein (HDL)-associated PON1 prevented low-density lipoprotein (LDL) oxidiation

– This sparked medical interest in PON1 as an atheroprotective enzyme.

– The findings were confirmed by Watson *et al.* and by Aviram *et al.*

– A *PON1*$^{-/-}$ mouse created by Shih and colleagues demonstrated accelerated atherosclerosis, especially in combination with *apoE*$^{-/-}$.

– Human transgenic *PON1* mouse showed higher PON1 activity and was more effective at protecting against LDL oxidation and atherosclerosis.

– Clinical studies using only *PON1* genotypes had mixed results for association with cardiovascular disease. This highlights the importance of using PON1 status, rather than genotypes alone.

■ PON1 is closely associated with apoA1 and found in the small, dense subfractions of HDL.

■ HDL can deliver PON1 to sites of cellular oxidative stress

– This finding may underlie the association of PON1 variability with human diseases related to oxidative stress, such as cancer, Parkinson's disease and many other diseases.

Drug targets of paraoxonase hydrolysis

■ PON1 has extremely broad substrate specificity and metabolizes numerous drugs

– It bioactivates fluoroquinolone and prulifoxacin to their active forms.

– Inactivates glucocorticoid compound by limiting the systemic side effects.

– PON1 and PON3 hydrolyze atorvastatin δ-lactone (inactive statin metabolite associated with statin-related toxicity).

– PON3 hydrolyzes bulkier substrates, such as statins and aldosterone antagonists (spironolactone and canrenone).

■ Although PON1 has recently been linked to clopidogrel hydrolysis, numerous conflicting results have been reported

– Authors believe that PON1 probably does not metabolize clopidogrel.

Lifestyle determinants of PON1 activity

■ Nonmodifiable factors

– Age/development.

– PON1 activity in both serum and the liver increases with fetal development.

– PON1 activity is low at birth and increases to a plateau at approximately 6–15 months of age.

– There is a progressive decline in PON1 in geriatric stages of human life.

Lifestyle determinants of PON1 activity (cont.)

■ Gender

– Females have slightly higher PON1 activity/levels.

– Gonadectomy of male mice resulted in higher PON1 mRNA.

– Lower levels of *PON1* gene expression in males may be mediated by male-pattern growth hormone.

– Estrogen supplementation likely stabilizes or regulates PON1 protein stability and/or function.

■ Modifiable lifestyle factors

– Alcohol use.

– Mild-to-moderate (~40g/day) alcohol use generally increases PON1.

– Heavy alcohol use decreases PON1 activity.

– PKC/Sp1 transcription pathways may provide a mechanism for findings.

– Smoking.

– Cigarette smoke is inhibitory to PON1 activity.

– This is likely through modification of free thiol group, required for antioxidant effects on LDL peroxidation.

– Inhibition is reversible with *N*-acetyl cysteine or glutathione.

– Only current smoking affects PON1 activity (likely direct mechanism).

■ Lipid-rich diets

– Cholesterol.

– Dietary cholesterol found to be positively correlated with PON1 activity.

– Dietary cholesterol was not found to be acting as a proxy for HDL or apoA1; further investigation to elucidate the exact pathway is needed.

– Fatty acids.

– Polyenoic fatty acids are inhibitory of PON1 activity.

– Monoenoic fatty acids slightly increase PON1 activity, although conflicting results have been reported.

– Sugar intake.

– Subjects with diabetes have lower PON1 activity than control subjects.

– Hyperglycemic conditions in hepatocytes leads to enhanced PON1 promoter activity and *PON1* expression through PKC/Sp1.

– This may represent a compensatory mechanism to diabetes, a physiologic state of high oxidative stress (which inactivates PON1).

■ Iron and other metal intake

– Dietary iron is negatively correlated with PON1 activity in normal subjects.

– Prior *in vitro and in vivo* studies report PON1 inhibition by metals, including lead.

– Inhibition may occur through binding to free sulfhydryl group at residue 284.

Pharmacologic determinants of PON1 levels and/or activity

■ Cardiovascular drugs

– Statins likely increase PON1 activity.

– The *PON1* −108C polymorphism may be required for statin-mediated increase in PON1 expression.

– Statins may mediate effects on PON1 activity through Sp1 and SREBP-2.

– Statins possibly act through the p44/p42 signaling pathways to activate Sp1 and SREBP-2.

– The *PON1* −108C/T mutation may be necessary for statin-mediated increase in PON1 expression.

■ Other lipid-lowering medications.

– Fibrates likely increase PON1 expression.

– Probucol likely increases PON1 expression.

– Ezetimibe likely increases PON1 activity.

– Berberine likely increases PON1 expression.

– These increases are possibly caused via the JNK/c-Jun signaling pathways and AP1 promoter.

■ Other cardiovascular drugs

– Aspirin likely increases PON1 activity and expression.

– Arylhydrocarbon receptor likely necessary for aspirin-mediated increase in PON1 expression.

– Eplerenone likely increases PON1 activity.

– Erythropoietin-β likely increases PON1 activity.

■ Antidiabetic drugs

– Rosiglitazone – PPAR-γ agonist likely increases PON1 activity and expression.

– Sulfonylureas – likely increase PON1 activity.

Antioxidant determinants of PON1 activity

■ Vitamins C and E likely increase PON1 activity; possiblly by direct effect on the PON1 protein.

■ Quercetin and resveratrol likely increase PON1 activity and expression

– Arylhydrocarbon receptor and xenobiotic response element in *PON1* promoter may be necessary for quercetin- and resveratrol-mediated increases in *PON1* expression.

– Quercetin may also act through the transcription factor SREBP2.

Antioxidant determinants of PON1 activity (cont.)

- Polyphenols in pomegranate juice
  - Likely increase PON1 activity and expression.
  - Directly stabilize PON1 enzyme binding to HDL and overall stabilize PON1.
  - May increase *PON1* expression through PKA and PPAR-γ pathways.

Table 1

Targets of paraoxonase family enzyme activity.

Substrate	Enzyme(s)	Ref.
Organophosphates		
Sarin/soman (nerve gas)	PON1	[42]
Paraoxon (metabolite of parathion, insecticide)	PON1 > PON3	[16,37,38,47]
Chlorpyrifos oxon (metabolite of chlorpyrifos)	PON1	[16,38,41-43,47]
Diazoxon	PON1	[42,47]
Aryl esters (phenylacetate)	PON1 > PON3 > PON2	[43,47]
L-homocysteine thiolactone	PON1	[4,46]
Quorum sensing factor of *Pseudomonas aeruginosa*	PON1 and PON2	[5,6]
Oxidized low-density lipoprotein	PON1	[8,40,48,52,54]
Pharmacologic agents		
Statins (lovastatin)	PON3	[47]
Statin adducts (atorvastatin lactone)	PON1 and PON3	[79]
K+ sparing diuretics (spironolactone, canrenone)	PON3	[47]
Fluoroquinolone (prulifloxacin)	PON1	[77]
Glucocorticoids	PON1	[78]
Clopidogrel†	PON1	[80]

†Although PON1 was reported to bioactivate dopidogrel, recent evidence has placed this conclusion in serious doubt. Please see the ‘Clopidogrel (Plavix®) controversy’ section.

Table 2

Lifestyle determinants of PON1 activity.

Lifestyle component	Increased PON1 references	Decreased PON1 references	Potential mechanism
Female gender	[25,89,92]		Androgen production [92]
Age			
Fetal development to approximately 15 months	[86,87]		
Geriatric age		[88]	
Alcohol intake			
Moderate alcohol intake	[25,95,96,98,99]	[97]	PKC/Sp1 activation [99,100]
Heavy alcohol intake		[30,99]	PKC inhibition of *PON1* promoter [100,101]
Smoking status			
Current smoking status		[25,101,102]	PON1 enzyme free thiol modification [101]
Lipid-rich diets			
Cholesterol intake	[25,103]		
Fatty acid intake			
Monoenic acids	[108,109]		Stabilize PON1 *in vitro* [109]
Polyenic acids		[109]	
High glucose intake	[111]		PKC/Sp1 activation [100,111]
Dietary iron		[25,112,113]	Direct inactivation of enzyme [112]

Table 3

Pharmacological determinants of PON1 activity.

Drug	Increased PON1 references	Decreased PON1 references	Potential mechanism
Cardiovascular drugs: lipid lowering			
Statins (atorvastatin and simvastatin)	[116-119,120-126]	[120,121]	p44/42 MAPK activating SREBP2/Sp1 [124,125]
Fibrates (gemfibrozil, bezafibrate and ciprofibrate)	[121,122,127]	[128]	
Probucol	[130]		
Ezetimibe	[130]		
Berberine	[131]		JNK/c-Jun binding AP1 site in *PON1* promoter [131]
Aspirin	[132,133]	[134]	AhR-mediated signaling [133]
Eplerenone	[135]		
Erythropoietin-β	[29]		
Antidiabetic drugs			
Rosiglitazone	[136-138]		PPAR-*7* signaling pathways
Sulfonylureas	[139]		

Table 4

Antioxidant determinants of PON1 activity.

Antioxidant	Increased PON1 references	Decreased PON1 references	Potential mechanism
Vitamin C	[25,141,143]	[144]	
Vitamin E	[25,141,142]	[144]	
Quercetin	[140,145,146,148]	[144]	AhR-mediated signaling; SREBP2 [146,148]
Resveratrol	[147]		AhR-mediated signaling [147]
Pomegranate polyphenols	[149-152]		PKA; PPAR-γ pathways [152]