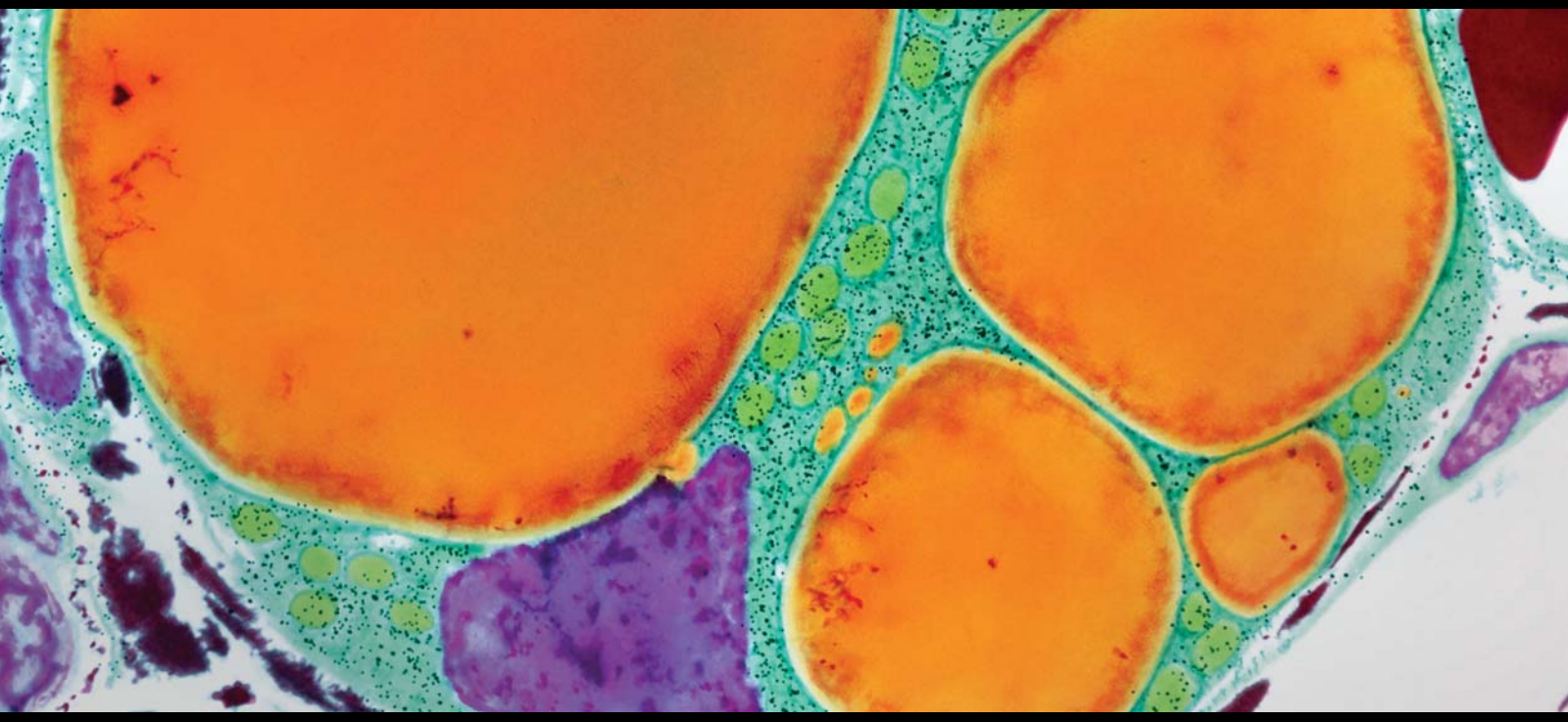
Journal of Lipids

Paraoxonases

Guest Editors: Michael Aviram, Mira Rosenblat, Bianca Fuhrman, and Alejandro Gugliucci



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Editorial Board

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Editorial

Introduction to Paraoxonases

Michael Aviram

The Lipid Research Laboratory, Rambam Medical Center, Bat-Galim, 31096 Haifa, Israel

Correspondence should be addressed to Michael Aviram, aviram@tx.technion.ac.il

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The objectives of this special issue on paraoxonases (PONs) are to bring the latest aspects of paraoxonases (PON 1, 2, 3) research on the genetics, biochemistry, cell biology, and structural biology of PONs. The issue also addresses the role of PONs in human diseases (cardiovascular, cancer, renal failure, and gastrointestinal disorders). Inflammatory and oxidative stress-related diseases, such as diabetes or rheumatoid arthritis are also favorably affected by PON. PONs also provide microbial protection by hydrolyzing bacterial quorum lactone.

The pivotal role of the PON family in a variety of inflammatory diseases, and in preventing the toxicity of organophosphorus insecticides and nerve agents, has made PONs an interesting target for both clinicians and scientists alike. Research into the paraoxonase family of enzymes has increased dramatically, especially following the initiation of the paraoxonase conferences. Five international conferences on paraoxonases were organized between 2004 and 2012, where the PON scientific community gathered to discuss PONs research achievements and future scientific directions. These meetings took place in Ann Arbor (MI), Debrecen (Hungary), Los Angeles (CA), La Pineda (Spain), and Columbus (OH). According to PubMed, only few PON papers were published before 1980. From 1980 till these days, about 3000 papers were published.

The PONs gene cluster contains three adjacent gene members, and all of the three PON genes share high sequence, indentify a similar β propeller protein structure, and hydrolyze esterase/lactonase activities.

PONs play a clear protective role against cardiovascular diseases. Major cardioprotective PON characteristics include, beside their potent antioxidant properties, the following: for PON 1, favorable effects on macrophage cholesterol metabolism, for PON 2, attenuation of macrophage triglyceride accumulation, and for PON 3, improvement in bile acids metabolism.

Human serum HDL-associated paraoxonase (PON1) is an esterase that possesses cardiovascular protective properties which result in the following antiatherogenic functions: (1) attenuated oxidative stress in serum, in lipoproteins, in macrophages, and in atherosclerotic lesions; (2) decreased oxidized LDL uptake by macrophages; (3) inhibited macrophage cholesterol biosynthesis rate; (4) stimulated HDL-mediated cholesterol efflux from macrophages.

Major PON1 inactivators (and reversal of their action) include (1) oxidative stress (and reversal effect by antioxidants such as the pomegranate polyphenolic tannin punicalagin); (2) high cholesterol (and reversal effect by statins); (3) high triglycerides (and reversal effect by fibrates); (4) high glucose (and reversal effect by insulin).

Macrophage PON2 regulation (as related to atherogenesis) differs from that of serum PON 1 in the following characteristics: (1) stimulation (not inhibition as shown for PON1) by oxidative stress and also by anti oxidants (like PON1); (2) stimulation by high glucose (PON1 is inhibited) and also by insulin (like PON1); (3) stimulation by urokinase plasminogen activator (uPA); (4) stimulation by high triglycerides (PON1 is inhibited); (5) stimulation by arachidonic acid and also by its product prostaglandin E2 (like PON1); (6) inhibition by high cholesterol and reversal effect by statins (like PON1).

Figure 1 summarizes our current view on the antiatherosclerotic properties of circulatory HDL-bound PON1 and those of cellular PON2. Reduction in specific oxidized lipids in the blood, in arterial lipoproteins (LDL, HDL), and in macrophages, by overexpressing humoral PON1 (and also

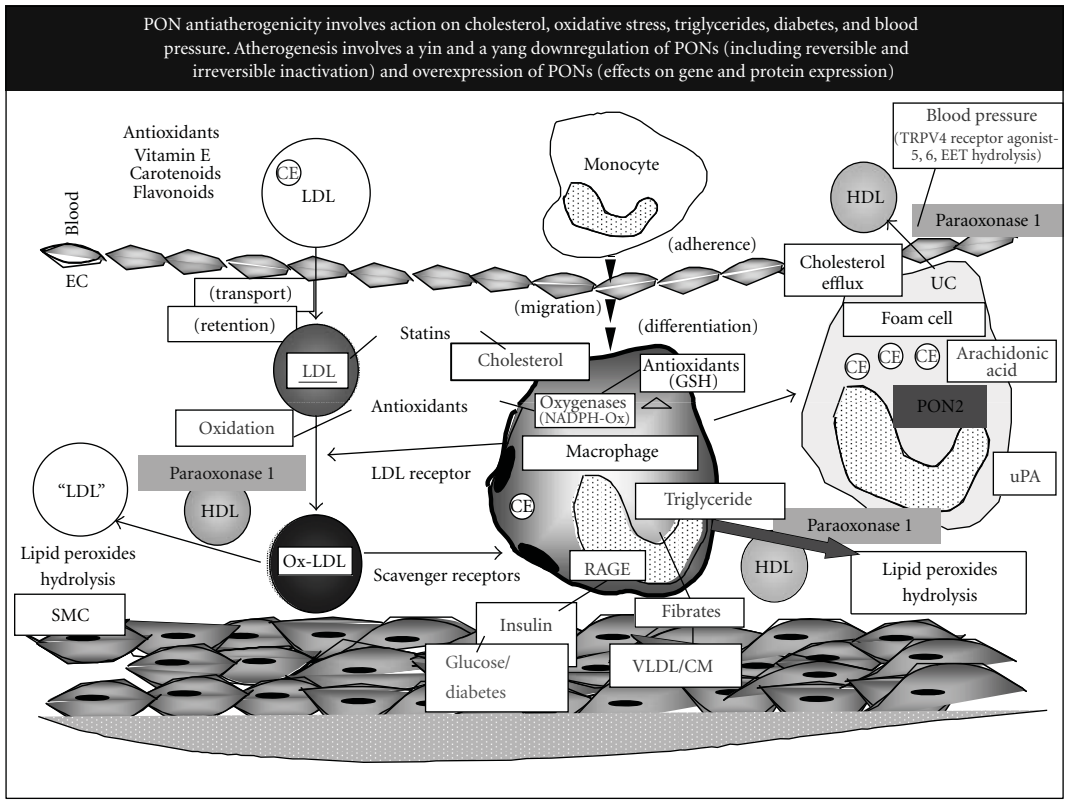

Figure 1

cellular PON2), could thus be important target for the attenuation of atherosclerosis development.

Michael Aviram

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Research Article

Solubilization and Humanization of Paraoxonase-1

Mohosin Sarkar,[1] Christina Keventzidis Harsch,[1] George T. Matic,[1] Kathryn Hoffman,[1] Joseph R. Norris III,[2] Tamara C. Otto,[2] David E. Lenz,[2] Douglas M. Cerasoli,[2] and Thomas J. Magliery[1,3]

[1] *Department of Chemistry, The Ohio State University, Columbus, OH 43210, USA*
[2] *Physiology and Immunology Branch, Research Division, United States Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD 21010, USA*
[3] *Department of Biochemistry, The Ohio State University, Columbus, OH 43210, USA*

Correspondence should be addressed to Thomas J. Magliery, magliery.1@osu.edu

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Paraoxonase-1 (PON1) is a serum protein, the activity of which is related to susceptibility to cardiovascular disease and intoxication by organophosphorus (OP) compounds. It may also be involved in innate immunity, and it is a possible lead molecule in the development of a catalytic bioscavenger of OP pesticides and nerve agents. Human PON1 expressed in *E. coli* is mostly found in the insoluble fraction, which motivated the engineering of soluble variants, such as G2E6, with more than 50 mutations from huPON1. We examined the effect on the solubility, activity, and stability of three sets of mutations designed to solubilize huPON1 with fewer overall changes: deletion of the N-terminal leader, polar mutations in the putative HDL binding site, and selection of the subset of residues that became more polar in going from huPON1 to G2E6. All three sets of mutations increase the solubility of huPON1; the HDL-binding mutant has the largest effect on solubility, but it also decreases the activity and stability the most. Based on the G2E6 polar mutations, we "humanized" an engineered variant of PON1 with high activity against cyclosarin (GF) and found that it was still very active against GF with much greater similarity to the human sequence.

1. Introduction

Paraoxonase-1 (PON1) is a mammalian serum protein, the activity of which is related to cardiovascular health and the toxicology of organophosphorus (OP) compounds [1–3]. PON1 is thought to be synthesized mostly in the liver, and it is associated with high-density lipoproteins (HDLs) in serum [4]. The exact function of PON1 is not known, but it is an efficient hydrolase of lactones and esters and an inefficient hydrolase of OP compounds, including pesticide metabolites such as paraoxon (from parathion) and chlorpyrifos oxon, and nerve agents such as sarin, tabun, and VX [1, 5]. Increased PON1 activity appears to be related to lower levels of oxidation of low-density lipoprotein (LDL) particles, and its hydrolytic activity has been suggested to be directed at oxidized fatty acids and homocysteine thiolactone [6–8]. Its increased activity has been shown to be related to decreased atherosclerosis, and it has been implicated in mechanisms of cholesterol efflux [9, 10]. PON1 also efficiently hydrolyzes bacterial lactones involved in quorum sensing, and it may contribute to innate immunity through this activity [11]. Although the hydrolysis of OP compounds is almost certainly a promiscuous activity of the enzyme, it contributes to the susceptibility to OP intoxication [12], and PON1 has been suggested as a lead molecule for a prophylactic or therapeutic bioscavenger of OP toxins [13, 14]. Human PON1, particularly the R192 alloform, is already sufficiently active to protect against chlorpyrifos oxon and diazoxon exposures without engineering. The turnover of many other OPs by natural PON1 is not sufficient to afford significant protection, but a mammalian chimeric form of PON1 has recently been engineered for significant activity against some G-agents [15].

As a result of the physiological and toxicological correlations with increased PON1 activity, there is great motivation to develop PON1 as a therapeutic agent. There are significant

difficulties with this: PON1 has only moderate solubility; it has three Cys residues including two forming a disulfide bond, and it is glycosylated [16]. Human PON1 (huPON1) is very difficult to produce in soluble, folded form in *E. coli*. Large-scale fermentation has been used to produce soluble huPON1 successfully in *E. coli*, but in poor yields for pharmaceutical production [14]. This motivated Aharoni and colleagues to generate a chimeric mammalian PON1 by DNA shuffling of mouse, rat, rabbit, and human PON1 isoforms, resulting in a variant called G2E6 that could be expressed when fused to the C-terminus of thioredoxin in good yields in the soluble fraction of *E. coli* (Figure 1) [17]. The crystal structure of G2E6 was solved, revealing it to be a six-bladed β-propeller protein bound to two Ca^{2+} ions, one of which appears to play a more structural role and one of which is located in what is presumed to be the active-site pocket [16]. A further generation of DNA shuffling and selection yielded G3C9 PON1, which can be expressed in significant amounts in the soluble fraction of *E. coli* without a fusion partner (although it bears a C-terminal hexahistidine tag). Both of the proteins have greatest sequence similarity to the rabbit isoform of PON1, and they differ by 58-59 (G2E6) and 50-51 (G3C9) amino acids from huPON1, depending on the polymorph, mostly on the surface and essentially not at all in the putative active site. (Human PON1 is either Leu or Met at 55, while rabbit is only known to be Leu at this position).

The poor solubility of human PON1 is presumably in part a consequence of its ability to associate with HDL. The nature of this interaction is not clearly defined. PON1 has a signal sequence directing it for cellular export, and it is mutated at the cleavage site for the signal protease, resulting of retention of the hydrophobic signal peptide [18, 19]. That peptide is disordered in the structure of G2E6 [16]. There is a large hydrophobic patch on the surface of PON1 that is near the N-terminus, suggesting that this is the HDL interaction surface. The interaction of PON1 with HDL stimulates its activity towards lactones, and removal of the signal peptide (residues 1–20) has been shown to abrogate that stimulation, suggesting that it is critical for proper embedding in the apoA-I HDL particle [20]. It is not surprising that most of the differences between the very insoluble human PON1 and the more soluble G2E6 and G3C9 are on the surface of the protein, as this is where changes would be most expected to affect the solubility of the folded protein.

Despite this intuitive expectation, surprisingly little is known about how mutations affect the solubility of proteins or how to engineer proteins for greater solubility. Several studies have reengineered membrane proteins to render them soluble. Li and coworkers reengineered phospholamban (PLB), a protein that forms a stable helical homopentamer within the sarcoplasmic reticulum membrane, into a soluble pentameric helical bundle by replacing its lipid-exposed hydrophobic residues with charged and polar residues [21]. Based on computational design, Slovic and coworkers rationally engineered a water-soluble analog of PLB by changing membrane-exposed positions to polar or charged amino acids, while the putative core was left unaltered [22]. These constructs were based on the hypothesis that membrane proteins and water-soluble proteins share a similar core and it should be possible to solubilize membrane proteins by mutating only their lipid-exposed residues. The redesigned PLBs mimic all of the reported properties of PLB including oligomeric state, helical structure, and stabilization upon phosphorylation. Based on the same approach, Slovic and coworkers redesigned a water-soluble variant of a membrane protein, potassium channel KcsA, by mutating the lipid-contacting side chains to more polar groups [23].

We were interested in determining how mutations to huPON1 would affect its solubility and soluble expression in *E. coli*. We hypothesized that three different types of mutations might increase the solubility of human PON1. We speculated that (a) removal of the hydrophobic N-terminal leader sequence and (b) mutations of hydrophobic amino acids in the presumptive HDL binding site to polar residues would increase the solubility. We also speculated that (c) the surface residues which were mutated to be more polar amino acids during the directed evolution of G2E6 PON1 were mostly responsible for the increased solubility. To test these ideas, we constructed three mutants of human PON1 called ΔN-huPON1, ΔHDL-huPON1, and g2e6p-huPON1 (Figure 1). We also combined some of the mutations to look for additive effects on solubility.

To test the solubility of these proteins, we exploited the screen developed by Waldo and colleagues based on fusion of an analyte protein ("protein of interest" or POI) to the N-terminus of the "folding reporter" variant of green fluorescent protein (frGFP) [24]. Briefly, if the POI folds and is soluble, then the frGFP also folds and its chromophore develops, resulting in fluorescent cells. If the POI is insoluble, then the fusion is found in the membrane-associated fraction and little fluorescence develops. Waldo and colleagues demonstrated that the amount of cellular fluorescence is related to the amount of soluble protein. Consequently, we fused each of the huPON1 variants to the N-terminus of frGFP and determined the fluorescence level of the host bacterial cells. We also examined the activity and stability of the resulting proteins.

We were also interested in the determinants of huPON1 solubility because we wished to use that knowledge to generate variants of engineered PON1 that had significantly greater activity toward OP agents, but with a surface sequence significantly more like native huPON1. Little is known about the immunological effects of the administration of heterologous variants, but the large number of mutations on the surface of G2E6 and G3C9 relative to human PON1 is a cause for concern. In the field of antibody-based therapeutics, human anti-mouse antibody syndrome is a common effect of the administration of mouse-derived antibodies, and so variants of mouse antibodies have been successfully "humanized" by replacing their surface residues with a human sequence while maintaining the binding site residues elicited during affinity maturation [25]. We speculated that we might be able to "humanize" or at least partially humanize evolved variants of G3C9 PON1 with high OP activity by reverting the surface back to the human sequence, except for solubilizing mutations identified in the

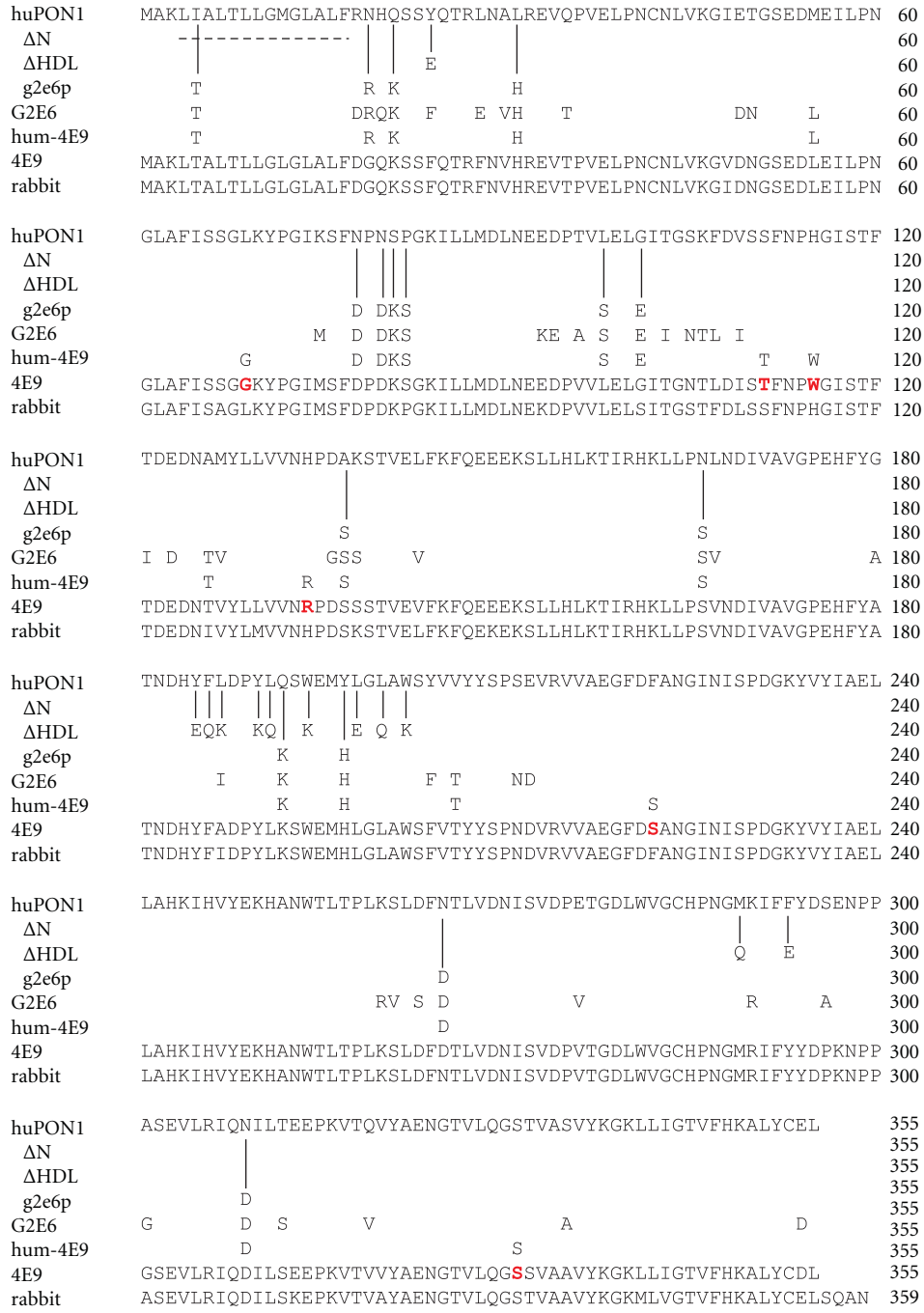

Figure 1: *Alignment of PON1 variants.* All differences are noted with respect to the human PON1 sequences (Q192/M55 polymorph); the 4E9 sequence and the similar rabbit PON1 sequence are shown in full for reference. Differences between G3C9 and 4E9 are noted in red in the 4E9 sequence. -: deletion.

first part of this work. We chose to humanize the recently reported 4E9 variant (Figure 1) [15], which has very high activity against the cyclosarin (GF) analog CMP, using the same strategy used to generate g2e6p-huPON1. Because the substrate specificities of huPON1 and G2E6 PON1 are quite different despite essentially identical active sites, it was not clear if humanization could yield an active enzyme [5]. Here we show that humanization of 4E9 was successful and suggests a path forward for improved therapeutics based on engineered PON1 variants.

2. Materials and Methods

2.1. Cloning huPON1 and frGFP Fusions in pET11a. The frGFP gene was generated in our lab from the genes for

GFPuv [26] and EGFP [27] by overlap PCR, resulting in a GFP with mutations F64L S65T F99S M153T V163A. The frGFP gene was PCR amplified with primers coding for a 6×His tag and an AatII site at the 3′ end and an EcoRI site at the 5′ end. Wild-type human PON1 (Q192/M55) was PCR amplified from a mammalian expression vector, pcDNA3. The oligonucleotide (Sigma Genosys, The Woodlands, TX) primers 5′-AATAATTATC ATATGGCTAA GCTGATTGCG CTCACCC-3′ with an NdeI site, and 5′-ATAATGAATT CGCCGCTGCT TCCGCTCTGA AAATACAGAT TCTCACCGCC GGTACCGAGT TCGCAGTAAA GAGCTTTGTG AAACAC-3′ coding for a KpnI site, TEV protease (ENLYFQG) site, linker (GSSG) and EcoRI site, were used for amplification. A fusion of huPON1-(KpnI-)TEV-Linker-(EcoRI-)frGFP (Figure 2) was cloned into a pET11a vector between the NdeI and AatII sites using a three-piece ligation. For reference, the G2E6 gene was analogously cloned into this construct (the gene was kindly provided by Dan Tawfik, Weizmann Institute of Science). The sequence of the fusion construct was confirmed by DNA sequencing (Genewiz, South Plainfield, NJ).

2.2. Rationally Engineered huPON1 Variants. To generate ΔHDL-huPON1 (Figures 1 and 3), twelve Glu, Gln, or Lys mutations were made at hydrophobic residues in the putative HDL binding site (Y24E, Y185E, F186Q, L187K, Y190K, L191Q, W194K, L198E, L200Q, W202K, M289Q, F293E) in the huPON1 construct. All of these mutations were introduced at once by overlap PCR. Three pairs of oligonucleotides were used to amplify three fragments of the huPON1 gene with all 12 mutations. The three fragments were then subjected to assembly and amplification similar to the final steps of DNA shuffling using two terminal primers, 5′-ATAGATATAC ATATGGCGAA GCTGATTGCA CTCACGCTCT TGGGGATGGG ACTGGC ACTC TTCAGGAACC ACC-3′ and 5′-CTCACCGCCG GTACCGAGTT CGCAGTAAAG AGCTTTG-3′. These primers coded for NdeI and KpnI sites. The amplified ΔHDL-huPON1 gene was cloned into pET11-huPON1-TEV-frGFP vector, replacing huPON1 between NdeI and KpnI, and the construct was confirmed by DNA sequencing.

The g2e6p-huPON1 was engineered by introducing surface mutations that became more polar in the directed evolution of G2E6 from huPON1. Fifteen such mutations (I5T, N19R, Q21K, L31H, N78D, N80D, S81K, P82S, L98S, G101E, A137S, Q192K, Y197H, N265D, and N309D) and an N166S compensatory mutation of the Q192K mutation were introduced into huPON1 by total gene synthesis using the TBIO method [28]. Thirty primers of 60 nt coding for all 16 point mutations were designed using DNAWorks [29]. The assembled full-length gene for g2e6p-huPON1 was amplified using two terminal primers, 5′-GTTTAACTTT AAGAAGGAGA TATACATATG GCAAAGCTGA CCGC-3′ and 5′-TGAAAATACA GATTCTCACC GCCGGTACCT AATTCACAG-3′, and cloned as described for ΔHDL-huPON1.

To generate the N-terminal deletion (ΔN) constructs, residues 4 to 17 (LIALTLLGMGLALF) from the leader sequence of PON1 were deleted by PCR amplification of the host genes. The forward primer 5′-AATAATAATC ATATGGCAAA GAGGAACCAC CAGTCTTCTT AC-3′ was used for huPON1 and g2e6p-huPON1; 5′-AATAATAATC ATATGGCGAA AAGGAACCAC CAGTCTTCAG AAC-3′ was used for ΔHDL-huPON1. The reverse primer 5′-AATAATGAAT TCGCCGCCGC TTCCGCTCTG AAAATACAG ATTCTC-3′ was used for huPON1 and ΔHDL-huPON1, and 5′-AATAATAATG GTACCTAATT CACAGTATAA TGCTTTATGG AAAACCG-3′ was used for g2e6p-huPON1. These were cloned as described for ΔHDL-huPON1.

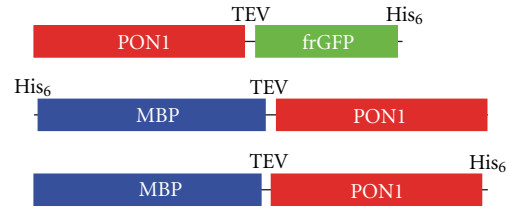

Figure 2: *Schematics of the fusions used in this study.* PON1, paraoxonase-1 variant; TEV, TEV protease site; frGFP, folding reporter GFP; His_6, hexahistidine tag; MBP, maltose-binding protein.

2.3. GFP-Fusion Assay for Solubility. BL21(DE3) cells were transformed with engineered pET11a-PON1-frGFP fusion constructs. LB media (100 mL) supplemented with ampicillin were inoculated with 2 mL of overnight saturated culture grown from a single colony. The cells were grown to OD_{600} ~0.7 and induced with 0.1 mM IPTG. The fusion proteins were expressed for 4 h at 30°C and the cells were incubated at 4°C for 6 h before harvesting by centrifugation. Cell pellets were resuspended in PBS and washed with PBS twice before the density of the cells was normalized by adjusting OD_{600} to 0.1. Whole-cell fluorescence was measured in a Perkin Elmer LS50B fluorimeter using 480 nm excitation and 509 nm emission.

2.4. Cloning into pHMT. Genes for full-length and N-terminal deletion variants were cloned into the pHMT vector [30] (kindly provided by Mark Foster, Ohio State Biochemistry) between the NcoI and PstI sites as a C-terminal fusion to maltose-binding protein (MBP). The vector encodes a 6×His tag at the N-terminus of MBP, a linker (EFGSSRVD), and a TEV protease site (ENLYFQG) between the MBP and fused protein (Figure 2). For the ease of cloning, the SalI site in the original vector was replaced with an NcoI site, and a fragment of unrelated DNA was inserted between NcoI and PstI sites. The huPON1 variant genes were amplified with PCR and cloned between the NcoI and PstI sites.

2.5. Cloning into pET11a with a C-Terminal 6×His Tag. The MBP fusions of the huPON1 variants were PCR amplified from pHMT using a 5′ primer that removes the N-terminal 6×His tag and a 3′ primer encoding a new C-terminal 6×His tag. Genes for the fusions with a TEV protease site

between the MBP and protein variants (Figure 2) were cloned into a pET11a vector between the NdeI and XhoI sites.

2.6. Fusion Protein Expression and Purification. Origami B (DE3) *E. coli* (Novagen, Madison, WI) were transformed with plasmids encoding the frGFP fusion or MBP fusion constructs and grown overnight to saturation. The MBP fusions were also expressed from cells containing the chaperone plasmid pKJE7 (encoding DnaK, DnaJ, and GrpE, from Takara Bioscience). One liter of LB media supplemented with appropriate antibiotics and 1 mM $CaCl_2$ (and 0.1% arabinose when pKJE7 was used) was inoculated with 20 mL of saturated culture and grown at 37°C to an OD_{600} of 0.8. Cells were induced with 0.1 mM IPTG, sometimes after brief incubation in an ice-water bath, and grown at 16–30°C for 4 h to overnight. (Material from different induction and growth conditions had identical specific activity for paraoxon and is presumed to be identical).

All purification was carried out at 4°C unless stated. Cells harvested by centrifugation at 6,000 g for 10 min were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM $CaCl_2$, with or without 10% glycerol) supplemented with 1 mM DTT and lysed by extrusion through a needle and sonication. The lysate was incubated with 0.1% Tergitol NP-10 (Sigma-Aldrich) on a nutator at 4°C for 2-3 h. After centrifugation at 27,000 g for 1 h, cleared lysate was mixed with Ni-NTA agarose resin (Qiagen, Valencia, CA) and incubated at 4°C for 3 h with gentle mixing. The slurry was poured into a chromatography column and the flow-through fraction was discarded. The resin was typically washed with lysis buffer containing 25–40 mM imidazole and eluted with lysis buffer containing 150 mM imidazole. It was then exchanged into buffer with 10% glycerol using dialysis or a PD10 desalting column (GE Healthcare) and then into buffer containing 50% glycerol by dialysis. The buffer used was 50 mM Tris-HCl, pH 7.4, 10 mM $CaCl_2$ for proteins assayed against paraoxon and phenyl acetate and 50 mM Tris-HCl, pH 8, 50 mM NaCl, 1 mM $CaCl_2$, 0.1% Tergitol NP-10, for proteins assayed against EMP and CMP. The difference in buffers was merely due to testing at different times by different researchers. Protein concentrations were determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA) and confirmed by SDS-PAGE. Samples were stored at −20°C.

For thermal inactivation studies (see below) the huPON1 variants were cleaved away from the MBP fusion protein. After elution from the NiNTA column, proteins were exchanged into a buffer containing 50 mM Tris-HCl, pH 7.4, 10 mM $CaCl_2$, 5 mM DTT using a PD10 desalting column. Samples were then treated with TEV protease for 4–6 h at room temperature before subjecting them to Ni-NTA resin binding again. After 4 h of binding at 4°C, resin slurry was poured into a chromatography column, washed with lysis buffer containing 20 mM imidazole, and eluted with lysis buffer containing 150 mM imidazole. Protein samples were exchanged into 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM $CaCl_2$ using a PD10 desalting column. Samples were further purified over an anion exchange column (Resource Q, GE Healthcare) to separate them from any coeluted proteins. Pure huPON1 and its variants were eluted using a gradient of from 50 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM $CaCl_2$ to the same buffer with 0.5 M NaCl over 50 mL. Pooled fractions were tested for aryl esterase activity and dialyzed overnight into 50 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$, 50% glycerol, before kinetic measurements.

2.7. Construction and Purification of Hum-4E9. The humanized 4E9 protein (hum-4E9) was designed by introducing the 4E9 mutations (L69G S111T H115W H134R F222S T332S) into the g2e6p-huPON1 sequence. Note that two additional nonpolar-to-polar mutations were made compared to g2e6p-huPON1 (A126T V206T) and that Leu was used at human polymorphic position 55. The amino acid sequence was reverse-translated to retrieve the gene sequence, which was codon-optimized for *E. coli* expression. The gene sequence was ordered from Genewiz, which provided the gene in a pUC57 plasmid. Using BamHI, the hum-4E9 was cloned into pET11a-MBP vector, yielding an MBP-tag at the N-terminus and a 6 × His tag at the C-terminus. The fusion was purified as described above. In order to remove the MBP fusion, the hum-4E9 variant was also cloned into pET32b using NcoI and XhoI. For comparison, we also purified 4E9, as described, from a plasmid kindly provided by Dan Tawfik [15].

2.8. Enzyme Kinetics. Kinetic parameters for the hydrolysis of phenyl acetate and paraoxon were determined as described [5] using an assay buffer containing 50 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.4. Paraoxon (Sigma) was used from 0.06 to 2.6 mM, phenyl acetate was used from 0.06 to 3.3 mM, and EMP (3-cyano-4-methyl-2-oxo-2*H*-chromen-7-yl ethyl methylphosphonate) and CMP (3-cyano-4-methyl-2-oxo-2*H*-chromen-7-yl cyclohexyl methylphosphonate) were used from 0.005 mM to 0.5 mM. The initial rate of formation of hydrolysis product at 25°C was monitored by following the absorbance at 405 nm for *p*-nitrophenolate from paraoxon (ε = 14,320 M^{-1} cm^{-1}), at 270 nm for phenol from phenyl acetate (ε = 1,310 M^{-1} cm^{-1}), and at 405 nm for 7-hydroxy-4-methyl-3-cyanocoumarin (MeCyC, ε = 37,000 M^{-1} cm^{-1}) from EMP and CMP, using an Agilent 8453 UV-Vis spectrophotometer or a SpectraMax M5 Pro multiwell plate reader (Molecular Devices, Sunnyvale, CA) and Greiner One UV Star plates. Stocks of phenyl acetate, paraoxon, EMP, and CMP were prepared in methanol. Kinetic parameters for EMP and CMP were determined at 2% constant methanol. Parameters were derived by fitting a Michaelis-Menten model of steady-state enzyme kinetics to the data with KaleidaGraph (Synergy Software, Reading, PA). EMP and CMP were kindly provided by Yacov Ashani, Weizmann Institute of Science, and were synthesized in house by the method of Ashani et al. [31] (S. Muthukrishanan, D. Mata, TJM and C. Hadad, unpublished).

To test activity against the more toxic S_P isomer of CMP, the protocol from Gupta et al. [15] was followed as described. In short, racemic CMP (0.025 mM) in the presence of 3B3 PON1 (provided by Dan Tawfik) was incubated in 50 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.4 for 30 min at 4°C to deplete the R_P-isomer from the reaction. The reaction mixture

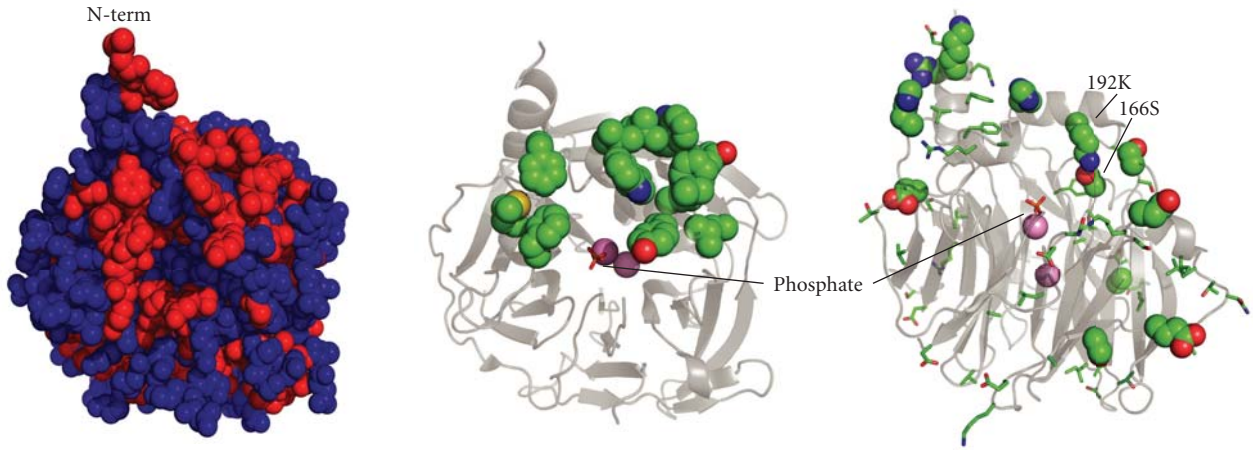

Figure 3: *PON1 solubilizing mutations.* (a) The surface of G2E6 is shown, with hydrophobic amino acids (VGMCILYFW) shown in red. Residues 1–15 are not resolved in the X-ray crystal structure, but the ΔN-huPON1 variant removed residues 4–17. (b) The positions modified in the ΔHDL-huPON1 variant are shown in spheres. These residues compose much of the hydrophobic surface patch near the N-terminus evident in (a). The Ca^{2+} ions are shown as pink spheres, and a phosphate bound in the presumed active site is shown in orange sticks. (c) The 59 positions that differ between huPON1 and G2E6 are shown; the positions that were modified in g2e6p-huPON1 are spheres, and the other 43 positions are sticks. Position 166, which was modified because of its proximity of 192, is noted. Rendered from PDB ID: 1V04 with PyMOL.

(150 μL) and 50 μL of diluted enzyme were added to a 96-well plate.

To determine the kinetics of cyclosarin (GF) hydrolysis, racemic cyclosarin was obtained from the US Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD). Analysis by NMR spectroscopy showed it to be >95% pure. Stock solutions of GF in saline were prepared at 2 mg mL^{-1} and stored at −70°C. Enzyme was incubated with 0.3125 mM GF in 10 mM MOPS, 2 mM $CaCl_2$ at room temperature. At specific time intervals, 100 μL aliquots were removed and inactivated through extraction with an equal volume of ethyl acetate containing 50 μM diisopropyl fluorophosphate (DFP; internal standard); this extraction both inactivates the enzyme and prevents racemization of nonhydrolyzed GF stereoisomers. The organic layer (containing nonhydrolyzed GF) was then removed and analyzed by gas chromatography/mass spectrometry (GC/MS).

Chiral gas chromatographic analysis of GF was performed using an Agilent 7890 gas chromatograph (Foster City, CA) fitted with a 20 m × 0.25 mm internal diameter ASTEC G-TA column (Astec, Whippany, NJ). Helium was used as the carrier gas at an average linear velocity of 54.5 cm s^{-1}. The oven temperature was held initially at 70°C for 1 min and then ramped from 70 to 160°C at a rate of 10°C min^{-1}. Split injections (50 : 1) of 1 uL volume were made using an Agilent 7693 autosampler. The injection port temperature was 210°C and the split vent delay was set at 1 min. The GC was interfaced to an Agilent 5975 mass spectrometer (MS) with an electron impact ion source. The MS operating conditions were as follows: ion source pressure approximately 1.0×10^{-5} Torr; source temperature, 230°C; quadrupole temperature, 150°C; electron energy, 70 eV; transfer line temperature, 265°C. The MS was operated using selected ion monitoring. Ion pairs m/z 99 and 67 and m/z 101 and 127 were monitored for GF and DFP, respectively. A dwell time of 100 ms for each ion pair resulted in a scan rate of 8.26 cycles s^{-1}. Rate constants (k_{app}) for hydrolysis of racemic GF were derived by using nonlinear regression to fit hydrolysis progress curves to a single-phase decay model using Prism 4.03 (GraphPad Software, La Jolla, CA). Relative stereoisomeric preference was calculated by determining the ratio of the rates of hydrolysis of each enantiomer of GF.

2.9. Thermal Inactivation and Residual Activity Determination. Protein samples were heated for 10 min at different temperatures ranging from 25°C to 80°C. After a brief incubation on ice and centrifugation in a picofuge at 2,200 g for 2 min, their activities were determined from EMP (0.35 mM) or phenyl acetate (3.6 mM) hydrolysis. The residual hydrolysis activity from incubation at 20°C (phenyl acetate) or 25°C (EMP) was taken as 100%.

3. Results

3.1. Design of Rationally Engineered Variants. Based on what is known about the leader sequence, the crystal structure of G2E6, and sequence comparison of G2E6 to huPON1, we designed three variants of huPON1 to examine their effects on the solubility of the protein. The first variant, ΔN-huPON1, is a deletion of residues 4–17, which includes most of the leader sequence. The first residue resolved in the crystal structure of G2E6 (PDB ID: 1V04 [16]) is Leu16. The first three residues, MAK, are fairly soluble and the small size of Ala2 likely contributes to homogeneous demethioninylation in *E. coli*. Residues 16 and 17 are Leu and Phe, so the 4–17

deletion results in removal of basically all of the N-terminal hydrophobic residues.

Our second hypothesis was that increasing the polar character of the putative HDL binding site, which is defined by a large number of surface hydrophobic residues, would increase the solubility of the protein. We speculated that this might not affect the structure or activity of the protein significantly since it is on the surface and pointed away from the active site. Residues proposed to be involved in HDL anchoring lie principally in Helix 2 and the adjacent loops, as well as Helix 1 [16] (Figure 3). We modified this surface based on inspection of the crystal structure of G2E6 with a limited library of polar amino acids (Glu, Gln, and Lys) to yield ΔHDL-huPON1 (Y24E Y185E F186Q L187K Y190K L191Q W194K L198E L200Q W202K M289Q F293E).

Our third hypothesis was that only a subset of the surface changes in the directed evolution of G2E6 was responsible for increasing the solubility of the protein. In particular, we speculated that sites that became significantly more polar (either nonpolar to polar or charged, or polar to charged) would contribute to most of the solubility increase seen with G2E6 [17]. We chose 15 sites where residues became significantly more polar from huPON1 to G2E6 (I5T N19R Q21K L31H N78D N80D S81K P82S L98S G101E A137S Q192K Y197H N265D N309D). We then examined whether any of the mutations were in the vicinity of any of the other 44 mutations present in G2E6, and we found a single case wherein Lys192 made a hydrogen bond to Ser166. Position 192 is an interesting site because it is a site of human polymorphism, where Gln and Arg are common [32]. Also, of all 59 mutations in G2E6, only five positions are within 9 Å of the active-site His115 residue, and of those only the side chains of Ser166 and Lys192 point toward the active site. Consequently, with the idea that coupling between these two positions could be important, we also included the N166S mutation to yield g2e6p-huPON1.

We also engineered several combinations of these variants, by combining the N-terminal deletion to ΔHDL-huPON1 (to yield ΔN-ΔHDL-huPON1) and the g2e6p-huPON1 (to yield ΔN-g2e6p-huPON1), to examine the potential for additivity in increasing solubility.

3.2. GFP-Fusion Screen for Solubility. To assess the solubility of these engineered variants, we turned to the GFP-fusion screen developed by Waldo and colleagues [24]. In this screen, a protein of interest is fused to the N-terminus of "folding reporter" GFP, and cellular fluorescence develops in proportion to the solubility of the POI. In reality, the cellular fluorescence is related to the amount of soluble protein, but in this case all of the tested variants of PON1 expressed in similar significant quantities in whole-cell lysate (not shown), so we can assume that increased fluorescence is due to increased partitioning into the soluble fraction. We constructed frGFP by combining EGFP (F64L S65T) [27] with Stemmer's "cycle 3" GFPuv (F99S M153T V163A) [26], and we generated our own fusion construct in a pET11a plasmid. We also tested the screen by assaying unfused frGFP as well as fusions of T4 lysozyme, yeast triosephosphate isomerase (TIM), G2E6 PON1, and human PON1. Cells with the T4 lysozyme fusion were more fluorescent than those with the unfused GFP; yeast TIM resulted in comparable fluorescence, G2E6 with lower fluorescence, and huPON1 with even lower fluorescence than G2E6 (data not shown). Cells were washed with phosphate-buffered saline and normalized for cell density before fluorescence was measured.

The solubility results are shown in Figure 4. As we observed in our controls, the fluorescence was about 4.5-fold greater for G2E6 than huPON1. The three engineered variants were all more soluble than huPON1: the fluorescence for ΔN-huPON1 was about twice that of huPON1, g2e6p-huPON1 was about four times as fluorescent, and ΔHDL-huPON1 was about six times as fluorescent. Interestingly, g2e6p-huPON1 had nearly the same fluorescence level as G2E6, consistent with our hypothesis that the increased solubility of G2E6 was essentially entirely due to the subset of residues that became more polar on the surface of PON1. Mutation of the hydrophobic residues in the putative HDL binding site to polar and charged residues (ΔHDL-huPON1) had the greatest effect on the protein solubility, exceeding that of G2E6 by almost 50%.

The removal of the hydrophobic N-terminal leader had the least effect of the three solubilizing concepts (~2.4-fold). However, the effects of the N-terminal deletion were mostly additive with the ΔHDL and g2e6p mutation sets. The fluorescence increased an additional 2-fold for ΔN-g2e6p-huPON1 over g2e6p-huPON1 and an additional ~1.8-fold for ΔN-ΔHDL-huPON1 over ΔHDL-huPON1.

3.3. Expression and Purification of Engineered Variants. We wished to purify the huPON1 solubilized variants to measure their activity and stability, as well as to verify their soluble expression. However, we found that the frGFP fusions were expressed at such low levels that it was inconvenient to work with them. As a result, we recloned the constructs, fusing a hexahistidine tag and maltose-binding protein to the N-terminus of the huPON1 variants. When these variants were purified by NiNTA affinity chromatography, they appeared to copurify with a significant number of smaller proteins at reduced but significant levels (Figure 5). Note that, for the MBP fusions, the amount of soluble protein captured in the purification was greatest for ΔN-ΔHDL-huPON1 and then ΔHDL-huPON1; g2e6p-huPON1 and ΔN-huPON1 were purified in comparable lower amounts, consistent with the screening data. It proved difficult to purify the proteins with increasingly stringent washes, which made us suspect that the other bands on the SDS-PAGE gel were truncation products of the full-length constructs. A blot with the anti-His_6 reagent HisProbe-HRP confirmed that the smaller proteins contained a hexahistidine tag.

These truncation products likely arose from proteolysis due to poor protein stability or inefficient folding of the huPON1 variants. We speculated that moving the $6\times$ His tag to the C-terminus might allow us to capture the full-length proteins. Consequently, we recloned selected constructs into a pET11a vector as fusions with MBP on the N-terminus and a hexahistidine tag on the C-terminus. However, the yields of these full-length proteins were low. We previously observed a similar result with huPON1 and found that

coexpression with DnaK/DnaJ/GrpE chaperones as well as expression at lower temperatures enhanced the production of full-length protein (V. Shete, B. Competty, TJM, manuscript in preparation). When the MBP-PON1-His_6 constructs were expressed in *E. coli* overexpressing the DnaK chaperone system, full-length huPON1 variants could be purified at higher yields with no significant evidence of truncation (Figure 5). A protein the same size as DnaJ was found to copurify with the MBP fusions. We verified that no significant hydrolytic activity above background could be observed from the lysates of cells overexpressing the DnaK chaperones.

Because G2E6 can be purified as a thioredoxin or MBP fusion (not shown) with no evidence of these truncation products, it is likely that all of the huPON1 derivatives fold less well than G2E6.

3.4. Activity and Stability of Engineered Variants. The activity of our engineered PON1 variants against phenyl acetate and the OP compounds paraoxon and EMP was determined and compared with huPON1 and G2E6 (see Figure 6 for structures of the substrates). Because the activity levels were modest and initial trials suggested that the K_m values were likely to be near the top of the concentration range that could be tested with each substrate, specific activities are reported for some substrates (Table 1).

Assuming that 3.3 mM phenyl acetate nearly saturates our PON1 variants, we can calculate a comparable specific activity for huPON1 based on the k_{cat} value we reported previously [5]. The huPON1 used in that study was not an MBP fusion, but if we calculate the specific activity using the mass of the fusion for comparison, we arrive at a specific activity of 690 μmol min^{-1} mg^{-1}. The corresponding value for G2E6 is 820 μmol min^{-1} mg^{-1}. When we expressed huPON1 as an MBP fusion exactly as we did for the huPON1 variants here, the specific activity was 4-fold lower than that reported previously for enzyme purified from 293T cells. All of the variants engineered here had considerably lower activity. The ΔN-huPON1 and g2e6p-huPON1 were about 10-fold lower in activity than huPON1. The ΔHDL-huPON1 variant was about another 20-fold lower than the other two variants. Moreover, the N-terminal deletion reduced the activity of g2e6p-huPON1 by 5-fold, and it approximately halved the already-low activity of the ΔHDL-huPON1. While all three of these methods of solubilization did in fact produce more soluble material, the protein produced had lower specific activity, with the most significant reduction in activity for the ΔHDL-huPON1.

The activity of huPON1 against paraoxon is considerably lower than against phenyl acetate (the corresponding specific activity is 0.3 μmol min^{-1} mg^{-1}), so it is not surprising that we were only able to detect activity against paraoxon with a single variant, the g2e6p-huPON1. The g3e6p-huPON1 activity was about 30-fold below the calculated specific activity for huPON1 expressed in 293T cells, on par with the corresponding reduction in phenyl acetate activity. However, when huPON1 was expressed the same way, the activity against paraoxon was actually 35-fold lower than g2e6p-huPON1 and about 100-fold lower than the 293T-expressed material. The reason for this reduction against paraoxon is not clear. Using EMP, which is an excellent OP substrate for PON1, we also observed that g2e6p-huPON1 was the most active variant, and it was about 2.5-fold more active than ΔN-huPON1 and 100-fold more active than ΔN-g2e6p-huPON1. We could not detect activity against EMP with ΔHDL-huPON1. The activity of g2e6p-huPON1 was also slightly higher than huPON1 with EMP (~1.5-fold), although much less so than with paraoxon.

We were also interested in the effects of each of these solubilizing sets of mutations on the stabilities of the resulting proteins. Because PON1 denatures irreversibly upon heating, thermal inactivation is a good measure of the relative stability of the variants [33]. With phenyl acetate activity as the read-out, the $T_{1/2}$ for huPON1 was roughly 55–60°C (Figure 7). The ΔN-huPON1 was increased slightly in stability, to about 60°C. Both g2e6p-huPON1 and ΔN-g2e6p-huPON1 have $T_{1/2}$ values close to 55°C, and both ΔHDL-huPON1 and ΔN-ΔHDL-huPON1 have $T_{1/2}$ values close to 40°C. A similar experiment using EMP as the substrate produced similar results, with huPON1 showing a midpoint in the inactivation curve around 55°C and ΔN-huPON1 and g2e6p-huPON around 50°C. Overall we conclude that the N-terminal deletion had little effect on the stability, the g2e6p mutations reduced the stability of huPON1 slightly, and the ΔHDL mutations reduced it significantly.

3.5. Humanization of 4E9. We wished to test whether we could produce a solubilized huPON1 with significant activity toward an OP compound, effectively "humanizing" an engineered PON1. The g2e6p-huPON1 variant appeared to afford the best combination of solubility, activity, and stability from among our original variants, so we elected to use those mutations to humanize an engineered PON1. Gupta and colleagues recently reported the engineering of a PON1 variant called 4E9 that has significant activity toward the cyclosarin (GF) analog CMP and notably increased activity against the more toxic S_P enantiomorph of CMP [15]. It also has significant activity against authentic GF, as determined from an AChE inactivation assay in which GF is generated *in situ* at low concentrations. 4E9 is derived from G3C9, with the mutations L69G S111T H115W H134R F222S T332S, all of which are in the presumed active site except for S111T. Therefore, we generated a variant of huPON1 with surface solubilizing mutations derived from G2E6 (akin to the g2e6p-huPON1 described above and in Figure 1) and the 4E9 mutations obtained during directed evolution. The resulting variant, hum-4E9 (Figure 8), has two additional nonpolar-to-polar mutations that we did not elect to make in the g2e6p-huPON1: A126T and V206T. As an additional test of the solubilization afforded by the surface polar mutations, we not only expressed hum-4E9 as an MBP fusion with C-terminal 6 × his tag, but we also produced it with no fusion partner (with a C-terminal 6 × His tag only, which is also present in G3C9 and 4E9). The MBP fusion of hum-4E9 was purified in similar yield to the other MBP fusions of the huPON1 variants and G2E6. The unfused hum-4E9 was produced in about 5-fold lower yield than this and approximately 25-fold lower yield than 4E9 itself.

Table 1: *Aryl esterase and OPase activity of huPON1 variants.* Phenyl acetate and paraoxon specific activities were determined at 3.3 mM and 2.6 mM substrate, respectively. Errors for the specific activities are the standard errors from 3 trials. EMP values were determined at constant 2% methanol, and the errors are with respect to the fit. *n.d.*: not detected. —: k_{cat}/K_m is reported from a linear fit of initial rate versus substrate concentration when the enzyme could not be saturated under experimental conditions.

Variant	Phenyl acetate specific activity μmol min^{-1} mg^{-1}	Paraoxon specific activity μmol min^{-1} mg^{-1}	EMP k_{cat} s^{-1}	EMP K_m mM	EMP k_{cat}/K_m M^{-1} s^{-1}
huPON1	160 ± 20	0.003 ± 0.001	0.027 ± 0.001	0.14 ± 0.01	190 ± 20
ΔN-	17 ± 9	*n.d.*	—	—	117 ± 8
g2e6p-	20 ± 5	0.011 ± 0.002	0.041 ± 0.003	0.14 ± 0.02	300 ± 50
ΔN-g2e6p-	4 ± 1	*n.d.*	—	—	2.2 ± 0.1
ΔHDL-	1.2 ± 0.2	*n.d.*		*n.d.*	
ΔN-ΔHDL-	0.8 ± 0.2	*n.d.*		*n.d.*	

Table 2: *Activity of hum-4E9.* k_{cat}/K_m values were determined under constant 2% methanol conditions for all three substrates. None of the enzymes could be saturated with those substrates under experimental conditions. S_P-CMP specific activities were determined at ~0.0125 mM S_P-CMP. GF k_{app} hydrolysis rate constants and stereoisomeric preferences were determined using GF at 0.315 mM. ND: not determined.

Variant	paraoxon k_{cat}/K_m M^{-1} s^{-1}	EMP k_{cat}/K_m M^{-1} s^{-1}	CMP k_{cat}/K_m M^{-1} s^{-1}	S_P-CMP specific activity nmol min^{-1} mg^{-1}	GF k_{app} M^{-1} s^{-1}	GF $R_P : S_P$ ratio
4E9	3,100 ± 200	24,000 ± 1,000	17,000 ± 3,000	1.1 ± 0.1	ND	ND
hum-4E9	750 ± 20	7,500 ± 600	3,200 ± 300	1.10 ± 0.05	8,700	3.5 : 1
MBP-hum-4E9	230 ± 80	6,800 ± 300	6,000 ± 2000	ND	5,400	6 : 1

3.6. Characterization of Hum-4E9. Not only was hum-4E9 expressed in significant quantities both with and without an MBP fusion tag, but the enzyme was also very active (Table 2). The k_{cat}/K_m for hydrolysis of EMP was 6,800 M^{-1} s^{-1} for the MBP fusion protein and surprisingly was slightly higher (7,500 M^{-1} s^{-1}) for the protein produced without the fusion partner. This represents more than a 20-fold increase over g2e6p-huPON1. We have observed that the activity of G3C9 and 4E9 against EMP is similar (within 2-fold, CKH and TJM, unpublished), suggesting that the increase in EMP activity between g2e6p-huPON1 and hum-4E9 cannot be attributed entirely to the active-site mutations.

The rates of hydrolysis of CMP were also high. For this substrate, the MBP fusion protein had slightly higher activity than the untagged version (6,000 M^{-1} s^{-1} versus 3,200 M^{-1} s^{-1}). The value for the MBP fusion is within 3-fold of the CMP activity of 4E9 measured under identical conditions. There is also significant activity against authentic GF (~6,000 M^{-1} s^{-1}). Tawfik reports that G3C9 has no detectable activity against the S_P isomer of CMP, and we have also observed this (CKH and TJM, unpublished). In the R_P-CMP depletion assay, we found that 4E9 and hum-4E9 had the same level of activity against S_P-CMP, despite the overall lower level of activity for hum-4E9 against CMP. The ratio of the rate constants for hydrolysis of the $R_P : S_P$ isomers of GF was about 4 : 1, suggesting that hum-4E9 displays substantial activity against the more toxic isomer of GF, even though it is still selective for the less toxic isomer.

We also examined the stability of hum-4E9 by thermal inactivation, using EMP as a substrate (Figure 8). The apparent $T_{1/2}$ values were ~50°C for both the MBP fusion and the unfused protein. This level of stability is consistent with that of g2e6p-huPON1.

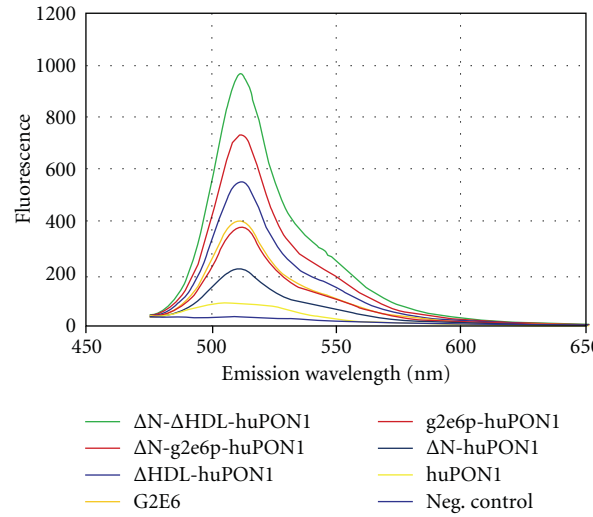

Figure 4: *GFP-fusion screening for solubility.* The fluorescence level is shown for normalized numbers of cells in PBS with frGFP fusions of the PON1 variants. Results from multiple trials typically varied by less than 10%. Negative control cells contained no frGFP fusion. For clarity, the constructs are listed in the legend in the same order from top to bottom as the maxima of the emission spectra.

4. Discussion

There is relatively little known about the effects of mutations on protein solubility or about how to engineer proteins

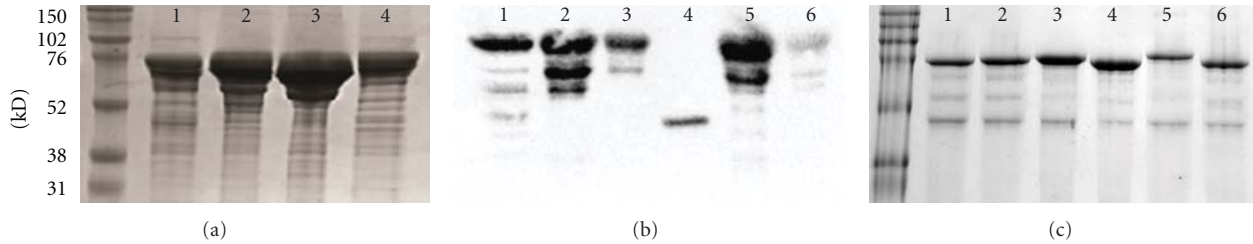

Figure 5: *Purification of huPON1 variants from E. coli.* (a) SDS-PAGE of purification from His_6-MBP-PON1 fusions from the pHMT plasmid using NiNTA chromatography. (1) ΔN-huPON1; (2) ΔHDL-huPON1; (3) ΔN-ΔHDL-huPON1; (4) g2e6p-huPON1. (b) Blot of SDS-PAGE of PON1 variants as His_6-MBP-PON1 fusions with HisProbe-HRP (Pierce), demonstrating that many of the smaller proteins bear the $6\times$His tag and are likely proteolytic fragments. (1) ΔHDL-huPON1 lysate; (2) and (3), purified ΔHDL-huPON1; (4) cleaved MBP; (5) purified g2e6p-huPON1; (6) purified ΔN-huPON1. (c) SDS-PAGE of purified proteins from the MBP-PON1-His_6 constructs in pET11a with coexpression of DnaK/DnaJ/GrpE from pKJE7 (Takara Bioscience). (1) huPON1; (2) ΔN-huPON1; (3) ΔHDL-huPON1; (4) ΔN-ΔHDL-huPON1; (5) g2e6p-huPON1; (6) ΔN-g2e6p-huPON1.

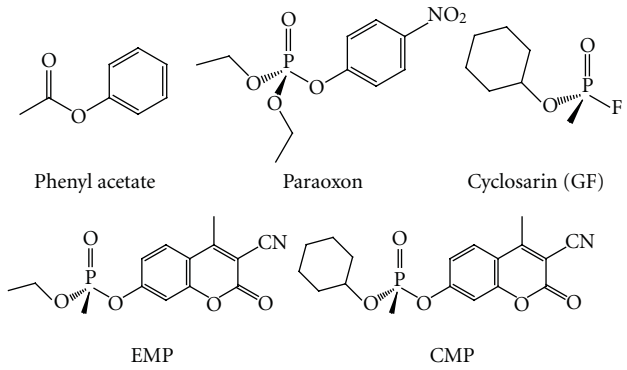

Figure 6: *Structures of the substrates used in this study.* CMP, EMP, and GF are shown as the S_P enantiomorphs.

for increased solubility. This is one reason that Waldo and colleagues invented their GFP-fusion screen, so that directed evolution from random mutagenesis could be used to select for more soluble proteins [24]. It seems intuitively reasonable that increasing the fraction of polar and charged residues on the surface of a protein would increase solubility, but natural proteins generally have the same fraction of polar residues on their surfaces as they do in overall composition, meaning that about half of surface residues are hydrophobic. Mutations to the surfaces of proteins are often naively thought of as neutral, but solubilizing mutations also affect the solubility of the unfolded state. Consequently, they can affect both the stability and folding of proteins. Proteins from thermophiles differ more from their mesophilic counterparts on their surfaces than in factors such as hydrophobic core packing [34].

Here we explored the effects of three different kinds of mutations to PON1, to assess their impact on solubility, activity, and stability. Two of the approaches were rational—we removed the hydrophobic signal sequence from the N-terminus of the proteins, and we solubilized the putative HDL binding site by replacing a large number of closely grouped surface hydrophobic residues with polar and charged amino acids. The third approach was based on the directed evolution of G2E6. We postulated that most of the increased solubility of G2E6 over huPON1 arose from substitution of residues on the surface to more polar amino acids. We constructed the ΔHDL and g2e6p mutants of huPON1, as well as the ΔN mutants of huPON1 and the other two variants. We examined the solubility of the variants using GFP-fusion screening. These fusions were produced at low levels, so we recloned the variants as MBP fusions for higher yield expression and *in vitro* characterization.

All three of the sets of mutations increased the solubility relative to huPON1, but to different degrees. The deletion of the N-terminal signal sequences had the least effect and afforded roughly the same amount of solubilization to the other variants as it did to huPON1. These results suggest that the effects of the different sets of solubilizing mutations can be at least partially additive. The N-terminal deletion also resulted in only a modest decrease in the activity and little change in the stability of huPON1. It is interesting that there is any decrease in activity for this variant (ΔN-huPON1), since the N-terminus is disordered in the crystal and not near the active site of the protein. The ΔHDL mutant afforded the greatest solubilization, but at the highest cost to both activity and stability. While these mutations were a much more radical change to the protein than the N-terminal deletion, it is still somewhat surprising that they have such a profound impact on the activity of the protein. It is known that the activity of PON1, particularly lactonase activity, is stimulated by binding to HDL [20]. While there is no HDL in these preps, either remnants of bacterial cellular lipids (such as lipopolysaccharide) or detergent added to the purification may partially substitute for HDL. It is possible that binding to lipid has either a direct effect on the structure of the active site or that it affects other properties such as enzyme dynamics that cannot be easily recapitulated in mutants that do not bind lipid.

Perhaps the single most important result of this work is the demonstration that the subset of surface mutations that become more polar in G2E6 entirely account for the increased solubility of G2E6 over huPON1. This is potentially a useful approach for minimizing the number of mutations that arise from random mutagenesis and directed

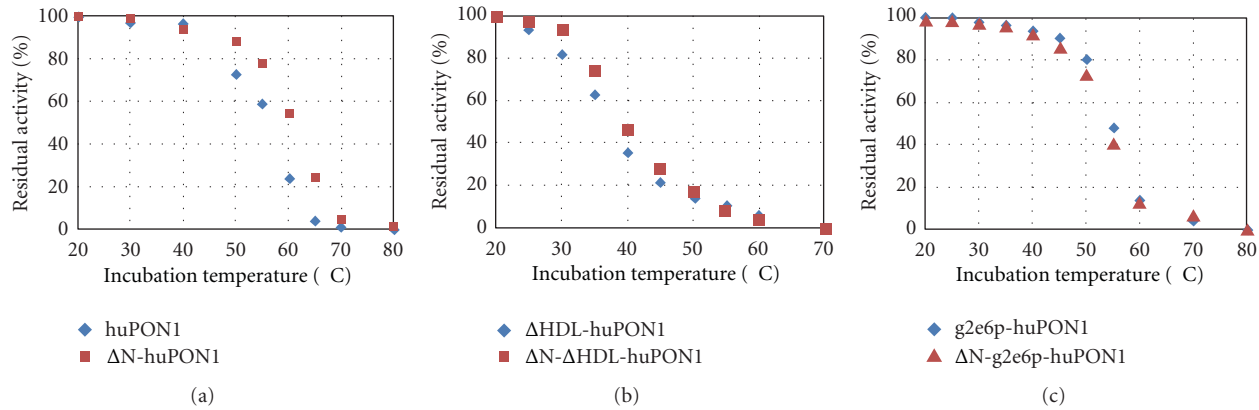

Figure 7: *Stability of huPON1 variants.* The residual activities against phenyl acetate after 10 min of incubation at the indicated temperatures are shown. The residual activity after incubation at 20°C was taken as 100%. (a) huPON1 and ΔN-huPON1; (b) ΔHDL-huPON1 and ΔN-ΔHDL-huPON1; (c) g2e6p-huPON1 and ΔN-g2e6p-huPON1.

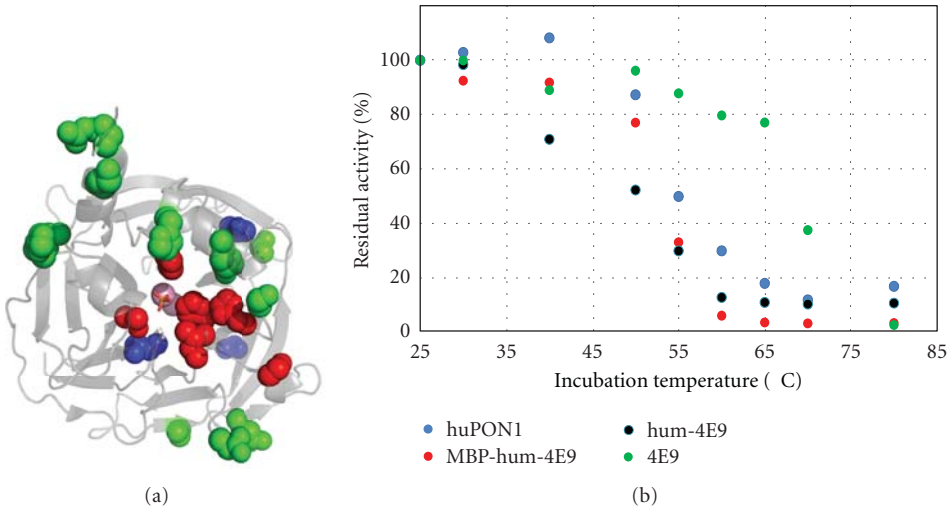

Figure 8: *Humanization of 4E9.* (a) The g2e6p mutations are shown in green spheres; the three additional mutations M55L, A126T, and V206T, are shown in blue spheres; the 4E9 mutations (with respect to G3C9) are shown in red spheres. (b) Thermal inactivation of hum-4E9 produced with and without MBP fusion is compared to huPON1 and 4E9. Residual activity here was from EMP hydrolysis rather than phenyl acetate because the H115W mutation renders PON1 inactive against phenyl acetate.

evolution of other proteins. The g2e6p-huPON1 variant was only slightly destabilized relative to huPON1, and it was reduced in activity by only a small amount. Mutations from huPON1 to G2E6 mostly arose from residues that are found in other mammalian paraoxonases, which likely aided in making them minimally detrimental to the structure and function. Still, we can conclude that some of the other 43 mutations from huPON1 to G2E6 must be important for other parameters, such as the higher activity of G2E6 relative to g2e6p-huPON1.

For huPON1 and all of the soluble variants, we observed that considerable amounts of proteolytic products were purified and that these could be eliminated by coexpression of the DnaK chaperone system. Such products are not observed with thioredoxin or MBP fusions of G2E6, which suggests that one of the major effects of the directed evolution to G2E6 was an increase in the folding rate of the protein. Since the mutations that solubilized the g2e6p-huPON1 were alone not sufficient to eliminate these products, we can conclude that this effect also arises from some of the other 43 residues that change from huPON1 to G2E6. It will be interesting to determine which mutations are responsible for this effect. Overall, our results emphasize that surface mutations, aside from having solubility effects, are often far from neutral on the stability and folding of proteins.

The idea of "humanization" of engineered proteins was conceived in the development of antibody drugs, where it is necessary to raise antibodies in mice or other animals, but a consequence of which is immune response to the constant regions of the heterologous antibodies. It is often possible to engineer molecules with human antibody surfaces but affinity matured binding sites, albeit sometimes with loss of affinity or stability [25]. There are not yet sufficient data to know if mammalian chimeric PON1 variants like

G3C9 and its progeny will elicit immune responses, but it is a concern given the degree of sequence divergence. Using an approach reminiscent of antibody humanization, we implanted the active site of the engineered 4E9 variant into huPON1 and solubilized huPON1 with the surface polar mutations from G2E6. We have shown the activity and the effects of mutations in the huPON1 and G2E6 backgrounds to be highly divergent, so it was not clear that introduction of the 4E9 active-site mutations would afford high CMP and GF activity as observed for 4E9. Interestingly, the engineered variant does exhibit 4E9-like activity, including similar stereochemical preferences for CMP and GF. Our hum-4E9 still differs in a significant number of positions from huPON1 and expresses at lower levels than 4E9, so further engineering may be required to afford an ideal molecule. Nonetheless, the current data present a promising proof of principle that protein solubility can be altered in a controlled and rational way.

Finally, it is of note that two of the mechanisms that we chose to solubilize huPON1 (ΔN and ΔHDL) are likely to reduce or exclude HDL binding, while the g2e6p mechanism is likely to be compatible with it. These mutants, and perhaps related mutants in G3C9, may be useful for examining the role of HDL binding in the function and regulation of PON1. For example, we have found that mutation of a single Trp in the putative HDL binding site of G3C9 dramatically increases the solubility of G3C9 and consequently may reduce its binding to HDL (R. Baldauff and TJM, unpublished). It is unclear what effect untethering PON1 from HDL will have on its physiological function, its serum lifetime, or its recognition by the immune system, but it may help solve the puzzle of exactly what PON1 is doing *in vivo*.

Conflict of Interests

The authors declared that there are no conflicts of interest.

Disclosure

The views expressed in this paper are those of the authors and do not reflect the official policy of the United States Department of Army, Department of Defense, or Government.

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Research Article

Paraoxonase 1 Phenotype and Mass in South Asian versus Caucasian Renal Transplant Recipients

Philip W. Connelly,[1] Graham F. Maguire,[2] Michelle M. Nash,[2] Lindita Rapi,[2] Andrew T. Yan,[3] and G. V. Ramesh Prasad[1]

[1] *Department of Medicine, Keenan Research Centre, Li Ka Shing Knowledge Institute, St. Michael's Hospital, University of Toronto, Toronto, ON, Canada M5B 1W8*
[2] *Keenan Research Centre, Li Ka Shing Knowledge Institute, St. Michael's Hospital, Toronto, ON, Canada M5B 1W8*
[3] *Division of Cardiology, Keenan Research Centre, Li Ka Shing Knowledge Institute, St. Michael's Hospital, University of Toronto, Canada*

Correspondence should be addressed to Philip W. Connelly, connellyp@smh.ca

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South Asian renal transplant recipients have a higher incidence of cardiovascular disease compared with Caucasian renal transplant recipients. We carried out a study to determine whether paraoxonase 1, a novel biomarker for cardiovascular risk, was decreased in South Asian compared with Caucasian renal transplant recipients. Subjects were matched two to one on the basis of age and sex for a total of 129 subjects. Paraoxonase 1 was measured by mass, arylesterase activity, and two-substrate phenotype assay. Comparisons were made by using a matched design. The frequency of PON1 QQ, QR and RR phenotype was 56%, 37%, and 7% for Caucasian subjects versus 35%, 44%, and 21% for South Asian subjects ($\chi^2 = 7.72$, $P = 0.02$). PON1 mass and arylesterase activity were not significantly different between South Asian and Caucasian subjects. PON1 mass was significantly associated with PON1 phenotype ($P = 0.0001$), HDL cholesterol ($P = 0.009$), LDL cholesterol ($P = 0.02$), and diabetes status ($P < 0.05$). Arylesterase activity was only associated with HDL cholesterol ($P = 0.003$). Thus the frequency of the PON1 RR phenotype was higher and that of the QQ phenotype was lower in South Asian versus Caucasian renal transplant recipients. However, ethnicity was not a significant factor as a determinant of PON1 mass or arylesterase activity, with or without analysis including PON1 phenotype. The two-substrate method for determining PON1 phenotype may be of value for future studies of cardiovascular complications in renal transplant recipients.

1. Introduction

Kidney transplant recipients (KTRs) are known to be at increased risk for cardiovascular disease and by three years after transplant about 40% of KTRs have experienced a cardiovascular disease-related event [1]. The incidence of disease is occurring in the context of best medical practice for treatment of established cardiovascular risk factors. Thus there is a need to identify novel risk factors with the objective of improving treatment and reduction of the incidence of cardiovascular disease.

End-stage renal disease, the precursor to KTR, is increasing in individuals of South Asian ethnicity relative to subjects of Caucasian ethnicity [1]. There is a need to characterize these subjects and determine whether novel risk factors for cardiovascular disease are present in an ethnicity-dependent manner. The current study was designed to compare two novel risk factors, adiponectin and paraoxonase 1 (PON1), as candidates for differing risk between South Asian and Caucasian KTR. We observed significantly lower total and high molecular weight adiponectin in South Asian KTR [2]. Stage 3 chronic renal disease, defined as cystatin C-based

estimated glomerular filtration rate (eGFR) less than 60, was associated with lower PON1 mass in subjects with diabetes [3]. In this paper we present the analysis for PON1 and report that PON1 Gln192Arg (Q192R) phenotype is different by ethnicity.

2. Methods

2.1. Patient Selection. Subjects were recruited with age (±10 years) and gender matched in a 1 : 2, South Asian : Caucasian ratio. Inclusion criteria were KTR of stated ethnicity with stable renal function (less than 20% fluctuation of creatinine in the 2 months preceding recruitment), within 3 to 60 months of transplant, and able to provide informed consent. Subjects were excluded if ethnicity was uncertain, if they were multiorgan transplant recipients, if they had unstable renal function, or if they were unable to understand the consent process. Subjects who consented provided a blood sample at the time of clinic visit in the nonfasting state. The study was approved by the Human Research Ethics Board of St. Michael's Hospital. A total of 60 Caucasian males, 30 South Asian males, 26 Caucasian females, and 13 South Asian females were studied, slightly exceeding the study design target sample of 120 subjects. A complete description of the subjects can be found in Prasad et al. [2].

2.2. Measurements. Routine clinical laboratory measurements were performed for standard blood clinical chemistry analytes, including creatinine, glucose, and hemoglobin A1c. Body mass index was determined from weight and height. Clinical records were used for determination of the presence of diabetes according to the 2008 Canadian Diabetes Association guidelines. Lipoproteins were analyzed by ultracentrifugation according to the Lipid Research Clinics protocol. High-density lipoprotein cholesterol was determined by a homogeneous assay on the Beckman LX-20 instrument (Beckman, Mississauga, ON). Cystatin C, apolipoprotein B, and apolipoprotein AI were measured using the Dade-Behring BN Prospec (Dade-Behring, Mississauga, ON).

PON1 mass was measured on serum samples by Western blot with a recombinant standard and calibrated with a normal serum sample [4]. PON1 arylesterase activity was measured using 3 mM phenyl acetate to determine the arylesterase activity and expressed in U/mL. PON1 phenotype was determined using the two-substrate assay of Richter et al. [5] Data for the hydrolysis of 4-(chloromethyl)phenyl acetate (CMPA) was plotted on the x-axis and data for hydrolysis of phenyl acetate in the presence of 2 M NaCl (high salt conditions) was plotted on the y-axis. Data was transformed using the arctangent of the ratio of CMPA/arylesterase activity to derive the radians from the origin. For convenience in presentation, these values were converted to degrees. The activity for purified PON1 Q192 and PON1 R192 was used to confirm identity of the QQ and RR samples. The PON1 phenotypes were assigned as follows: QQ, arctangent range from 11.8 to 17.08 degrees; QR, arctangent range from 20.7 to 34.9 degrees; and RR, arctangent range from 51.3 to 57.4 degrees.

2.3. Statistical Analyses. Analyses were carried using SAS 9.3. Analysis of variance with the matched design was performed using Proc Mixed. A P value < 0.05 was used for statistical testing.

3. Results

Demographic data and biochemical data are summarized in Table 1. Subjects were well matched by age at transplant (50 versus 52 years for South Asian versus Caucasian) and time after transplant (65 versus 71 months for South Asian versus Caucasian). None of the established risk factors were significantly different between South Asian and Caucasian subjects by univariate analysis. PON1 mass and arylesterase activity were similar between the two groups and had a similar distribution as seen by comparison of the 25th and 75th percentiles.

Univariate Spearman correlation was determined for PON1 mass and arylesterase activity by gender. Among women, arylesterase activity correlated with PON1 mass ($r = 0.71$, $P < 0.0001$), HDL cholesterol ($r = 0.45$, $P = 0.004$), and apoAI ($r = 0.35$, $P = 0.03$). Among men, arylesterase activity correlated with PON1 mass ($r = 0.78$, $P < 0.0001$), but not with HDL cholesterol ($r = 0.14$, $P =$ NS) and weakly with apoAI ($r = 0.21$, $P = 0.05$). PON1 mass was not significantly correlated with either HDL cholesterol or apoAI in either gender.

PON1 phenotype was determined as described by Richter et al. [5] using the two-substrate method. We observed clear separation of the results into three phenotypes representing the QQ, QR, and RR phenotypes (Figure 1). The frequency of QQ, QR, and RR was significantly different ($\chi^2 = 7.72$, $P = 0.02$) between South Asian and Caucasian subjects with the RR phenotype being 3 times more prevalent in South Asian subjects (Table 2).

The determinants of PON1 mass were determined with ethnicity and the covariates PON1 phenotype, lipoprotein concentrations, diabetes status, and renal status as covariates. In spite of the difference in PON1 phenotype, ethnicity was not a significant determinant of PON1 mass. In contrast, PON1 phenotype, diabetes status, HDL cholesterol, and LDL cholesterol were all significantly associated with PON1 mass (Table 3).

Next the determinants of serum arylesterase were studied (Table 4). Again, ethnicity was not significantly associated with arylesterase activity. Among the variables tested, only HDL cholesterol was significantly associated with arylesterase activity ($P = 0.003$). When regression analysis was performed including PON1 mass as an independent variable, PON1 mass had a partial r^2 of 0.67 ($P < 0.001$), PON1 phenotype had a partial r^2 of 0.11 ($P < 0.001$), and HDL cholesterol had a partial r^2 of 0.007 ($P = 0.05$). Thus 77% of the variation on arylesterase activity was accounted for by PON1 mass and phenotype and less than 1% was accounted for by HDL cholesterol.

Neither creatinine-based (MDRD) nor cystatin C-based (Arnal-Dade) estimates of glomerular filtration rate (eGFR) showed a relationship with PON1 mass or arylesterase activity (data not shown).

Table 1

Parameter	All patients		P-value
	South Asian ($N = 43$)	Caucasian ($N = 86$)	
Age at transplant (years)	48 ± 11	50 ± 11	NS
Age at study visit (years)	50 ± 10	52 ± 11	NS
Gender (M/F)	30/13	60/26	NS
Time post transplant (months)	65 ± 47	71 ± 48	NS
BMI (kg/m^2)	26.9 ± 4.1	28.0 ± 6.0	NS
Total cholesterol (mmol/L)	4.8 ± 1.6	4.4 ± 1.1	NS
HDL cholesterol (mmol/L)	1.1 ± 0.4	1.1 ± 0.3	NS
LDL cholesterol (mmol/L)	3.1 ± 1.2	2.7 ± 0.9	0.07
Triglycerides (mmol/L)	1.8 ± 0.9	1.6 ± 0.7	NS
VLDL cholesterol	0.5 ± 0.4	0.5 ± 0.3	NS
VLDL triglycerides	1.3 ± 0.8	1.2 ± 0.6	NS
ApoAI (mg/L)	1.4 ± 0.3	1.4 ± 0.3	NS
ApoB (mg/L)	0.9 ± 0.3	0.8 ± 0.2	NS
eGFR by MDRD (mL/min/1.73 m^2)	66 ± 21	60 ± 19	NS
Cystatin C (mg/L)	1.2 ± 0.4	1.2 ± 0.3	NS
eGFR by Cystatin C (mL/min/1.73 m^2)	65 ± 22	62 ± 20	NS
C-reactive protein (mg/L)	4.5 ± 7.9	6.0 ± 13	NS
PON1 (μg/mL) median (quartiles)	100 (84, 122)	105 (81.7, 127)	NS
PON1 arylesterase (U/mL) median (quartiles)	79.5 (72.7, 90.4)	81.4 (72.2, 91.8)	NS
PON1 CMPA activity (U/mL) median (quartiles)	19.4 (15.3, 26.8)	16.5 (13.6, 22.8)	0.08
PON1 arylesterase activity salt stimulated (U/mL) median (quartiles)	45.4 (35.0, 54.7)	47.8 (37.3, 56.7)	NS

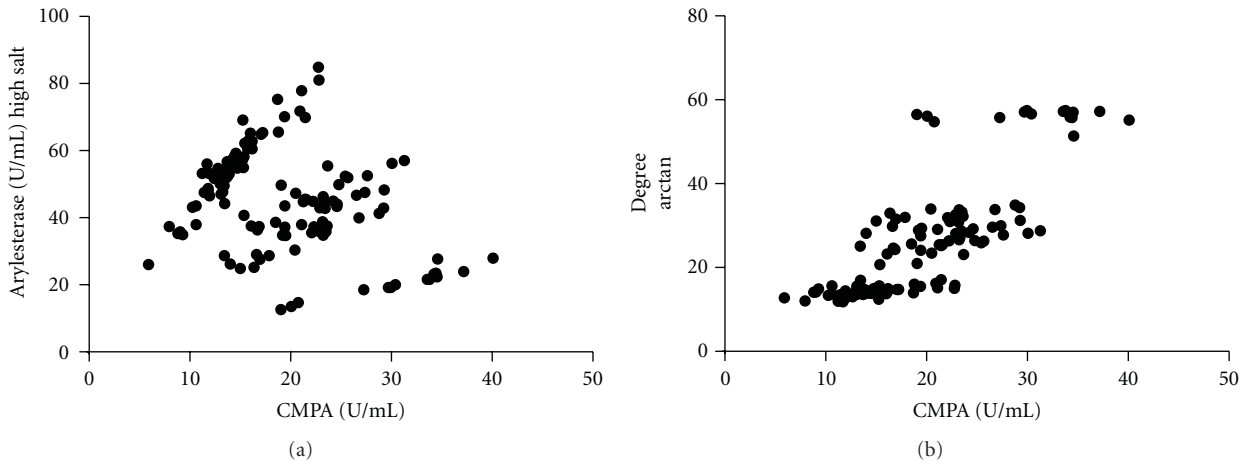

Figure 1: (a) Activity measured using phenyl acetate at high salt conditions versus CMPA. (b) Transformation of activities using arctangent $(x/y) * 180/3.14$ versus CMPA. This transformation converts x and y coordinates into the angle in degrees.

4. Discussion

PON1 has been extensively studied as a risk factor for cardiovascular disease independent of lipoprotein concentrations [6]. Differences in PON1 between subjects have been shown to be due to genetic polymorphisms [7, 8], presence of diabetes [9], presence of hepatic disease [10], and presence of renal disease [3, 11–15]. The complexity of the genetic polymorphism at the PON1 locus may confound studies of PON1 and thus it has been suggested that determination of the PON1 phenotype, mass, and activity be measured [5, 16]. We report that the phenotypic prevalence of the QR

Table 2: Frequency of PON1 phenotypes by ethnicity.

Ethnicity	PON1 QQ (n, %)	PON1 QR (n, %)	PON1 RR (n, %)
Caucasian	48 (55.8%)	32 (37.2%)	6 (7%)
South Asian	15 (34.9%)	19 (44.2%)	9 (20.9%)

Table 3: Mixed model ANOVA analysis with PON1 mass as the dependent variable.

Variable	P value
Ethnicity	NS
PON1 phenotype	0.0001
Diabetes	<0.05
HDL cholesterol	0.009
LDL cholesterol	0.02

Table 4: Mixed model ANOVA analysis with arylesterase activity as the dependent variable.

Variable	P value
Ethnicity	NS
PON1 phenotype	NS
Diabetes	NS
HDL cholesterol	0.003
LDL cholesterol	NS

and RR phenotype is higher in South Asian compared with Caucasian KTR subjects. The frequency of the phenotypes in Caucasians is similar to that reported for renal transplant patients in Debrecen, Hungary [17, 18]. The frequency of the phenotypes in South Asians is consistent with recently published data on the frequency of the of Gln192Arg polymorphism in North-West Indian Punjabis [6]. It is also similar to that reported for patients with CAD or T2DM from Hyderabad, India [19]. Interestingly, the frequency of the RR was 11.7% in control subjects from Hyderabad, similar to the frequency we observed for Caucasians.

In contrast to the difference in phenotype by ethnicity, there was no difference in the mass or arylesterase activity of PON1 by ethnicity in KTR. Further, there was a difference in PON1 mass, but not arylesterase activity by diabetes status. Analysis of indices of diabetes (glucose, hemoglobin A1c) did not show significant associations with PON1 mass or arylesterase activity (data not shown). Post hoc analyses for an interaction between ethnicity and diabetes status were also negative. Thus, in this group of KTR subjects, diabetes was a limited determinant of PON1 status. Similarly, an a priori hypothesis was that renal function would be a determinant of PON1. However, neither creatinine nor cystatin C-based estimates of glomerular filtration rate were associated with PON1. This may be due to other factors in KTR being primary determinants of PON1. It should also be noted that the study design was to restrict subjects to a range of post renal transplant from 3 to 60 months. Thus although most of the subjects with diabetes had pre-transplant diabetes, the duration of posttransplant factors was limited to the relatively early time points. Most previous studies of non-KTR have been of subjects with an average duration of diabetes greater than 15 years [20, 21]. Thus one explanation for the absence of associations with diabetes and eGFR could be the shorter duration after transplant in this study.

PON1 mass, but not arylesterase activity, has been reported to be inversely correlated with mortality in patients on dialysis [22]. In contrast, PON1 genotypes have not been associated with increased cardiovascular risk in renal transplant recipients [23]. Future studies will require measurement of PON1 phenotype by enzymatic activity and PON1 concentration in order to fully evaluate the predictive value of PON1 as a cardiovascular risk factor in kidney transplant recipients.

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Research Article

Increased Levels of Human Carotid Lesion Linoleic Acid Hydroperoxide in Symptomatic and Asymptomatic Patients Is Inversely Correlated with Serum HDL and Paraoxonase 1 Activity

Elad Cohen,[1,2] Michael Aviram,[2] Soliman Khatib,[1] Asaf Rabin,[3] Dalit Mannheim,[3] Ron Karmeli,[3] and Jacob Vaya[1]

[1] *Oxidative Stress Research Laboratory, Migal-Galilee Technology Center and Tel Hai College, P.O. Box 831, Kiryat Shmona 11016, Israel*
[2] *Rappaport Family Institute for Research in the Medical Sciences, Rambam Medical Center, Haifa 31096, Israel*
[3] *Department of Vascular Surgery, Carmel Medical Center, Haifa, Israel*

Correspondence should be addressed to Jacob Vaya, vaya@migal.org.il

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Human carotid plaque components interact directly with circulating blood elements and thus they might affect each other. We determined plaque paraoxonase1 (PON1) hydrolytic-catalytic activity and compared plaque and blood levels of lipids, HDL, PON1, and HbA1c, as well as plaque-oxidized lipids in symptomatic and asymptomatic patients. Human carotid plaques were obtained from symptomatic and asymptomatic patients undergoing routine endarterectomy, and the lesions were ground and extracted for PON activity and lipid content determinations. Plaque PONs preserved paraoxonase, arylesterase, and lactonase activities. The PON1-specific inhibitor 2-hydroxyquinoline almost completely inhibited paraoxonase and lactonase activities, while only moderately inhibiting arylesterase activity. Oxysterol and triglyceride levels in plaques from symptomatic and asymptomatic patients did not differ significantly, but plaques from symptomatic patients had significantly higher (135%) linoleic acid hydroperoxide (LA-13OOH) levels. Their serum PON1 activity, cholesterol and triglyceride levels did not differ significantly, but symptomatic patients had significantly lower (28%) serum HDL levels and higher (18%) HbA1c levels. Thus LA-13OOH, a major atherogenic plaque element, showed significant negative correlations with serum PON1 activity and HDL levels, and a positive correlation with the prodiabetic atherogenic HbA1c. Plaque PON1 retains its activity and may decrease plaque atherogenicity by reducing specific oxidized lipids (e.g., LA-13OOH). The inverse correlation between plaque LA-13OOH level and serum HDL level and PON1 activity suggests a role for serum HDL and PON1 in LA-13OOH accumulation.

1. Introduction

The human atherosclerotic plaque is characterized by increased levels of oxidized lipoproteins, such as LDL, HDL, phospholipids, triglycerides [1, 2], oxidized cholesterol products (oxysterols) [3], FFAs, and fatty acid derivatives [4], as well as proteins such as fibrinogen, apolipoprotein A-I (apoA-I), clusterin, and paraoxonase (PON) [5, 6]. Accumulating cholesterol in the plaque tends to precipitate and crystallize, forming sharp edges with increased volume leading to rupture-prone fibrous caps [7]. These "vulnerable plaques" have several features that differentiate them from their nonvulnerable counterparts: vulnerable plaques are less stable, characterized by a large lipid core, thin fibrous cap and less collagen, intraplaque hemorrhaging and infiltration of inflammatory cells without calcification [8]. These unstable plaques are associated with increased ischemic events, and about 20% of ischemic strokes are attributed to carotid artery atherosclerosis. Differentiating asymptomatic from symptomatic patients is important in determining the appropriate treatment. Major serum biomarkers of plaque vulnerability are related to inflammatory and proteolytic markers, yet

no biomarkers exist for regular clinical use to indicate high risk of neurological events and to select patients for carotid surgery [8].

Mammalian PONs (PON1, PON2, and PON3) are a unique family of calcium-dependent esterases/lactonases. Many of the antiatherogenic properties of HDL are attributed to PON1. This enzyme reduces macrophages' cellular oxidative stress, decreases cholesterol-biosynthesis rate, and stimulates HDL-mediated macrophage cholesterol efflux, thus protecting against foam-cell formation and atherogenesis [9, 10]. PON1-deficient mice are susceptible to the development of atherosclerosis [11], whereas overexpression of human PON1 in mice inhibits atherosclerosis development [12]. Epidemiological evidence demonstrates that low PON1 activity is associated with increased risk of cardiovascular disease [13]. HDL-PON1 antioxidant activity has been correlated with carotid intima-media thickness [14]. The hydrolytic lactonase, arylesterase, and paraoxonase activities of PON1 are all inactivated under oxidative stress [15]. Immunohistochemical analysis has shown accumulation of PON1 in the human lesion as it progresses from fatty streak to advanced lesion [5, 16].

PON2 is expressed in most tissues, including macrophages [5]. Like PON1, PON2 has also been shown to protect vascular cells from oxidative stress, decrease triglyceride accumulation in macrophages [10, 17, 18], play a role against inflammation, and exhibit high acylhomoserine lactone hydrolysis. PON3, like PON1, is associated with HDL but does not exhibit paraoxonase activity and is 200 times less abundant [19].

We recently reported that lipids derived from carotid atherosclerotic plaque (lesion lipids extract—LLE) can accelerate macrophage and lipoprotein oxidation and possess atherogenic properties [20], with the formation of macrophages with foam cell-like appearance [21]. Incubation of recombinant PON1 (rePON1) with LLE reduces lipid carotid plaque atherogenicity [20, 21] but at the same time, linoleic acid hydroperoxide (LA-13OOH) present in the LLE inhibits rePON1 paraoxonase and lactonase activities via reaction of LA-13OOH with the enzyme's cysteine at position 284 (Cys284) [22]. These dual effects between plaque constituents and the elements that are in contact with them, such as blood components (lipids, proteins) circulating via the lesion, led us to further investigate (a) if PONs present in the human carotid plaque [16, 23] are still active and (b) if differences between symptomatic and asymptomatic patients occur within the plaque constituents and between plaque and blood elements.

2. Materials and Methods

2.1. Materials. N,O-bis(trimethylsilyl) acetamide (BSA), cholesterol, 2-hydroxyquinoline (2HQ), diethyl p-nitrophenyl phosphate (paraoxon), and 4-nitrophenyl acetate were purchased from Sigma-Aldrich. 5-(Thiobutyl)butyrolactone (TBBL) was synthesized in our laboratory by a previously described method [24]. 7α-Hydroxycholesterol, 7β-hydroxycholesterol, β-epoxycholesterol, α-epoxycholesterol, 26-hydroxycholesterol, and 7-ketocholesterol were purchased from Steraloids Inc. (Wilton, NH). Recombinant PON1 from *Escherichia coli* was purchased from the Structural Proteomics Center, Weizmann Institute of Science (Rehovot, Israel).

2.2. Carotid Plaques. Human carotid plaques were taken from patients undergoing routine endarterectomy in the Department of Vascular Surgery in Carmel Hospital (Haifa, Israel). Both symptomatic and asymptomatic patients underwent surgery under local anesthesia. Patients were considered symptomatic if they had experienced stroke, transient ischemic attack, or amaurosis fugax ipsilateral to the carotid lesion being studied. Complete atherosclerotic plaques were removed, including the common internal and external carotid sections and were immediately placed in saline and kept at -80°C. All plaques were approved for research by the Helsinki Committee regulations, with patient consent (Helsinki approval number 3071). Lesion samples were laid on filter paper to absorb the liquid and then weighed and ground to a powder under liquid nitrogen. The powder was extracted at 4°C for 30 min with PON activity buffer (1 mM $CaCl_2$ in 50 mM Tris-HCl, pH 8.0) with 0.1% (v/v) Protease Inhibitor Cocktail and 1 mM PMSF (1 mL for 400 mg tissue), and centrifuged at 10,000 g for 10 min at 4°C. The supernatant was removed and used for determination of protein levels by DC protein assay (Bio-Rad) and for PON activities. Precipitate was ground again under liquid nitrogen and extracted with ethyl acetate. Ethyl acetate was evaporated, and the LLE was dissolved in DMSO to a final concentration of 50 mg/mL and used for detection of lipids and oxidized lipids by liquid chromatography-mass spectrometry (LC-MS) or gas chromatography (GC-MS).

2.3. Recombinant PON1 (rePON1). rePON1 was generated in *E. coli* by directed evolution as described previously [25]. PON1 storage buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 1 mM $CaCl_2$, and 0.1% v/v tergitol) was supplemented with 0.02% (w/v) sodium azide and stored at 4°C.

2.4. Lactonase Activity. Protein (40 μg) from the plaque homogenate, 0.1 μg/mL rePON1, or 1 : 20 diluted serum was taken for a total reaction volume of 200 μL. Lactonase activity was measured using TBBL as the substrate [24]. Initial rates of hydrolysis were determined spectrophotometrically at 405 nm. The assay mixture included 1 mM TBBL and 1 mM $CaCl_2$ in 50 mM Tris-HCl, pH 8.0. Nonenzymatic hydrolysis of TBBL was subtracted from the total rate of hydrolysis. One unit of lactonase activity was equal to 1 μmol of TBBL hydrolyzed/min mL.

2.5. Paraoxonase Activity. Protein (100 μg) from the plaque homogenate or 0.2 μg/mL rePON1 was taken for a total reaction volume of 200 μL. Paraoxonase activity was measured using paraoxon as the substrate. Initial rates of hydrolysis were determined spectrophotometrically at 405 nm. The basal assay mixture included 2 mM paraoxon and 1 mM

$CaCl_2$ in 50 mM glycine/NaOH buffer, pH 10.5. Nonenzymatic hydrolysis of paraoxon was subtracted from the total rate of hydrolysis. One unit of PON1 paraoxonase activity was equal to 1 nmol of paraoxon hydrolyzed/min mL [26].

2.6. Arylesterase Activity. Protein (60 μg) from the plaque homogenate or 0.1 μg/mL rePON1 was taken for a total reaction volume of 200 μL. Arylesterase activity was measured using 4-nitrophenyl acetate as the substrate. Initial rates of hydrolysis were determined spectrophotometrically at 405 nm. The assay mixture included 3 mM 4-nitrophenyl acetate and 1 mM $CaCl_2$ in 50 mM Tris-HCl, pH 8.0. Nonenzymatic hydrolysis of 4-nitrophenyl acetate was subtracted from the total rate of hydrolysis. One unit of arylesterase activity was equal to 1 μmol of 4-nitrophenyl acetate hydrolyzed/min mL.

2.7. Plaque Triglyceride Mass. Plaque triglyceride mass was determined using the Serum Triglyceride Kit from Sigma (Rehovot, Israel). In short, plaque lipids were extracted in ethyl acetate and dried under a nitrogen stream. Dry samples were dissolved in DMSO (50 mg/mL), and 50 μL was added to 1 mL of glycerol reagent and 4 mL triglyceride reagent, vortexed and incubated for 10 min at 37°C. Triglyceride levels were determined at 540 nm according to a glycerol standard.

2.8. Oxysterol Detection by GC-MS. Samples were first subjected to hydrolysis to convert all sterol esters into their free form. The dry residue of the extracted sample was dissolved in 0.5 mL KOH solution (20% KOH in a mixture of MeOH : DDW 70 : 30) and mixed for 3 h at 21°C. Two volumes of diethyl ether were added and the pH was adjusted to 5 with 0.5 mL of citric acid (20% in DDW). The upper organic phase was removed and the liquid phase was extracted with another portion of 2 mL diethyl ether. The organic layers were combined, treated with sodium sulfate, and evaporated to dryness under nitrogen purge. Dried extracts were subjected to the silylating reagent BSA dissolved in 1,4-dioxane (dried on 4 Å molecular sieves and passed through aluminum oxide) and heated to 80°C for 60 min. Oxysterol was detected as previously described [3]. Briefly, standards or dried extracts were subjected to 200 μL BSA, followed by the addition of 300 μL 1,4-dioxane (treated as before) and heated to 80°C for 60 min. Samples were detected by GC-MS in a total ion monitoring (TIM) mode, and 2–4 of the most representative ions were selected for reinjection in single ion monitoring (SIM) mode. The mean quantity of each oxysterol was calculated from calibration curves of its standard.

2.9. Analyzing FFAs and Oxidized FFAs by LC-MS/MS. FFAs were quantified by LC-MS/HPLC (Waters 2790) connected to an MS (Micromass Quattro UltimaMS, UK). The HPLC column was a 3.5 mm C18 ODS XTerra column (Waters). MS analysis of the FFAs was performed in SIM mode, using electron spray negative ions. MS/MS analysis of the oxidized products was performed in scan and daughter modes, using ES^-. The source temperature of the MS was set at 120°C, with a cone gas flow of 22 L/h and a desolvation gas flow of 400 L/h. Peak spectra were monitored between *m/z* 50 and 350. Collision-induced dissociation MS was performed, with a collision energy of 20 eV and 3–3.5 kV capillary voltage. Multiple-reaction monitoring was performed under the same conditions used to quantify the oxidized products.

2.10. Statistical Analysis. Statistical analysis was performed using the Student paired or heteroscedastic *t*-test when comparing the means of two groups. Linear regression was calculated using GraphPad Prism 4 software. Each experiment was repeated, separately, at least three times ($n \geq 3$). Results are presented as mean ± SEM. Clinical parameters were analyzed using GraphPad Prism 4 software by Contingency table with chi-square test.

3. Results

3.1. Plaque PON Activity. Immunohistochemical analysis has recently revealed the presence of PON1, PON3 [16], and PON2 [23] in carotid lesions. However, such analyses do not indicate whether the enzymes in the plaque still possess hydrolytic activity. As part of our ongoing research into the chemical composition of the human carotid plaque, the mechanism by which plaque components interrelate, and their possible dual effects with blood components, we tested whether plaque PONs are still active. Human carotid lesions were ground to a powder under liquid nitrogen. The powder was extracted with Tris buffer and then centrifuged (see Section 2). The supernatant was used for determination of PON paraoxonase, lactonase and arylesterase activities, with or without the addition of the PON1 inhibitor 2HQ. All three PON activities were preserved in the plaque homogenate, and 2HQ almost completely inhibited paraoxonase and lactonase activities (from 2.9 to 0.014 and from 0.115 to 0.008 U/mg protein, resp., Figures 1(a) and 1(b)), whereas it only slightly decreased arylesterase activity (from 3.3 to 2.5 U/mg protein) (Figure 1(c)). The effects of 2HQ on PON's hydrolytic properties were reexamined with rePON1. 2HQ inhibited rePON1 paraoxonase, lactonase, and arylesterase activities almost completely (from 9006 ± 161 to 1819 ± 30, 365 ± 6 to 65.6 ± 0.7, and 118 ± 14 to 9.9 ± 2.8 U/mg rePON1 protein, resp.) (Figures 1(d)–1(f)). These results indicated that PONs that are present in the plaques preserve all three of their hydrolytic activities.

3.2. Symptomatic versus Asymptomatic Patients. Atherosclerotic patients have been shown to have less PON1 activity in their blood than healthy subjects [27]. Since symptomatic patients have some features of vulnerable plaques, their plaques are considered to be more severe than those of asymptomatic patients [7, 28, 29]. In the present study, clinical parameters were compared between patients with hypertension, hyperlipidemia, or being treated with statins or antihypertensive drugs: no significant differences were found between symptomatic and asymptomatic patients in these criteria. The symptomatic group included more

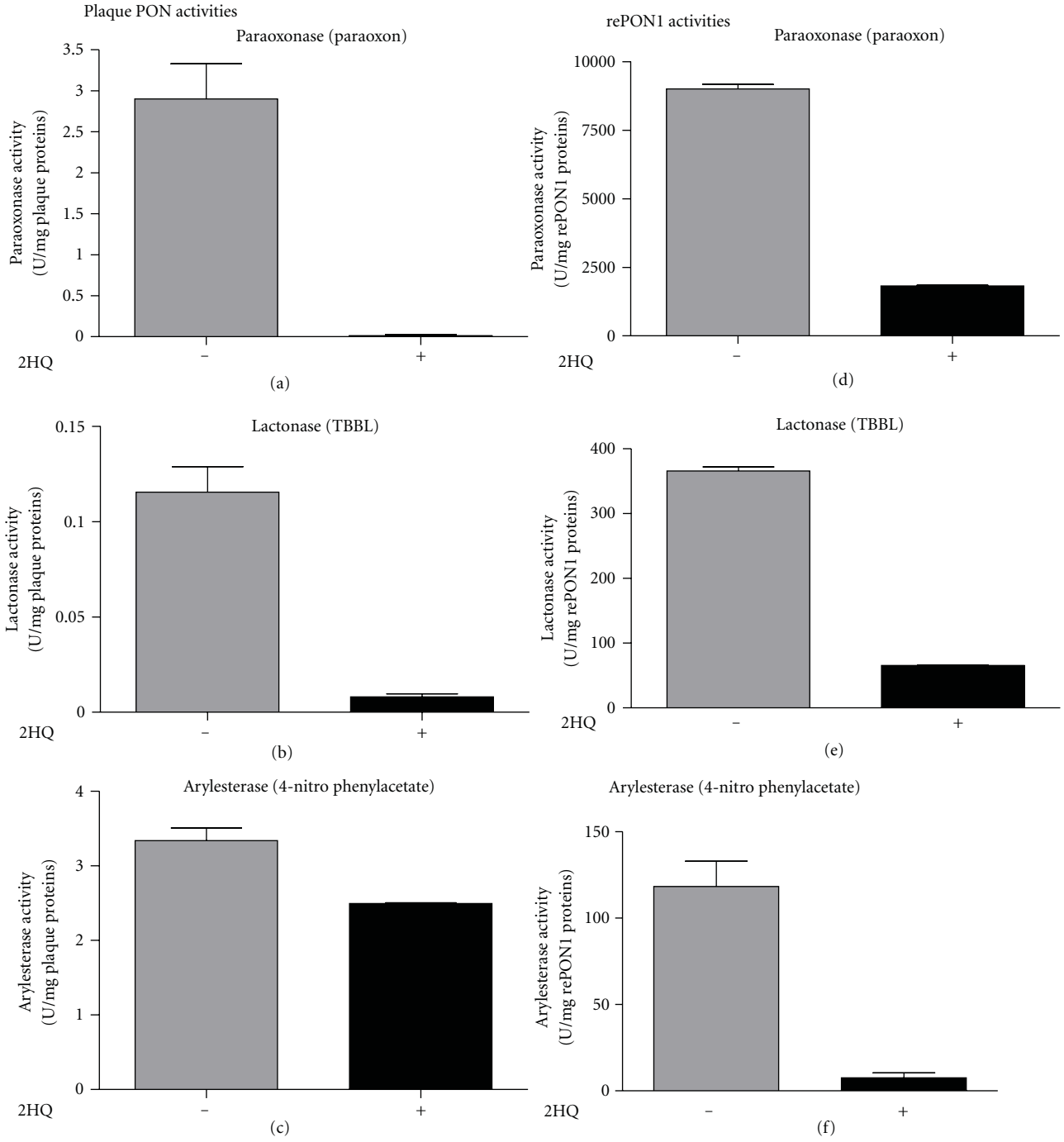

Figure 1: Hydrolytic activities of paraoxonases (PONs) in human carotid plaque homogenate and of recombinant PON1 (rePON1). (a): Homogenate paraoxonase (paraoxon), (b): lactonase (TBBL), and (c): arylesterase (4-nitrophenyl acetate) activities; (d): rePON1 paraoxonase, (e): lactonase, and (f): arylesterase activities, with or without 2-hydroxyquinoline (2HQ).

diabetic subjects than the asymptomatic group (Table 1). Differences in plaque PON activity between symptomatic and asymptomatic patients were assessed. Surprisingly, there was no significant difference in plaque lactonase activity between the two groups (Table 2). Serum PON1 activity, as expected, was lower (15%) in the symptomatic patients than in the asymptomatic patients, but the difference was not significant ($P = 0.14$) (Table 2). Atherogenic components were then compared between symptomatic and asymptomatic patients, including levels of triglyceride, oxysterol, and LA-13OOH in the plaque lipid extract, and levels of lipoproteins and hemoglobin A1c (HbA1c) in individual patients' blood. The levels of various lipids and oxidized lipids in the plaque extracts were determined by MS. No

Table 1: Symptomatic and asymptomatic clinical parameters.

	Symptomatic	Asymptomatic	*P* value
Age (y)	68 ± 1.6	72 ± 2.5	0.2
Hypertension	5/6 (83%)	10/13 (77%)	0.75
Hyperlipidemia	4/6 (67%)	10/13 (77%)	0.64
Treated with statins	5/6 (83%)	11/13 (85%)	0.94
Treated with antihypertensive drugs	4/6 (67%)	9/13 (69%)	0.91
Diabetes	3/6 (50%)	1/13 (8%)	0.035

Table 2: Atherogenic and antiatherogenic elements in the plaques and blood of symptomatic and asymptomatic patients.

		Symptomatic ($n = 6$)	Asymptomatic ($n = 13$)	*P* value
Plaque	PON lactonase activity (U/mg proteins)	0.16 ± 0.035 ($n = 8$)	0.18 ± 0.054 ($n = 9$)	N.S.
	Triglyceride (% of LLE)	3.4 ± 1.07	2.37 ± 0.37	N.S.
	7-keto (% of cholesterol)	0.38 ± 0.19	0.25 ± 0.05	N.S.
	26-OH (% of cholesterol)	1.16 ± 0.27	1.29 ± 0.19	N.S.
	7α-OH (% of cholesterol)	0.087 ± 0.015	0.085 ± 0.01	N.S.
	7β-OH (% of cholesterol)	0.075 ± 0.01	0.08 ± 0.009	N.S.
	β-epoxy (% of cholesterol)	0.096 ± 0.01	0.114 ± 0.02	N.S.
	α-epoxy (% of cholesterol)	0.055 ± 0.004	0.069 ± 0.008	N.S.
	LA-13OOH (μg/mg plaque)	0.012 ± 0.002	0.0053 ± 0.0009	0.019
Serum	PON1 lactonase activity (U/mL)	38 ± 3.2	45 ± 2.9	N.S.
	HbA1c (%)	7.17 ± 0.48	6.04 ± 0.15	0.065
	HDL (mg/dL)	37.8 ± 3.35	51.7 ± 3.06	0.009
	LDL (mg/dL)	79 ± 13.2	90.9 ± 4.4	N.S.
	Cholesterol (mg/dL)	158.6 ± 11.2	168.08 ± 6.4	N.S.
	Triglyceride (mg/dL)	164.8 ± 27	127.2 ± 15.2	N.S.

LLE: lesion lipid extract. Results are presented as mean ± SEM.

significant differences were found in the amounts of detected oxysterols (5,6-α- and β-epoxy cholesterol, 7α-OH and 7β-OH cholesterol, 7-keto cholesterol, and 26-OH cholesterol) or triglycerides. In contrast, symptomatic patients had significantly higher (135%) amounts of LA-13OOH in their plaques than asymptomatic patients (Table 2). In addition, while there was no significant difference in the amounts of total cholesterol, LDL cholesterol, or triglyceride in the blood, symptomatic patients had significantly less (28%) HDL cholesterol than the asymptomatic group (Table 2). Symptomatic patients also had more (18%) HbA1c in their blood than asymptomatic patients, although this difference was only marginally significant (Table 2).

3.3. Correlations between Plaque LA-13OOH and Elements in the Blood of the Same Individual. Previous studies from our group have shown that LA-13OOH (with the hydroperoxide at position 13 of linoleic acid) inhibits PON1 by specific interaction with the enzyme's Cys284. Thus, LA-13OOH is considered an atherogenic factor in the plaque which can augment oxidative stress and progression of atherosclerosis [22]. This led us to correlate LA-13OOH level in the plaque with other atherogenic and antiatherogenic elements in the blood of the same patient, to assess the possible correlation between plaque status and a specific component in the blood. Results showed that plaque LA-13OOH level is indeed inversely correlated with two antiatherogenic elements in the blood: serum PON1 lactonase activity ($R^2 = 0.35$, $P = 0.01$, Figure 2(a)) and HDL cholesterol ($R^2 = 0.3$, $P = 0.027$, Figure 2(b)). In addition, a direct correlation was found between the two atherogenic elements, LA-13OOH and HbA1c ($R^2 = 0.27$, $P = 0.038$, Figure 2(c)).

4. Discussion

Atherogenesis is accompanied by the accumulation of oxidized lipids in the arterial wall. The antioxidant enzyme PON1 lowers lipid peroxide levels [30], protects arterial cell walls (as well as endothelial cells, smooth muscle cells, and macrophages) and lipoproteins (LDL and HDL) from oxidation, inhibits oxidized LDL uptake by macrophages [9, 10], and shows anti-inflammatory activities [31, 32]. Thus, the presence of PON1, PON2, and PON3 in the human plaque may have an important role in decreasing atherosclerotic progression. Immunohistochemical analysis of human plaque has revealed the presence of PONs in the lesion; during lesion progression, there is a shift of PON1 and PON3 from smooth muscle cells to macrophages [16],

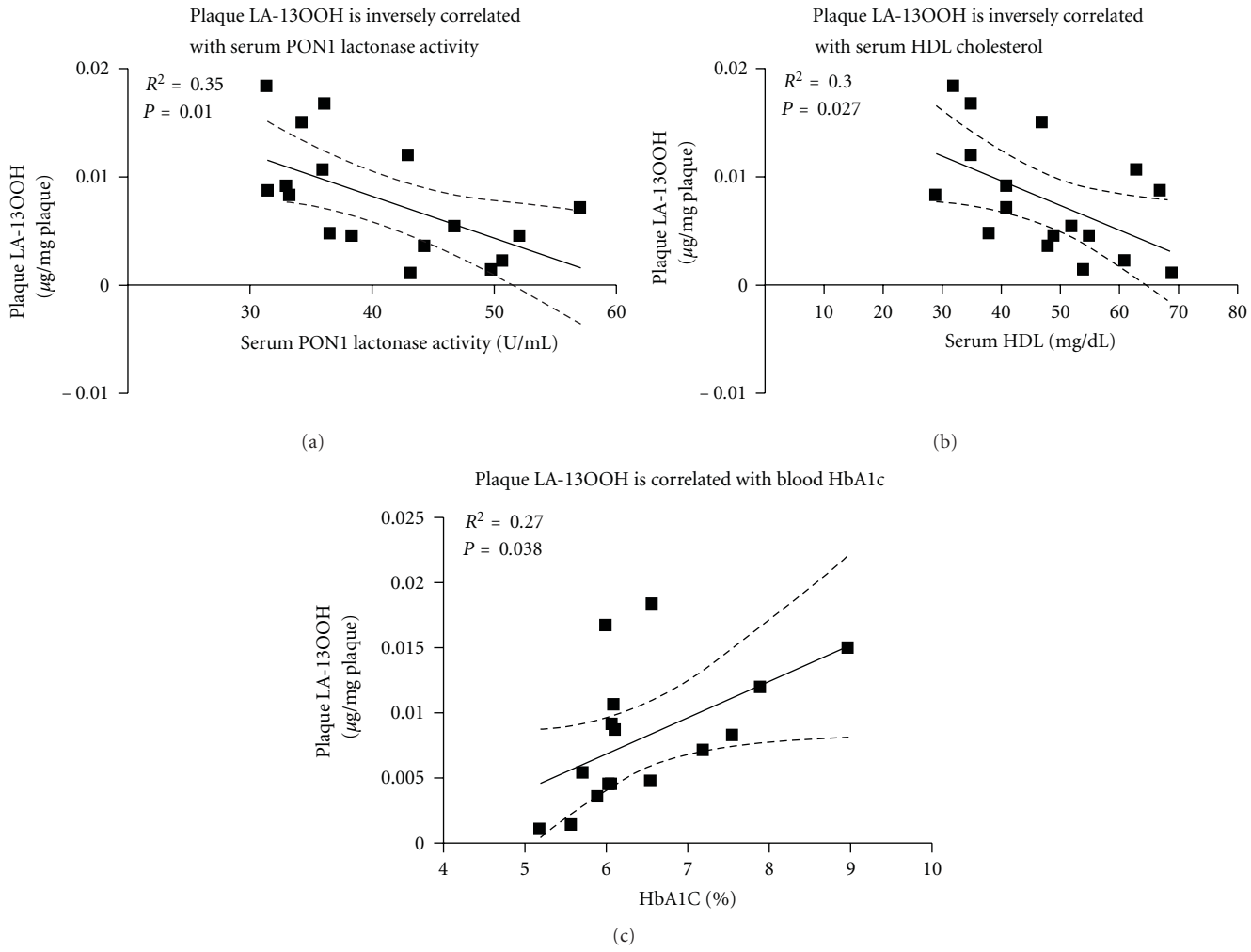

Figure 2: Human plaque linoleic acid hydroperoxide (LA-13OOH) level versus serum HDL, serum PON1 activity and blood hemoglobin (Hb) A1c. Human plaque LA-13OOH is inversely correlated with (a): serum PON1 lactonase activity and (b): serum HDL, but (c): positively correlated with blood HbA1c.

whereas arterial PON2 level decreases [23]. Hence, it is important to assess whether the PONs identified immunohistochemically in the plaque are still active. Our current results show that the PONs are indeed still active in the plaque homogenate, preserving their catalytic paraoxonase, arylesterase, and lactonase activities. Among these activities, only PON1 can hydrolyze paraoxon, hence we concluded that human carotid lesion has active PON1. PON1 may thus act as a potent reducing antioxidant enzyme not only in the serum, but also within the plaque, leading to attenuated atherosclerotic progression. In addition, while 2HQ inhibited rePON1 arylesterase almost completely but only slightly decreased arylesterase in the homogenate, we can conclude that in the latter, other esterases are present and PON's contribution is only minor.

A distinction between symptomatic and asymptomatic patients through the identification of biomarkers could provide information on symptom occurrence. Such biomarkers are not yet available but are needed to make appropriate decisions on the type of intervention required [8]. Previous studies from our group have shown that LA-13OOH is present in the lipid extract of the human carotid plaque, and that it specifically inhibits rePON1 activity in a dose- and time-dependent manner. During PON1's interaction with lesion LA-13OOH, the enzyme displayed a peroxidase-type of catalysis, reducing LA-13OOH to LA-OH (hydroxide) via the PON1 amino acid Cys284 [22]. Thus, the levels of LA-13OOH in symptomatic and asymptomatic patients were compared and correlated with antiatherogenic compounds in the plaque, or in the serum derived from these patients. Symptomatic patients had significantly higher levels of LA-13OOH in their plaques than their asymptomatic counterparts. In addition, LA-13OOH levels in the plaque were significantly inversely correlated with serum PON1 activity (Figure 2(a)) and with serum HDL cholesterol (Figure 2(b)). Although PON1 is an HDL-associated enzyme, HDL particles are highly heterogeneous in their structure, intravascular metabolism, and biological activities [33]. Furthermore, PON1 is only present on a relatively small fraction of the HDL particles, mostly on the HDL3 subfraction. Thus, the

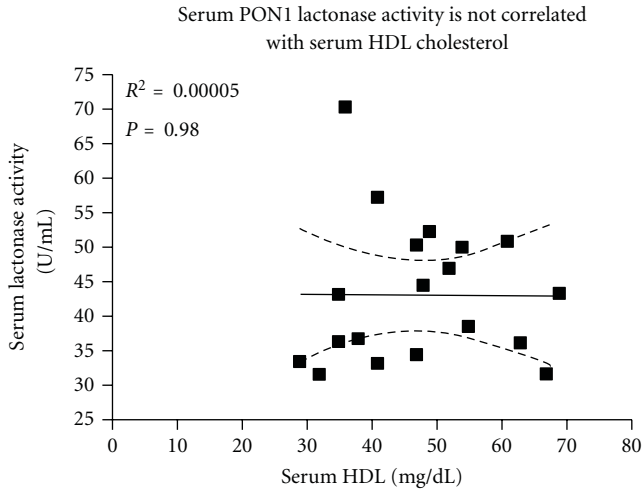

Figure 3: Serum PON1 activity versus serum HDL cholesterol. Serum PON1 activity is not correlated with serum HDL cholesterol levels.

amount of the HDL in the blood is not necessarily correlated with serum PON1 activity. Indeed, in this study, serum PON1 lactonase activity was not correlated with serum HDL levels (Figure 3). These results indicate that LA-13OOH might be affected, independently, by both serum PON1 and serum HDL level. As plaque LA-13OOH has been shown to interact with and inhibit rePON1 [22], and since PON1 is present and active, in both blood and the atherosclerotic plaque, we determined PON1 activity in serum and plaques derived from the same individuals and compared them in symptomatic versus asymptomatic patients. Our results showed no differences in plaque PON activity between symptomatic and asymptomatic atherosclerotic patients ($P = 0.8$). Serum PON1 activity in the symptomatic patients was lower than in the asymptomatic patients, although this difference was not significant ($P = 0.14$) (Table 2). Plaque PON activity may be attributed to both PON1 and the intracellular enzyme PON2, which may not be affected by the presence of LA-13OOH. In addition, it has been previously shown that inhibition of rePON1 by LA-13OOH can be prevented if certain thiols, such as the amino acid cysteine, are present, and that PON1 inhibition by LA-13OOH can be partially recovered if a thiol is added to the incubation system. LA-13OOH probably oxidizes the PON1 Cys284 to sulfenic acid, which can then be further oxidized to sulfinic and sulfonic acid derivatives. In the presence of thiol, the sulfenic acid derivative of Cys284 can be reduced back to thiol; however, if the oxidation proceeds further, addition of an external thiol can no longer reverse the reaction [22]. We can, therefore, hypothesize that the presence of free cysteine in the blood and in the plaque may also prevent LA-13OOH-induced PON1 inactivation.

In accordance with Carr et al. [29], we did not observe significant differences in serum cholesterol or triglyceride levels between symptomatic and asymptomatic patients, or in triglyceride or oxysterol levels in the plaques themselves (Table 2).

In addition to their higher levels of plaque LA-13OOH and lower levels of serum HDL relative to asymptomatic patients, there are more diabetics among the symptomatic patients (Table 1), as characterized by a higher concentration of blood HbA1c (Table 2). HbA1c is a glycated hemoglobin, which is highly prone to oxidation and its level is linked to oxidative stress. Diabetic patients are a heterogeneous population that may differ in their exposure to risk factors. For example, haptoglobin (Hp) genotype is an independent risk factor for vascular complications in diabetes [34]. This study suggests LA-13OOH as a possible risk factor for diabetic atherosclerosis development and complications, as analyses of plaque LA-13OOH and blood HbA1c revealed a direct correlation between these two atherogenic elements. Levels of HbA1c are strongly correlated with mean blood glucose [35], and high glucose concentration is known to increase oxidative stress [36]. HbA1c is also correlated with lipid peroxidation values, as shown in type 1 diabetic patients [37], as well as with plasma aldehydes (malondialdehydes) in type 2 diabetics [38, 39]. Furthermore, Hussein et al. [40] recently showed that the lag time required for the initiation of LDL oxidation (in the presence of copper ions) is inversely correlated with HbA1c concentration (mainly when HbA1c < 7.3%). Moreover, incubation of red blood cell hemolysate with increasing concentrations of glucose and with LDL or oxidized LDL results in an increased concentration of HbA1c. Thus, the increased tendency for LDL to undergo lipid peroxidation in diabetic patients contributes to increased levels of blood HbA1c. This further emphasizes the strong association between HbA1c and oxidative stress, which is in agreement with the present findings pointing to a direct link between the atherogenic compounds in the atherosclerotic lesions and serum, such as LA-13OOH and blood HbA1c. In addition, it is in line with the inverse relationships shown for serum HDL and PON1 activity, which are both antiatherogenic elements of the blood.

In conclusion, LA-13OOH levels in plaques from symptomatic patients are higher than those in asymptomatic patients' plaques. The high levels of plaque LA-13OOH are correlated with low levels of serum HDL, low levels of serum PON1 lactonase activity, and increased concentrations of blood HbA1c, all of which further accelerate atherosclerotic progression. Thus, the levels of PON1 activities, HDL concentration, and HbA1c content could serve as suitable biomarkers to assess LA-13OOH level in human carotid plaques.

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Research Article

Additional Common Polymorphisms in the *PON* Gene Cluster Predict PON1 Activity but Not Vascular Disease

Daniel S. Kim,[1] Amber A. Burt,[2] Jane E. Ranchalis,[2] Rebecca J. Richter,[2] Julieann K. Marshall,[2] Jason F. Eintracht,[3] Elisabeth A. Rosenthal,[2] Clement E. Furlong,[1,2] and Gail P. Jarvik[1,2]

[1] *Department of Genome Sciences, University of Washington School of Medicine, Seattle, WA 98195, USA*
[2] *Department of Medicine, Division of Medical Genetics, University of Washington School of Medicine, P.O. Box 357720, Seattle, WA 98195, USA*
[3] *Department of General Medicine, Virginia Mason Medical Center, Seattle, WA 98101, USA*

Correspondence should be addressed to Gail P. Jarvik, gjarvik@medicine.washington.edu

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Background. Paraoxonase 1 (PON1) enzymatic activity has been consistently predictive of cardiovascular disease, while the genotypes at the four functional polymorphisms at *PON1* have not. The goal of this study was to identify additional variation at the *PON* gene cluster that improved prediction of PON1 activity and determine if these variants predict carotid artery disease (CAAD). *Methods*. We considered 1,328 males in a CAAD cohort. 51 tagging single-nucleotide polymorphisms (tag SNPs) across the *PON* cluster were evaluated to determine their effects on PON1 activity and CAAD status. *Results*. Six SNPs (four in *PON1* and one each in *PON2/3*) predicted PON1 arylesterase (AREase) activity, in addition to the four previously known functional SNPs. In total, the 10 SNPs explained 30.1% of AREase activity, 5% of which was attributable to the six identified predictive SNPs. We replicate rs854567 prediction of 2.3% of AREase variance, the effects of rs3917510, and a *PON3* haplotype that includes rs2375005. While AREase activity strongly predicted CAAD, none of the 10 SNPs predicting AREase predicted CAAD. *Conclusions*. This study identifies new genetic variants that predict additional PON1 AREase activity. Identification of SNPs associated with PON1 activity is required when evaluating the many phenotypes associated with genetic variation near PON1.

1. Introduction

Paraoxonase 1 (PON1) is a liver-produced glycoprotein enzyme bound to the surface of high-density lipoprotein (HDL) whose activity is consistently correlated with atherosclerotic vascular disease and end-organ damage [1–3]. PON1 is at least partially responsible for the inhibitory effects of HDL on low-density lipoprotein (LDL) peroxidation [4–6] and also has been demonstrated to hydrolyze oxidized lipid or lipid hydroperoxides in LDL [7]. Accordingly, Watson et al. reported that inactivation of PON1 reduced the ability of HDL to inhibit both the oxidation of LDL and the interaction between macrophages and endothelium [6], both likely key factors in the inflammatory changes underlying atherogenesis. It has also been shown that PON1-deficient mice cannot neutralize the oxidized LDL lipids and have an increased susceptibility to organophosphate toxicity and coronary heart disease (CHD) [8, 9]. Finally, PON1 activity appears to play a role in maintaining the endothelial-atheroprotective effects of HDL [10].

There are four currently established functional common *PON1* single-nucleotide polymorphisms (SNPs) amongst the nearly 200 SNPs in the gene [11]: two missense mutations ($PON1_{Q192R}$[rs662] and $PON1_{M55L}$[rs854560]) and two that alter promoter activity ($PON1_{-108C/T}$[rs705379] and $PON1_{-162A/G}$[rs705381]). $PON1_{-108C/T}$ has the largest effect, altering expression likely due to modification of an Sp1 binding site [12]. Rare functional variants have also been identified [13].

While PON1 activity is predictive of vascular disease, studies investigating the role of *PON1* SNPs in vascular

disease have been contradictory [14–18]. A recent meta-analysis of 88 case-control studies by Wang et al. found that $PON1_{Q192}$ was correlated with CHD [19]. However, removal of smaller studies from the meta-analysis resulted in none of the functional *PON1* SNPs having significant association with CHD, thereby replicating the results of past meta-analyses [20–22]. Similarly, our own past investigations have found that while PON1 enzyme levels are predictive of carotid artery disease (CAAD), the genotypes at the four common functional SNPs fail to predict CAAD status [2, 3]. However, studies of CAAD or ischemic strokes are generally more positive for associations with the *PON1* functional SNPs [18, 23–26] than those for CHD. It should be noted that these studies generally have small sample size and several of such studies reported negative results [27, 28].

PON1 has broad substrate specificity and is protective against exposure to toxic organophosphorus insecticides [29]. For biological purposes, PON1 activity is generally measured with regard to the rate of hydrolysis of paraoxon, diazoxon, and phenylacetate (arylesterase activity) [30, 31]. These are termed POase, DZOase, and AREase activities, respectively. AREase enzymatic activity is unaffected by the functional $PON1_{Q192R}$ polymorphism, thus making it the best reflection of the levels of PON1 protein [32].

PON1 activity has also been linked to a number of other health-related phenotypes in addition to vascular disease and diabetes [33]. For example, PON1 also influences the metabolism of a variety of drugs, including statins, in addition to its aforementioned properties of reducing oxidized LDL and breaking down pesticides [34]. $PON1_{Q192R}$ is a reported determinant of clopidogrel efficacy [35], although this result has not been replicated [36] and remains controversial. PON1 has also been associated with diverse diseases [37]. The $PON1_{L55M}$ polymorphism has been repeatedly associated with Parkinson's disease [38–40], including a meta-analysis [41], but null results have also been reported [42]. Recent meta-analyses reported the association of *PON1* coding SNPs and breast cancer [43, 44]. Both PON1 activity and genotypes have been associated with age-related macular degeneration [45–51]. PON1 activity is reportedly lower in subjects with systemic lupus erythematosus (SLE) [48, 52–54]. Finally, diabetes is associated with both reduced PON1 activity and *PON1* genotypes [55].

PON1 is one of three paraoxonase gene family members, located in a gene cluster on chromosome 7q21.3-22.1. All of the paraoxonases have antioxidant activity [56]. *PON1* and *PON3* share similar functions in association with HDL as described previously; however, *PON3* has lower expression levels [57]. In contrast, *PON2* is ubiquitously expressed in human cells [58], particularly in endothelial and aortic smooth muscle cells [59]. *PON2* polymorphisms have also been associated with CHD [58, 60]. In addition, all three *PON* gene products have been reported to hydrolyze the quorum sensing factor of *Pseudomonas aeruginosa N*-3-oxododecanoyl homoserine lactone (3OC12-HSL)[61], with PON1 and 2 enzymes specifically being shown in animal knock-out studies to be protective against *p. aeruginosa* infection [62, 63].

Carlson et al. previously performed a tagSNP analysis of the *PON1, 2,* and *3* gene cluster for association with AREase activity and CAAD status in an overlapping, but much smaller, cohort ($n = 500$ versus 1328) [27]. That study found evidence that additional functional SNPs likely exist in *PON1*, but that the majority of the genetic effect on AREase variation was explained by the four functional SNPs previously described. They did not find evidence for *PON2* or *PON3* SNPs predicting additional AREase activity.

However, the investigation by Carlson et al. still left a large portion of the variation in PON1 activity unexplained. Thus, the goals of this study are to followup on these previous results and utilize an enlarged cohort and denser tagSNP genotyping to attempt to identify novel common SNPs in the *PON* gene cluster that associate with PON1 activity and/or predict CAAD.

2. Methods

2.1. Sample. The study population for this analysis consisted of 1,328 samples from the previously described Carotid Lesion Epidemiology And Risk (CLEAR) study [2, 3, 64]. Only Caucasian males were analyzed due to underrepresentation of female and minority samples in this primarily Seattle-Veterans-based cohort. Current smoking status and reported ancestry were obtained by self-report. Ancestry was confirmed using STRUCTURE with three ancestral groups [65]. CAAD status was determined via ultrasound of the internal carotid arteries, with cases defined as having >50% stenosis in either artery or a relevant procedure on their carotid arteries in their medical history. Controls had <15% stenosis in both arteries. 88 subjects had intermediate stenosis (15–49%) and were not included for prediction of CAAD, though they were included for prediction of PON1 enzyme activity.

2.2. Genotyping and PON1 Phenotypes. The four known functional *PON1* SNPs, $PON1_{Q192R}$, $PON1_{M55L}$, $PON1_{-108C/T}$, and $PON1_{-162A/G}$ and two SNPs identified as potentially predictive by Carlson et al. but not represented on the CVD chip, $PON1_{-909}$ (rs854572) and rs3917510 [27], were genotyped using previously described methods [12, 66]. An additional 86 SNPs in *PON1*, *PON2,* and *PON3* cluster were genotyped using the Illumina HumanCVD BeadChip (http://www.illumina.com/products/humancvd_whole_genome_genotyping_kits.ilmn). Duplicate genotyping for 34 individuals showed 99.7% consistency in calls. The *PON* cluster genotypes were filtered with a minor allele frequency cutoff of 1% and did not show deviation from Hardy-Weinberg equilibrium at the $P < 10^{-4}$ level. Rs3917564 was also found to be predictive by Carlson et al. and was genotyped by the CVD chip but was not included in the full analysis due to low minor allele frequency (C/T, C allele frequency = 0.008).

The PON1 POase, DZOase, and AREase activities were measured by a continuous spectrophotometric assay with lithium heparin plasma, as previously described [66]. AREase activity was measured in duplicate and averaged.

AREase was utilized as the primary measured outcome of *PON* gene cluster variation, due to its closer correlation with protein levels. POase activity is largely determined by the $PON1_{Q192R}$ missense polymorphism, which predicts over 70% of its variance [2].

2.3. Analysis. LDselect was used to create tagSNPs from the 86 *PON1*, *PON2,* and *PON3* SNPs genotyped on the Illumina HumanCVD chip [67]. Functional annotation for these SNPs were taken from SNP-Nexus [68]. 51 bins were created, using a linkage disequilibrium (LD) r^2 threshold of 0.64. The first 13 of these bins, corresponding to the *PON1* gene, had multiple SNPs within them, while the remaining bins consisted of singletons. One SNP from each bin was randomly included in the regression analysis for a total of 51 SNPs in the *PON* gene cluster. These 51 SNPs did not include the four functional SNPs, which were included in the analysis separately.

We also made an effort to independently replicate SNPs identified as predictive of PON1 activity by Carlson et al. [27]. As our full sample overlaps with that smaller sample, these were tested in a nonoverlapping sample of 523 subjects with complete genotype and phenotype data which were not available at the time of that study.

Regression analysis was done in R (http://www.r-project.org/) using the standard regression tools available. Genotypes were coded using an additive model. Stepwise linear regression was performed, and model comparison was done using Akaike's Information Criterion (AIC) to examine the fit of each model, beginning with a base model that included current smoking status, age, and the genotypes for the four functional PON1 SNPs as covariates [2, 3, 27]. SNPs that are included in the final model increased the ability of the model to predict the dependent variable. Statin drug use can influence *PON1* expression, and this appears to be influenced by $PON1_{-108}$ genotype [69]. However, statin drug use could not be included as a covariate due to confounding with CAAD status; the preferential use of statins in cases can lead to an erroneous estimation of statin effects on PON1 activity.

3. Results

The sample included 1,328 males with a mean age of 67.8 years; 16.5% of participants reported being current smokers. The subjects included 596 cases and 644 controls considered in the prediction of case status as well as 88 subjects with carotid stenosis between 15–49% who were considered only for the genotype effects on PON1 activity. Cases had a mean censored (CAAD onset) age of 66.5 years and mean current age at enrollment age of 70.9; controls had a mean current age of 64.6 years. The rates of current smoking and statin use, respectively, were 25.8% and 64.7% for cases and 9.6% and 19.5% for controls. Descriptions of the 51 tag SNPs for the *PON* gene cluster are available in Table 1. The AREase activity showed an approximately normal distribution, with a mean of 134 U/I and standard deviation of 51.8.

A regression model containing functional *PON1* SNPs ($PON1_{Q192R}$, $PON1_{M55L}$, $PON1_{-108C/T}$, and $PON1_{-162A/G}$), age, and current smoking status explained 25.2% of the variance in AREase activity. To explore the possibility of novel SNPs influencing AREase activity, we examined a best-fit model utilizing the stepwise regression including the aforementioned variables plus the 51 tagSNPs. AIC was used to assess whether the additional SNP provided a better fit to the prediction of AREase activity. Only SNPs that added to the predictive power of the best-fit model were kept; others that did not influence the model were discarded. In addition to the four functional SNPs, age, and current smoking, six SNPs were retained in the best-fit model. Together with the original 4 functional SNPs, these additional six SNPs in the *PON* gene cluster explained 30.1% of variance in AREase activity (see Table 2). Addition of these SNPs, rs854567, rs2299257, rs2237583, rs2375005, rs3917486, and rs11768074 serially explained an additional 2.34%, 0.85%, 0.5%, 0.34%, 0.58%, and 0.26% of total variance in PON1 activity. Amongst these six SNPs, four SNPs were in *PON1,* one was in *PON2* (rs2375005), and one was in *PON3* (rs11768074); all are intronic.

Five of the six SNPs found to predict PON1 activity were the only SNPs in their bin (singletons). The sixth SNP, rs854567, was binned with one other typed SNP, rs2299260, $r^2 = 0.80$. To observe whether it was superior at predicting PON1 AREase activity, we replaced rs854567 with rs2299260 in the complete model of 10 SNPs plus covariates. The model including rs2299260 did not predict additional AREase activity as compared to the model including rs854567, with a total of 30.1% of AREase variance explained in the full model. Therefore, either SNP or an untyped SNP in LD may be the functional SNP resulting in the association identified.

To address the potential that untyped SNPs are the functional SNPs that underlie the identified AREase associations, the 1000 Genomes database for European ancestry was consulted via SNP-Nexus [68] for these six SNPs. Five of the six SNPs we found to predict PON1 AREase activity were not in strong LD ($r^2 \geq 0.8$) with other regional SNPs, suggesting that they may be functional. Rs2375005, in contrast, is in strong LD with an additional five SNPs in *PON3* ($r^2 = 0.901$ with intronic rs978903 and synonymous A99A SNP rs1053275; $r^2 = 0.837$ with intronic rs10953146; $r^2 = 0.81$ for intronic rs11970910 and rs117154505) [10].

Prediction of POase activity utilizing these six SNPs that predicted AREase activity (including the base model with age, current smoking status, and the four functional *PON1* SNPs) resulted in 84.02% of POase enzymatic variance explained (see Table 3). This compared to 82.74% of variance explained with the base model with the four functional SNPs, age, and smoking status, with the high percentage of variation explained largely due to the effects of the PON_{Q192R} polymorphism on paraoxon catalytic efficiency. Five of the six SNPs (excluding rs2237583) showed the same directionality of their effects as seen in the AREase analysis, and three had significant effects on POase: rs854567, rs2299257, and rs3917486. When creating a best-fit model that allowed any of the 51 SNPs studied to enter regression in addition to the base model, 84.96% of POase variance in activity was explained.

Table 1: Characteristics of the 51 SNPs studied in the *PON* gene cluster.

SNP	Gene	Function[a]	Minor allele[b]	Major allele	MAF[c]
rs854549	*PON1*	3′-downstream	A	C	0.337
rs3735590	*PON1*	3′-UTR	A	G	0.060
rs3917577	*PON1*	3′-UTR	G	A	0.089
rs854552	*PON1*	3′-UTR	G	A	0.265
rs3917551	*PON1*	Intronic	A	G	0.051
rs3917550	*PON1*	Intronic	A	G	0.137
rs2269829	*PON1*	Intronic	G	A	0.278
rs3917542	*PON1*	Intronic	A	G	0.227
rs3917538	*PON1*	Intronic	A	G	0.236
rs2299257	*PON1*	Intronic	C	A	0.391
rs854560	*PON1*	Coding	T	A	0.360
rs3917498	*PON1*	Intronic	A	C	0.345
rs28699500	*PON1*	Intronic	G	A	0.289
rs854561	*PON1*	Intronic	A	G	0.357
rs854565	*PON1*	Intronic	A	G	0.294
rs2272365	*PON1*	Intronic	C	A	0.154
rs854567	*PON1*	Intronic	A	G	0.185
rs3917490	*PON1*	Intronic	A	G	0.490
rs2299261	*PON1*	Intronic	G	A	0.354
rs854568	*PON1*	Intronic	G	A	0.219
rs2299262	*PON1*	Intronic	A	G	0.399
rs854569	*PON1*	Intronic	A	C	0.216
rs2237583	*PON1*	Intronic	A	G	0.284
rs3917486	*PON1*	Intronic	A	G	0.054
rs3917481	*PON1*	Intronic	A	G	0.015
rs2237584	*PON1*	Intronic	A	G	0.058
rs3917478	*PON1*	Intronic	G	A	0.118
rs3917476	*PON1*	Intronic	A	C	0.031
rs854571	*PON1*	5′-upstream	A	G	0.289
rs13236941	*PON1*	5′-upstream	A	G	0.164
rs13228784	*PON1*	Intronic	G	A	0.255
rs17883513	*PON1*	Intronic	G	A	0.032
rs17886762	*PON1*	Intronic	A	G	0.072
rs17883952	*PON1*	Intronic	A	G	0.052
rs17884000	*PON3*	Intronic	G	A	0.202
rs9640632	*PON3*	3′-UTR	G	A	0.456
rs468	*PON3*	Intronic	G	A	0.066
rs11768074	*PON3*	Intronic	A	G	0.157
rs10487132	*PON3*	Intronic	G	A	0.390
rs740264	*PON3*	Intronic	C	A	0.254
rs17884563	*Intergenic*	*Intergenic*	T	A	0.109
rs17880030	*Intergenic*	*Intergenic*	A	G	0.199
rs17881071	*Intergenic*	*Intergenic*	A	G	0.198
rs2375005	*PON2*	Intronic	T	A	0.462
rs12026	*PON2*	Coding	C	G	0.240
rs2299264	*PON2*	Intronic	A	G	0.241
rs7803148	*PON2*	Intronic	A	G	0.405
rs2158806	*PON2*	Intronic	C	A	0.237
rs2286233	*PON2*	Intronic	A	T	0.131

Table 1: Continued.

SNP	Gene	Function[a]	Minor allele[b]	Major allele	MAF[c]
rs10259688	*PON2*	Intronic	G	A	0.179
rs730365	*PON2*	Intronic	A	G	0.132

Abbreviations: UTR = untranslated region, MAF = minor allele frequency, intergenic = located between two gene regions.
[a] SNP functional annotation from SNP-Nexus.
[b] Major and minor allele annotation from the Illumina HumanCVD Bead Chip.
[c] Minor allele frequencies calculated from the CLEAR study cohort.

Table 2: Best-fit model from stepwise linear regression predicting PON1 AREase activity.

Variable	Coefficient (± SE)	Gene[a]	MAF[b]	*t*-statistic[c]	AREase Variation %	*P*
(Intercept)	284.09 (±13.99)	—	—	20.304	—	$< 2.0 \times 10^{-16}$
$PON1_{C-108T}$	−24.82 (±2.61)	*(PON1)*	0.43	−9.498	14.10%	$< 2.0 \times 10^{-16}$
$PON1_{G-162A}$	4.61 (±4.60)	*(PON1)*	0.18	1.002	0.21%	0.317
$PON1_{Q192R}$	−22.09 (±4.20)	*PON1*	0.33	−5.258	1.17%	1.8×10^{-7}
$PON1_{M55L}$	−7.05 (±3.64)	*PON1*	0.42	−1.94	1.01%	0.053
Age	−1.33 (±0.15)	—	—	−9.014	4.29%	$< 2.0 \times 10^{-16}$
Current smoker	−28.25 (±3.63)	—	—	−7.776	4.42%	1.95×10^{-14}
rs854567	−8.19 (±4.77)	*(PON1)*	A = 0.185	−1.719	2.34%	0.086
rs2299257	12.66 (±3.57)	*(PON1)*	C = 0.391	3.546	0.85%	4.11×10^{-4}
rs2237583	11.36 (±3.12)	(*PON1*)	A = 0.284	3.645	0.5%	2.82×10^{-4}
rs2375005	−8.32 (±2.56)	*(PON2)*	T = 0.462	−3.25	0.34%	0.001
rs3917486	14.91 (±4.97)	(*PON1*)	A = 0.054	2.998	0.58%	0.003
rs11768074	8.42 (±4.48)	(*PON3*)	A = 0.157	1.878	0.26%	0.061

SE = standard error, MAF = minor allele frequency.
[a]Noncoding SNPs are presented in parentheses, for example, (*PON1*).
[b]Minor allele frequencies for the four functional SNPs reported through dbSNP. The remaining minor allele frequencies were calculated via the CLEAR cohort.
[c]*t*-statistics and *P* values were calculated from the coefficients (from all subjects) and standard errors within the best-fit multivariate model by the glm function in R.

Table 3: Application of best-fit model for PON1 AREase activity to predict PON1 POase activity.

Variable	Coefficient (± SE)	Gene[a]	MAF[b]	*t*-Statistic[c]	POase Variation %	*P*
(Intercept)	29.36 (±1.17)	—	—	24.986	—	$< 2.0 \times 10^{-16}$
$PON1_{C-108T}$	−1.91 (±0.22)	*(PON1)*	0.43	−8.762	11.78%	$< 2.0 \times 10^{-16}$
$PON1_{G-162A}$	0.78 (±0.39)	*(PON1)*	0.18	2.023	3.93%	0.043
$PON1_{Q192R}$	9.67 (±0.35)	*PON1*	0.33	27.27	65.61%	$< 2.0 \times 10^{-16}$
$PON1_{M55L}$	−1.59 (±0.30)	*PON1*	0.42	−5.133	0.35%	3.5×10^{-7}
Age	−0.09 (±0.01)	—	—	−7.475	0.78%	1.81×10^{-13}
Current smoker	−1.27 (±0.31)	—	—	−4.155	0.31%	3.56×10^{-5}
rs854567	−1.69 (±0.40)	*(PON1)*	A = 0.185	−4.246	0.54%	2.41×10^{-5}
rs2299257	0.92 (±0.30)	*(PON1)*	C = 0.391	3.085	0.15%	0.002
rs2237583	−0.35 (±0.26)	(*PON1*)	A = 0.284	−1.347	0.09%	0.179
rs2375005	−0.23 (±0.21)	*(PON2)*	T = 0.462	−1.081	0.00%	0.28
rs3917486	2.20 (±0.42)	(*PON1*)	A = 0.054	5.271	0.47%	1.7×10^{-7}
rs11768074	0.42 (±0.37)	(*PON3*)	A = 0.157	1.114	0.02%	0.266

SE = standard error, MAF = minor allele frequency.
[a]Non-coding SNPs are presented in parentheses, for example, (*PON1*).
[b]Minor allele frequencies for the four functional SNPs reported through dbSNP. The remaining minor allele frequencies were calculated via the CLEAR cohort.
[c]*t*-statistics and *P* values were calculated from the coefficients (from all subjects) and standard errors within the best-fit multivariate model by the glm function in R.

Similar application to the prediction of DZOase activity utilizing the six SNPs from the predictive AREase model plus the base model (age, current smoking status, and the four functional *PON1* SNPs) resulted in 54.85% of variance explained (see Table 4). Five of the six SNPs (excluding rs11768074) showed the same directionality of their effects, and 4 had significant effects (rs2299257, rs2237583, rs2375005, and rs3917486). When using the four functional SNPs, age, and sex alone, 50.99% of DZOase activity was explained. However, when allowing any of the 51 tagSNPs to enter the best-fit model, 55.60% of DZOase activity was accounted for, suggesting that different SNPs may affect DZOase.

We attempted to replicate SNPs previously identified by Carlson et al. as predicting PON1 activity in a nonoverlapping sample of 523 subjects (Table 5), because significance in an overlapping subset does not constitute replication. The SNPs identified by Carlson et al. were rs854549, rs3917564, rs2269829, rs854566, rs854572, and rs3917510. In our full analysis, rs854566 was tagged by rs854567 ($r^2 = 0.93$), which did enter the full model using the full sample and predicted 2.34% of AREase activity. Rs2269829 and rs854549 were not predictive of AREase in the full dataset. Rs3917564 was not included in the full model analysis due to minor allele frequency <0.01. Rs3917510 and rs854572 (PON_{-909} promoter) were not tagged in the CVD chip analysis and were genotyped separately for the replication analysis. When we considered the independent sample to test the six Carlson SNPs in a linear model predicting AREase, which also included age, current smoking status, and the four functional *PON1* SNPs, two of the six Carlson et al. findings were replicated. Both rs854566 (Carlson $P = 0.014$, current rs854567 $P = 1.64 \times 10^{-5}$) and rs3917510 (Carlson $P = 0.016$, current $P = 0.028$) were significant in predicting AREase. Moreover, the direction of effect for rs854566 (Carlson coefficient = −10.6, current coefficient = −20.4) and rs3917510 (Carlson coefficient = 16.6, current coefficient = 14.3) were the same in both analyses.

None of the 10 SNPs identified in our full analyses, including the four previously known and the six newly reported to predict AREase, predicted CAAD status, considering the covariates censored age and current smoking status. Moreover, none of the SNPs had a P value <0.10. However, AREase activity, adjusted by age and current smoking status, was highly associated with CAAD status ($P = 3.62 \times 10^{-6}$), as previously reported in a smaller sample.

4. Discussion

Only four *PON1* SNPs are well established to affect PON1 activity. These mutations alone account for approximately only 25% of PON1 AREase activity, leaving a large amount of variation left unexplained. In this study, we utilized denser tagSNP genotyping and a 2.65-fold increased sample size than those previously used in the Carlson et al. study [27] to examine the effects of common variants, demonstrating the presence of additional functional genetic variance within the *PON* gene cluster. We identified six additional SNPs that predicted AREase activity (rs854567, rs2299257, rs2237583, rs2375005, rs3917486, and rs11768074). All are intronic, with four in *PON1* and one each in *PON2* (rs2375005) and *PON3* (rs11768074). Of these, only rs2375005 was found to be in strong LD with other regional SNPs in the 1000 genomes data, which included a *PON3* synonymous SNP (rs1053275). This LD block SNP is also reported to be in weaker LD with a $PON1_{-1741GA}$ promoter region polymorphism (maximum $r^2 = 0.47$) [34]. The remaining 5 SNPs associated with AREase may be functional or in weaker LD with a functional site. Rs854567 alone predicted 2.3% of the additional variance in AREase; it lies in the first intron of *PON1*, a common regulatory area.

For the many phenotypes with genetic associations to the PON cluster, knowledge of which SNPs are associated with functional changes is helpful in determining true associations from spurious ones. As discussed above, rs2375005 is in strong LD with an additional five SNPs in *PON3* (rs978903, rs1053275, rs10953146, rs11970910, and rs117154505). These include SNPs that have a reported association with sporadic amyotrophic lateral sclerosis [10]. In addition, Riedmaier et al. have demonstrated that a haplotype block including rs2375005 was associated with atorvastatin lactose hydrolysis and increased *PON1* mRNA expression in liver tissue [34]. Our results validate the presence of a functional SNP in this haplotype block.

In comparing these results to the six SNPs identified by Carlson et al, we replicate the effects of two SNPs, rs85466 and rs3917510, while failing to replicate four (Table 5) in nonoverlapping data. Rs854566 was represented in our analyses by the tagSNP, rs854567 ($r^2 = 0.93$). In contrast, the effects of rs854549, rs854572, rs3917564, and rs2269829 are not replicated here. Rs854572 is 5′ SNP $PON1_{-909C/G}$; while it has been associated with AREase level, smaller studies suggested that all of its activity was attributable to LD with the four functional SNPs [66]. The Carlson et al. paper suggested that this site may have independent activity, but we find no additional effects of this site, in an independent sample of 523 subjects. In sum, our current study confirms both the effects of rs854566 or its bin-mate rs854567, predicting 2.3% of AREase activity and the effects of rs3917510, while also identifying five additional tagSNPs that accounted for approximately 2.7% of PON1 AREase activity that were not accounted for by Carlson et al.

The finding of *PON2* and *PON3* SNPs (rs2375005 and rs11768074, resp.) predicting PON1 AREase activity is intriguing. The *PON* genes are in a cluster and arranged in order from the centromere as *PON1, PON3*, and *PON2*. Each is transcribed in the same direction, toward the centromere. Therefore, variants in the *PON2* or *PON3* genes lie 5′ to *PON1*. Rs2375005 is in the sixth of eight *PON2* introns. Rs11768074 is in the last *PON3* intron. Neither PON2 nor PON3 has intrinsic AREase activity [70], suggesting that these SNPs tag effects on PON1. As noted above, SNPs in the *PON3* rs2375005 haplotype block have been described to affect *PON1* mRNA level [34], thus the effects of these SNPs, or SNPs in LD with them, may regulate *PON1* expression.

Recent research in a cohort investigating SLE has linked rs17884563 and rs740264 in the *PON3* region [53] and

Table 4: Application of best-fit model for PON1 AREase activity to predict PON1 DZOase activity.

Variable	Coefficient (± SE)	Gene[a]	MAF[b]	t-statistic[c]	DZOase Activity %	P
(Intercept)	154.26 (±4.24)	—	—	36.365	—	$< 2.0 \times 10^{-16}$
$PON1_{C-108T}$	−8.69 (±0.79)	*(PON1)*	0.43	−11.054	12.82%	$< 2.0 \times 10^{-16}$
$PON1_{G-162A}$	5.03 (±1.40)	*(PON1)*	0.18	3.597	5.10%	3.4×10^{-4}
$PON1_{Q192R}$	−20.41 (±1.28)	*PON1*	0.33	−15.944	23.71%	$< 2.0 \times 10^{-16}$
$PON1_{M55L}$	−5.03 (±1.12)	*PON1*	0.42	−4.498	3.39%	7.75×10^{-6}
Age	−0.44 (±0.44)	—	—	−9.797	4.21%	$< 2.0 \times 10^{-16}$
Current Smoker	−5.65 (±1.11)	—	—	−5.103	1.28%	4.08×10^{-7}
rs854567	−2.26 (±1.44)	*(PON1)*	A = 0.185	−1.566	1.74%	0.118
rs2299257	4.03 (±1.08)	*(PON1)*	C = 0.391	3.73	0.70%	2.03×10^{-4}
rs2237583	4.69 (±0.95)	(*PON1*)	A = 0.284	4.956	0.92%	8.57×10^{-7}
rs2375005	−1.73 (±0.76)	*(PON2)*	T = 0.462	−2.258	0.22%	0.024
rs3917486	3.42 (±1.51)	(*PON1*)	A = 0.054	2.275	0.27%	0.023
rs11768074	−0.64 (±1.36)	(*PON3*)	A = 0.157	−0.466	0.01%	0.641

SE = standard error, MAF = minor allele frequency.
[a]Noncoding SNPs are presented in parentheses, for example, (*PON1*).
[b]Minor allele frequencies for the four functional SNPs reported through dbSNP. The remaining minor allele frequencies were calculated via the CLEAR cohort.
[c]t-statistics and P values were calculated from the coefficients (from all subjects) and standard errors within the best-fit multivariate model by the glm function in R.

Table 5: Comparison of SNPs found significant in prior Carlson et al.[a] study with current, non-overlapping sample.

SNP	Seattle SNP annotation	Carlson coefficient (±SE)	Carlson t-Statistic [b]	Carlson P [c]	Current coefficient (±SE)[d]	Current t-Statistic [b]	Current P [c]
rs854566[e]	$PON1_{6842}$	−10.6 (±4.3)	−2.480	0.014	−20.4 (±4.68)	−4.353	$1.64 \times 10 - 5$
rs3917510	$PON1_{12471}$	16.6 (±6.9)	2.424	0.016	14.3 (±6.48)	2.208	0.028
rs2269829	$PON1_{19470}$	−16.5 (±10.8)	−1.520	0.129	13.6 (±21.82)	0.625	0.533
rs3917564	$PON1_{23887}$	−39.0 (±18.1)	−2.153	0.032	15.0 (±26.67)	0.564	0.573
rs854549	$PON1_{29021}$	9.2 (±4.5)	2.051	0.041	−1.3 (±4.90)	−0.260	0.795
rs854572	$PON1_{895}$	13.0 (±4.9)	2.677	0.008	−0.28 (±4.97)	−0.056	0.955

SE = standard error.
[a]Carlson et al. study $n = 500$ European male subjects [27].
[b]t-Statistics and P-values were calculated from the coefficients from each subgroup (Carlson $n = 500$, current study $n = 523$) and standard errors within the best-fit multivariate model by the glm function in R.
[c]Both Carlson and current study utilized a linear regression model adjusting for age, current smoking status, and the four functional PON1 SNPs.
[d]Current study subset of 523 European male subjects not considered by Carlson et al.
[e]Represented by proxy SNP, rs854457, with LD $r^2 = 0.93$ in the current study.

five *PON2* SNPs [52] (rs6954345, rs13306702, rs987539, rs11982486, and rs4729189) with PON1 POase activity [52, 53]. These investigations utilized POase rather than AREase activity [71]; this is not optimal, as the $PON1_{Q192R}$ activity accounts for most POase activity. Of the *PON3* SNPs found to predict POase activity [53], both rs17884563 (intergenic between *PON2* and *PON3* in our annotation) and rs740264 were directly genotyped and included in our regression model for PON1 AREase activity but were not predictive. When applying rs17884563 or rs740264 to POase activity, which the aforementioned investigators used as their PON1 phenotype, neither was predictive of POase activity. For the *PON2* SNPs predictive of POase in the SLE cohort, all five were represented by tag SNPs ($r^2 > 0.6$), but only rs2375005 ($r^2 = 1$ with rs987539) was predictive of PON1 AREase activity. Interestingly, none of these five *PON2* SNPs predict POase in our data, including rs2375005 ($P = 0.28$). The differences in *PON2* and *PON3* SNP associations between our data and the SLE cohort may reflect differences in cohort selection criteria (older male vascular disease versus younger female SLE, cases and controls) or sizes (1,322 in our data versus 922 in the SLE data).

Application of the six SNPs from the AREase best-fit model to predicting POase and DZOase activity resulted in the prediction of 98.89% and 98.65% of enzymatic activity predicted by models, where all 51 SNPs were allowed to enter. Three of these six SNPs, all in *PON1*, also predict both PON1 POase and DZOase activities. While it is clear why coding SNPs would differentially influence the PON1 degradation of these three substrates, it is less clear why regulatory variants

would. Further investigation is required to determine if and how these noncoding SNPs differentially influence PON1's multiple activities at the genomic, molecular, or cellular level.

None of the six new SNPs that predicted AREase activity were predictive of CAAD. In addition, none of the four functional *PON1* SNPs were predictive of CAAD, which is consistent with past findings with smaller sample sizes in this cohort [2, 3, 27]. Important sources of variance in AREase activity that are not captured by these genotypes or the covariates of age and current smoking must account for the strong association between this activity and CAAD. Possible sources of AREase variation include rare regional variants, regional gene regulation not captured by genotyping (such as methylation), variation in genes outside the *PON* cluster, nongenetic factors including statin drug use [72] and diet [73, 74], as recently reviewed [75], as well as interactions among these. Evidence of interactions includes the report of the association of *PON1* genotype and CHD only in subjects with diabetes [76]. These results emphasize the importance of researching the correlation of PON1 and cardiovascular disease more broadly by utilizing "PON status," taking into account both the genotype of *PON1* SNPs and the plasma activity [11, 77], as well as investigating factors which affect the specific activity of PON1. PON1 has been suggested as a drug target for vascular and other diseases, thus a clear understanding of its role in disease is crucial [78].

Some limitations of this study must be considered. First, the study was comprised entirely of males of European descent, thereby limiting the generalizations that can be drawn from these findings. Second, this investigation considered only SNPs from the *PON* gene cluster. Variation in other genes may influence PON1 activity [79]. For example, peroxisome proliferator-activated receptor gamma (PPARG) activates PON1 expression in hepatocytes [80], leading to the possibility that variation in the *PPARG* gene could alter levels of PON1 protein. However, the larger size of this study and the denser tagSNPing of the *PON* cluster, relative to the earlier Carlson et al. work [27], allowed us to detect novel genetic variation that predicts *PON1* AREase activity.

In conclusion, our analysis of the *PON* gene cluster identifies six additional common genetic variants that predict AREase activity: four are novel, predicting 2.4% of AREase activity and two replicate past findings. The replicated SNPs include rs854567, which tags 2.3% of AREase variance, rs3917510, and rs2375005, which tags 0.3% of AREase variance. We do not identify additional effects of the $PON1_{-909}$ polymorphism. Future studies to quantify the role of rare genetic variation and variation outside the *PON* cluster on PON1 activity will be important. Finally, the continued lack of an association between *PON1, 2,* or *3* genetic variants and CAAD, while PON1 activity is highly predictive, underscores the importance of utilizing PON status in future studies investigating the link between PON1 and vascular or other disease.

Abbreviations

AIC: Akaike's Information Criterion
CAAD: Carotid artery disease
CHD: Coronary heart disease
CLEAR: Carotid Lesion Epidemiology and Risk cohort
DZOase: Diazoxon enzymatic hydrolysis
POase: Paraoxon enzymatic hydrolysis
PON: Paraoxonase
tagSNP: Tagging single-nucleotide polymorphism.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Review Article

Protectors or Traitors: The Roles of PON2 and PON3 in Atherosclerosis and Cancer

Ines Witte,[1] Ulrich Foerstermann,[1] Asokan Devarajan,[2] Srinivasa T. Reddy,[2,3] and Sven Horke[1]

[1] *Department of Pharmacology, University Medical Center of the Johannes-Gutenberg University Mainz, Obere Zahlbacher Street 67, 55131 Mainz, Germany*
[2] *Department of Medicine, University of California, Los Angeles, CA 90095, USA*
[3] *Department of Molecular and Medical Pharmacology, University of California, Los Angeles, CA 90095, USA*

Correspondence should be addressed to Sven Horke, horke@uni-mainz.de

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Cancer and atherosclerosis are major causes of death in western societies. Deregulated cell death is common to both diseases, with significant contribution of inflammatory processes and oxidative stress. These two form a vicious cycle and regulate cell death pathways in either direction. This raises interest in antioxidative systems. The human enzymes paraoxonase-2 (PON2) and PON3 are intracellular enzymes with established antioxidative effects and protective functions against atherosclerosis. Underlying molecular mechanisms, however, remained elusive until recently. Novel findings revealed that both enzymes locate to mitochondrial membranes where they interact with coenzyme Q10 and diminish oxidative stress. As a result, ROS-triggered mitochondrial apoptosis and cell death are reduced. From a cardiovascular standpoint, this is beneficial given that enhanced loss of vascular cells and macrophage death forms the basis for atherosclerotic plaque development. However, the same function has now been shown to raise chemotherapeutic resistance in several cancer cells. Intriguingly, PON2 as well as PON3 are frequently found upregulated in tumor samples. Here we review studies reporting PON2/PON3 deregulations in cancer, summarize most recent findings on their anti-oxidative and antiapoptotic mechanisms, and discuss how this could be used in putative future therapies to target atherosclerosis and cancer.

1. Introduction

Most studies in the field of paraoxonases (PONs) deal with cardiovascular diseases, such as atherosclerosis and diabetes, where PONs exert protective functions in cell culture as well as animal studies. It has been anticipated that the known antioxidative functions of PONs, including PON2 and PON3, were central to their effects although underlying molecular mechanisms remained obscure. However, recent findings caused a significant progress in this field because molecular pathways of PON2 and PON3 functions have been largely revealed. Moreover, the result of the cell-protective function were shown to play a vital role in survival and stress resistance of cancer cells, along with the finding that numerous tumors overexpressed these enzymes. There, PON2 and PON3 appear to increase chemotherapeutic resistance and favor cell survival. In this review, we summarize the most recent findings and discuss the role of PON2/PON3 in atherosclerosis and cancer. A future perspective gives an outlook on how PONs may be targets of novel therapeutic approaches.

2. Altered Expression Levels of Paraoxonase Enzymes in Cancer

It is established that oxidative stress from mitochondria plays an important role in apoptosis and also leads to premature aging and cancer. There is growing scientific consensus that antioxidants or proteins with antioxidative functions, such as paraoxonases, can lower the incidence of, for example, cardiovascular and neurodegenerative diseases. On the other hand, recent studies have shown that various types of cancer obviously take advantage of this protection by enhanced expression of the antioxidative paraoxonase proteins. In the following section, we give an overview of studies that

assessed expression of PON1, PON2, or PON3 in various cancers, with the majority of studies seemingly reporting a deregulation of these proteins.

PON1 levels and activity are lower in many inflammatory and oxidative stress-associated diseases [1]. Also, serum PON1 and arylesterase activities were reduced in patients with epithelial ovarian cancer [2] and lung cancer [3]. Uyar et al. found that Q allele of PON1 was more frequent in renal cancer patients [4], and Antognelli at al. reported that certain PON1 genotypes were prone to increased risk of prostate cancer [5]. More recently, the presence of the variant alleles of the Q192R and L55M SNPs of PON1, both of which result in an amino acid replacement that alters PON1 activity, were found associated with a 18–29% increased risk of aggressive prostate cancer [6]. These studies clearly demonstrate a link between PON1 and cancer etiology; however, PON1 is not the scope of this review. We will focus on the role of PON2 and PON3 in cancer based on recent discoveries on the mechanism of action of these proteins in proliferation and apoptosis.

Research on paraoxonases is a relatively young field, and still much of our understanding comes from findings related to PON1. Back in 1999, our knowledge about PON2 and PON3 was extremely limited although few studies emerged that reported genetic associations with metabolic diseases [7]. There are two common single nucleotide polymorphisms (SNPs) in PON2—G148A and C311S—that have been associated with disease phenotypes. In essence, an association between these SNPs and several diseases was demonstrated. For PON2-G/A148 this is true, for instance, for higher plasma glucose [8], higher plasma HDL cholesterol [9], and lower plasma LDL cholesterol [10]. With respect to S/C311, Stoltz et al. reported that this mutation determines the lactonase activity of PON2 which links to hydrolysis of important bacterial virulence factors [11]. However, a subsequent study from our lab did not confirm this finding [12]. The impact of PON2-S/C311 on lactone hydrolysis thus merits further investigation. This may similarly apply for its role in coronary heart disease, where at least one study reported an association [13] that was not found in a subsequent meta-analysis [14].

Despite the established and prevailing role of paraoxonases in cardiovascular diseases and relevant parameters, more recent studies revealed an emerging association of PONs with cancer. For example, microarray studies observed an overexpression of PON2 in some solid tumors like hepatocellular carcinoma, prostate carcinoma [15, 16], and several others, which are illustrated in Table 1. Additionally, in various leukemia gene expression profiling studies, an upregulation of PON2 could be demonstrated; an example is pediatric acute lymphoblastic leukemia (ALL) [17]. Importantly, a subsequent study identified PON2 as member of a very small group of upregulated genes that characterized pediatric ALL patients with very poor outcome prognosis [18]. In another form of leukemia, chronic myeloid leukemia (CML), PON2 was also identified in an outcome-specific gene expression signature of primary imatinib-resistant patients [19]. Moreover, a marked overexpression of PON2 was observed in lymphocytes infected with T-cell leukemia virus [20].

In contrast to PON2, there are fewer studies for PON3 with the tendency of more diverse results (see Table 2). For instance, a downregulation was demonstrated in a meta-analysis of expression profiles in hepatocellular carcinomas (HCC) [21] and in ovarian serous papillary carcinomas (OSPCs) [22] shown by oligonucleotide microarrays. However, there are various other such analyses, which showed altered expression of PON3 (up as well as downregulated) in different types of cancers. For overview, consult the Gene Expression Atlas found at http://www.ebi.ac.uk/gxa/. In general, it should be noted that these association studies show no direct proof for a physiological relevance of these proteins in cancer, nor do such studies give any clues about their functions and mechanisms.

In addition to the listed microarray data, our very recent analyses showed that the PON2 level is increased in some tumors at the protein level (Table 1). We showed a moderate PON2 overexpression in pancreas, liver, kidney, and lung tumors and an over 10-fold upregulation of PON2 in thymus tumors and non-Hodgkins lymphomas [23]. Assessment of PON2 protein levels is not feasible in hundreds of cancer samples. Therefore, we previously used cDNA arrays, developed for differential gene expression analysis and validation of hundreds of different human tissues. We showed that PON2 is ~2–4-fold overexpressed in the tumors from urinary bladder, liver, kidney, lymphoid tissues, and endometrium/uterus in comparison to normal tissue [23], which are in accordance with western blot analyses. Despite some other tissues, where no increase in the expression level was observed, human tumors of the thyroid gland, testis, prostate, and pancreas showed a slight upregulation of PON2 (Table 1).

Using the same cDNA arrays as for PON2, our group showed a considerably increased PON3 expression in all tested cancer types, except cervix [24]. Remarkably, the intensity of PON3 overexpression was markedly enhanced compared to that of PON2. In this array over 10-fold upregulation of PON3 in tumors from endometrium/uterus and stomach was shown and over 3-fold induction in samples from pancreas, urinary bladder, thyroid, prostate, pancreas, liver testis, and lung cancers. These results could be verified with another matched array particularly for lung cancer (normal versus diseased samples from the same patient). But in contrast to PON2, PON3 expression appears to be largely restricted to cells derived from solid tumors [24]. One reason for the high expression level of PON3 in cancer tissue is certainly the low basal expression level of PON3 in healthy tissues but may nevertheless suggest a role for PON3 in cell death escape.

An interesting phenomenon is obvious upon closer inspection of the array data. A tumor subtype and stage-specific analysis revealed that both PON2 and PON3 are upregulated rather in the early stages and some subtypes of cancer, whereas the expression in the late stages of the tumor seems to be declining (see Figure 1). This could indicate that, especially in the early stages of tumor formation, the antioxidative and antiapoptotic function of PON2 and

Table 1: Expression levels of PON2 in various tumor tissues and/or cancer cell cultures. Microarray experiment (array express) listings are according to the Gene Atlas Database. Protein and cDNA levels according to [23]. Cell culture expression levels were roughly estimated as relative level comparing to A549, grouped into *low, medium,* or *high*.

Tissue (cancer)	Protein level (fold of normal tissue)	cDNA array (fold of normal tissue)	Microarray studies	Cell culture (expression level in cell line)
Kidney	2	2.2	Upregulated in renal carcinoma (E-MTAB-37)	Medium (HEK293)
Liver	1.7	2.2	Overexpressed (Li et al. [15])	High (Huh7/HepG2)
Lung	1.3	1	Upregulated in lung adenocarcinoma (E-MEXP-231/E-MTAB-37) Downregulated in small cell lung carcinoma (E-GEOD-4127)	High (A549; H661; H1299)
Spleen	0.5	n/a		
Pancreas	1.4	1.6	Upregulated in pancreatic carcinoma (E-MTAB-37)	
Thymus	11.5	n/a		
Urinary bladder	n/a	4.1		High (HT1367/RT112)
Esophagus	n/a	0.6	Upregulated in esophageal cancer (E-MTAB-62)	
Stomach	n/a	1	Upregulated in gastric carcinoma (E-GEOD-2685)	
Ovar	n/a	1	Upregulated (E-MTAB-62)	
Cervix	n/a	1	Upregulated (E-MTAB-37/E-MTAB-62)	Medium (HeLa)
Adrenal gland	n/a	1	Downregulated in adrenocortical carcinoma (E-TABM-311)	
Thyroid gland	n/a	1.4	Upregulated (E-GEOD-3467/E-GEOD-3678)	
Prostate	n/a	1.6	Overexpressed (Ribarska, T. et al. [16] E-MTAB-62)	
Testis	n/a	1.7		Low (SuSa/GCT27/833K)
Uterus/endometrium	n/a	2.1		
Lymphoid tissue	n/a	2.5		
Leukemias (various)	n/a		Upregulated in pediatric ALL (Ross et al. and Kang et al. [17, 18])	Low in AML-like Nalm6/EOL; Jurkat Tcells; PML-like HL60/HCW2; CML-like KCL Medium in blast crisis line K562; CML-like lama; AML-like THP1/MonoMac6/HEL
Non-Hodgkin	11.9	n/a	Downregulated (E-MTAB-37)	

PON3 is important and beneficial as it helps generating the platform for malignant transformation. This could represent a potential approach of innovative therapies trying to normalize the otherwise overexpressed PONs.

A first direct hint to this theory came from our recent study demonstrating that PON2 increased chemoresistance in leukemic cells [23], which is in line with genetic association studies where PON2 upregulation was associated with imatinib resistance in CML patients [19] and poor prognosis in cohorts of pediatric ALL [17, 18]. In support of the hypothesis, the same study [23] revealed that knockdown of endogenous PON2 caused spontaneous apoptosis of several human cancer cell lines—an intriguing but somewhat unexpected finding given the viability of PON2-deficient mice (the residual PON2 expression in these mice [25] may be comparable to efficient cell culture RNAi experiments).

An exciting question is how tumors achieve an increase in PON2 and/or PON3 expression, and this should be a major goal of future studies. Certainly there is no general answer to this question. Most likely, underlying mechanisms are individual for each given tumor. One simple explanation could be that, in some tissues, for example, papillary renal cell kidney carcinoma or prostate adenocarcinoma, chromosome 7, which contains the PON cluster, is amplified [16]. Another reason might be that the regulation depends on several signaling pathways, which are linked to reactive oxygen species and cancer, for example, PPAR-γ, AP-1, β-catenin/Wnt, NF-κB, HIF-1α, PI3K, and Nrf2 [26]. In accordance, earlier studies showed that PON2 expression is enhanced by oxidative stress [27], PI3K/PDGFR, PPARγ, and NADPH oxidase activation as well as by AP-1 activation [28, 29]. The urokinase plasminogen activator (uPA) system

Table 2: Expression levels of PON3 in various tumor tissues and/or cancer cell cultures. Microarray experiment (array express) listings are according to the Gene Atlas Database. cDNA levels according to [24]. Cell culture expression levels were roughly estimated as relative level comparing to A549, grouped into *low, medium,* or *high*.

Tissue (cancer)	cDNA array (fold of normal tissue)	Microarray studies	Cell culture (expression level in cell line)
Kidney	2.2	Downregulated in clear cell sarcoma of the kidney (E-GEOD-2712/E-TABM-282)	Not detectable (HEK293)
Liver	4.9	Downregulated in hepatocellular carcinoma (HCC) (Choi et al. [21])	High (Huh7) Medium (HepG2)
Lung	3.4	Upregulated in lung adenocarcinoma (E-MTAB-37/E-MTAB-62)	Medium (A549)
Pancreas	3.2	Upregulated in pancreatic carcinoma (E-MTAB-37)	
Urinary bladder	3.8		Not detectable (HT1367/RT112)
Esophagus	1.8		
Stomach	9.5		
Ovar	2.1	Downregulated in ovarian serous papillary carcinomas (OSPCs) (Santin et al. [22])	
Cervix	0.5	Downregulated in cervical carcinoma (E-MTAB-62) Upregulated in cervical carcinoma (E-MTAB-37)	Not detectable (HeLa)
Adrenal gland	1.5		
Thyroid gland	2.6	Downregulated in papillary thyroid carcinoma (E-GEOD-3467)	
Prostate	4.5	Downregulated in prostate carcinoma (E-MTAB-62)	
Testis	5.3		Not detectable (SuSa/GCT27/833K)
Uterus/endometrium	16.2		
Lymphoid tissue	2.3		
Leukemias (various)	n/a		Not detectable in AML-like Nalm6; Jurkat Tcells; PML-like HL60/HCW2 blast crisis line K562; CML-like lama; AML-like THP1 high in CML-like KCL
Non-Hodgkin	n/a	Downregulated (E-MTAB-37)	

may also be relevant, as this is increased in numerous cancers and upregulates PON2 [29].

A point of interest is why some tumors upregulate PON2 or PON3. One of the hallmarks of cancer is resistance to cell death [30]. It has been found that paraoxonases 2 and 3 provide a protection against mitochondrial cell death signaling [23, 24]. Their overexpression lowered susceptibility to different chemotherapeutics (e.g., imatinib, doxorubicin, and staurosporine) in cell culture models via diminishing proapoptotic mitochondrial O_2^- formation. It is established that oxidative stress and chronic inflammation are closely linked to cell death and cancer [26]. Therefore, it appears conceivable that tumors take advantage of the antioxidative function of PON2/PON3 to escape cell death.

3. The Antioxidative Mechanisms of PON2/PON3

Inflammation and oxidative stress contribute to the etiology of almost every known disease. Reactive oxygen species generated by enzymatic and nonenzymatic systems modify lipids and sterols, producing oxidized lipids and oxidized sterols that, if unchecked, produce a vicious cycle of undesirable inflammation and more oxidative stress. Atherosclerosis is a chronic inflammatory disease characterized by the focal accumulation of numerous cells, lipids, and extracellular matrices in the intima of arteries. Although reduced levels of high density lipoprotein (HDL) and elevated levels of low density lipoprotein (LDL) cholesterol are accepted

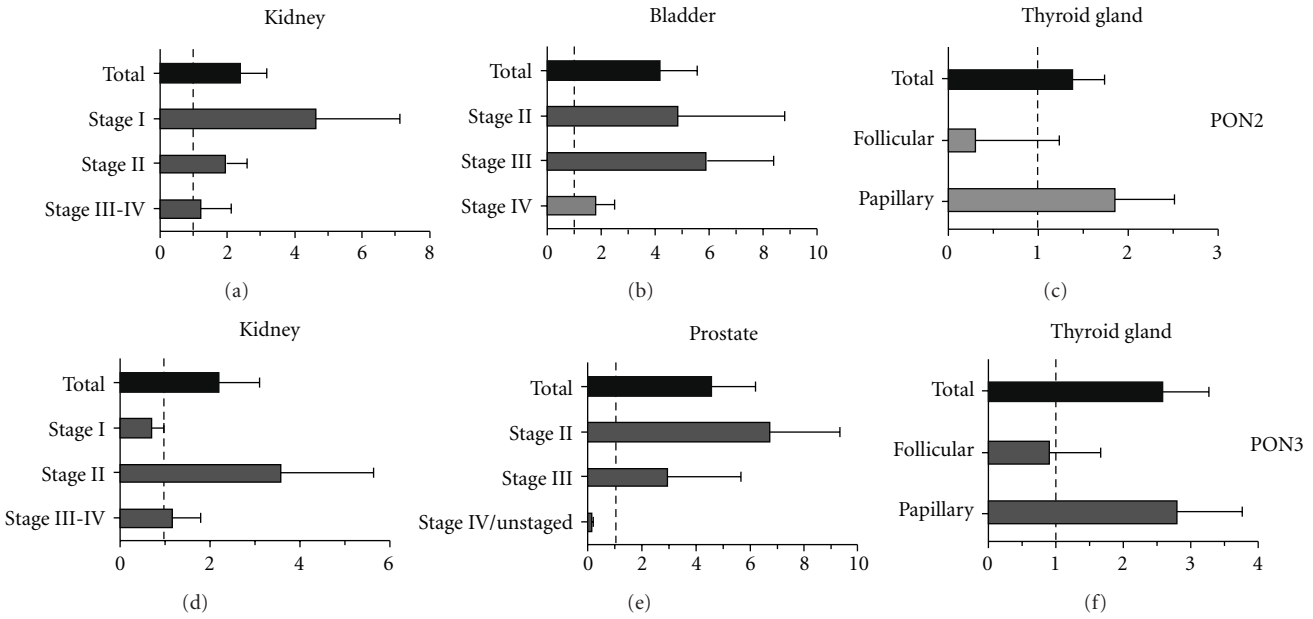

Figure 1: PON2 and PON3 are found overexpressed in early rather than late stages of tumors. Indicated cancer tissues were analyzed for PON2/PON3 cDNA levels (normalized to GAPDH) relative to healthy controls. Values were taken from recently performed arrays [23, 24].

risk factors for this disease, atherogenesis cannot solely be explained by cholesterol or lipid deposition in the arterial wall. Accumulating evidence suggests that oxidative stress plays a fundamental role in atherosclerosis. In particular, the oxidation theory for atherosclerosis proposes that LDL is a major target of oxidation and is involved in both the initiation as well as progression of atherosclerosis [31].

Although there has been a focus on PON1 due to its association with HDL, a number of studies demonstrated that PON2 and PON3 protect cells and tissues from oxidative stress by reducing reactive oxygen species [1, 25, 32–37]. PON2 and PON3 can inhibit LDL oxidation and enhance the antioxidant properties and cholesterol efflux capacity of HDL even though they are not readily found on the lipoproteins [1, 25, 32–37]. Moreover, in animal models, both PON2 and PON3 have been shown to abrogate the development of atherosclerosis [25, 35, 38]. These preclinical studies clearly demonstrated that PON2 and PON3 (similar to PON1) are (a) anti-atherogenic and (b) targets for therapy. However, to date, the physiological substrates and roles for PON2 and PON3 have not been elucidated, which similarly applies to PON1.

Recent studies suggest that PON2 [12, 38, 39] and PON3 [24] modulate the levels of reactive species in cells and in animal models demonstrating for the first time a physiological molecular link between PON proteins and oxidative stress. Based on the earlier result that PON2 was found in subcellular mitochondrial fractions [40], Altenhöfer et al. demonstrated that PON2 prevents the ubisemiquinone-mediated mitochondrial superoxide generation and apoptosis independent of its lactonase activity [12]. During Q cycle, unstable intermediate ubisemiquinone (coenzyme Q_{10} [CoQ_{10}^{-}]) can donate electron to molecular oxygen (instead of cytochrome c) leading to superoxide production and reduced ETC activity [41–43]. Devarajan et al. reported that (a) PON2 is present in the inner mitochondrial membrane (IMM), and (b) binds with high affinity to coenzyme Q10 (CoQ10), an important component of the ETC [38]. Steady-state concentrations of ubisemiquinone are increased in the IMM resulting in superoxide formation when treated with inhibitors of ETC, antimycin, or rotenone [43]. Devarajan et al. demonstrated that overexpression of PON2 reduces superoxide levels induced by either antimycin or rotenone suggesting that PON2 sequesters ubisemiquinone. Moreover, PON2-deficient mice harbour reduced ETC complex I + III activities, oxygen consumption, ATP levels, and enhanced mitochondrial oxidative stress further suggesting that PON2 maintains the respiratory chain by promoting the sequestration of the unstable reactive intermediate ubisemiquinone, thereby preventing the superoxide production. Supporting our hypothesis, previously, it has been shown that mitochondrial superoxide is inversely related to the amount of CoQ10 bound to membrane proteins [44]. Similar to PON2, Schweikert et al. have demonstrated that PON3 is also localized to mitochondria, protects against mitochondrial oxidative stress, and demonstrated that Q10 is associated with purified PON3-GFP protein [24]. This illustrates that the antiatherogenic effects of PON2/3 are, in part, mediated by their role in mitochondrial function (Figure 2). Since increased production of reactive oxygen species (ROS) as a result of mitochondrial dysfunction play a role in the development of many inflammatory diseases including atherosclerosis, the recent data on PON2 and PON3 provide a mechanistic direction for the scores of epidemiological studies that show a link between PON proteins and numerous inflammatory diseases including Type II diabetics and cancer.

Atherosclerosis and insulin resistance are multifactorial diseases that are commonly associated with dyslipidemia, oxidative stress, obesity, hypertension, and chronic inflammation. The liver is not only the primary site of lipid metabolism, but is a major site for glucose uptake, production, and storage. Its role in glucose metabolism is strongly influenced by systemic as well as local oxidative and inflammatory stimuli [45, 46], which in turn influences whole-body insulin responsiveness [47]. Hepatic glucose metabolism is strongly influenced by oxidative stress and proinflammatory stimuli. Given the elevated oxidative stress levels and abnormal lipid metabolism reported previously in PON2-deficient mice [25, 38], Bourquard et al. hypothesized that atherosclerosis may be accompanied by impaired hepatic insulin signaling and showed that PON2 deficiency is associated with inhibitory insulin-mediated phosphorylation of hepatic insulin receptor substrate-1 (IRS-1) [39]. Factors secreted from activated macrophage cultures derived from PON2-deficient mice are sufficient to modulate insulin signaling in cultured hepatocytes in a manner similar to that observed *in vivo* [39]. It was further demonstrated that modulation of hepatic insulin sensitivity by PON2 is mediated by a shift in the balance of NO and $ONOO^-$ (peroxynitrite) formation. These studies show that PON2 plays an important role in insulin sensitivity by its ability to modulate reactive species most likely as a result of PON2's association with mitochondrial function.

Oxidative stress has long been associated with the pathophysiology of cancer. In particular, enhanced ROS formation increases DNA damage, genome instability, and cell proliferation especially during cancer initiation. On the other hand, oxidative stress also counteracts tumorigenesis, as it induces senescence and drives apoptosis and other cell death pathways [48]. The precise spatiotemporal control of ROS generation is therefore a critical regulator of cell survival and death, for instance since overwhelming mitochondrial oxidative stress exerts apoptotic rather than protumorigenic functions. Nevertheless, reactive oxygen species may be conducive to the vitality of cancer cells and drive signaling transduction pathways, which lead to activation of redox-sensitive transcription factors and genes involved in cancer cell growth, proliferation, and survival [26]. In conclusion, PON2 and PON3 reduce oxidative stress and inflammation and thus act as central regulators of diseases, including cancer and atherosclerosis.

4. Paraoxonases and the Regulation of Cell Death

The antioxidative effects of PON2 and PON3 were reported long ago, but underlying mechanisms were uncovered just recently [12, 24, 38]. This similarly applies to the cell death-reducing activity of PON2, where discovery [40] and mechanistic realization [23] were separated by years. Based on the latest knowledge, these enzymes modulate execution of the apoptotic program. In this chapter, we review their involvement in apoptosis and discuss their putative functions in other cell death pathways.

Tumor cells evolve a plethora of strategies to resist cell death with the intrinsic apoptotic program being implicated as a major barrier to cancer formation. Execution of intrinsic cell death is mainly controlled by the balance of pro- and antiapoptotic Bcl-2 protein family members [30, 49], because they regulate mitochondrial pore opening and cytochrome C release. Importantly, it also requires intramitochondrial redox signaling to liberate cytochrome C from its membrane attaching molecule, cardiolipin [50, 51]. In fact, this is a two-step process because neither mitochondrial membrane permeabilization alone nor redox-triggered disruption of the cytochrome C/cardiolipin interaction sufficiently activates the cascade. Recent studies revealed that PON2 and PON3, due to interaction with coenzyme Q10, diminish O_2^- release on either side of the inner mitochondrial membrane [23, 24]. This results in both lowered cardiolipin peroxidation and cytochrome C release, providing a marked resistance against apoptosis. Thus, if a cancer cell needs to escape from mitochondrial redox-dependent cell death, it appears beneficial to increase PON2 or PON3 expression. In accordance, both enzymes protected against a range of chemotherapeutics when overexpressed [23, 24]. In contrast, receptor-mediated apoptosis was unchanged, at least in type-I cells, where stimulation with TRAIL or TNF-α directly activated caspases 8 and 3. This may be different for type-II cells, which involve mitochondrial actions.

Another important stress and cell death pathway is the unfolded protein response (UPR) as a result of insurmountable ER stress [52]. Both PON2 and PON3 protected against UPR-mediated apoptosis in a similar manner, that is, by negative modulation of JNK signaling, CHOP induction, and subsequent caspase activation [12, 23, 24]. Canonical UPR signaling (via ATF6, XBP1, or p-eIF2a) was unchanged, at least by PON2, so their precise mechanisms of protection remain uncertain. Future studies must reveal if PON2/PON3 act just through their mitochondrial effects or if they modulate signaling from IRE1 to TRAF2/ASK1, from the ER to mitochondria or local ROS/Ca^{2+} responses and how they reduce JNK phosphorylation. Interestingly, PON2 overexpression was induced by ER stress and protected against UPR-triggered cell death, but this was lost upon major disturbances of Ca^{2+}—homeostasis, presumably by calpain-dependent PON2 degradation [53]. Our current studies suggest that this similarly applies to PON3 ([54] this issue and data not shown). The postulated functions of PON2 and PON3 in apoptosis and ER stress-induced cell death are summarized in Figure 3.

A vital physiologic response that regulates cellular metabolism and survival is autophagy. This pathway operates at low basal levels but can be markedly increased under specific stress conditions. It enables breakdown of macromolecular structures and organelles to allow recycling of catabolites. Therefore, autophagy may alleviate nutrient limitation as experienced by many cancer cells. However, autophagy has opposing effects on different tumor cells and may cause survival of one but death of the other [55]. Whether paraoxonases modulate this pathway is unknown and no interaction with Bcl-2 family/autophagy-related

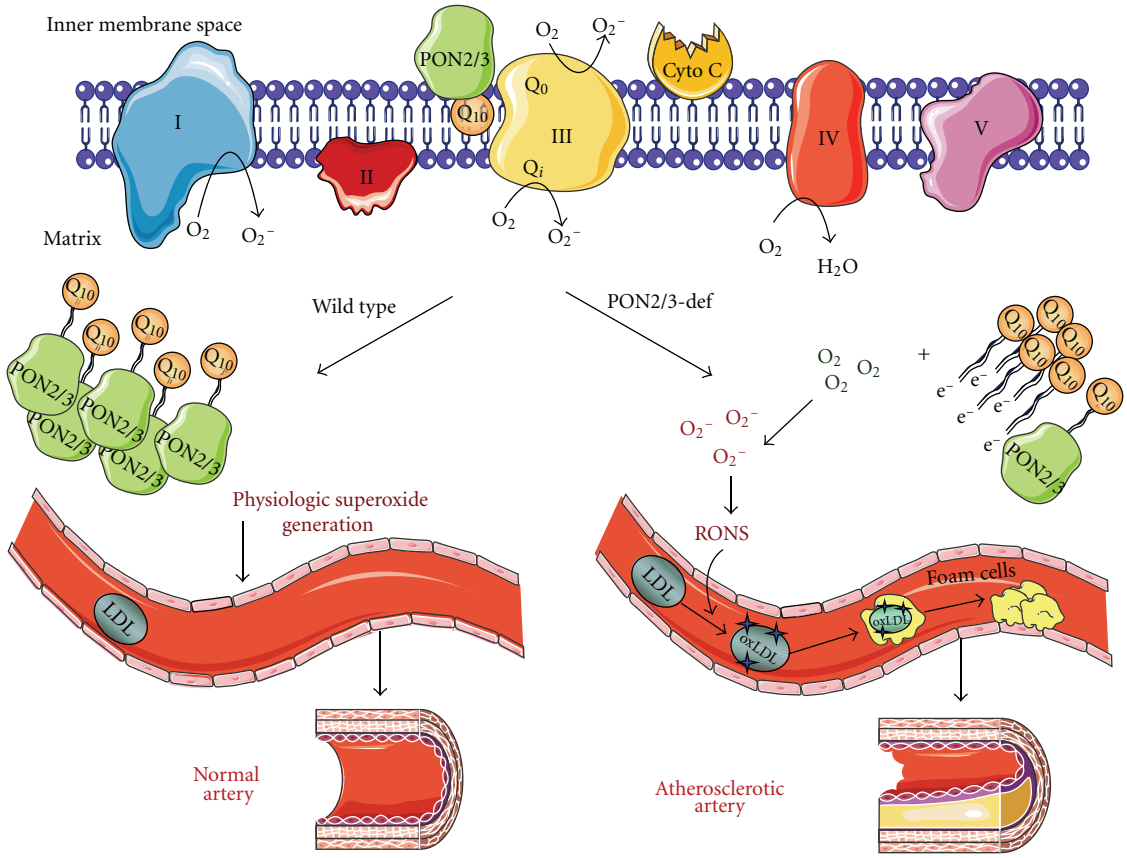

Figure 2: Schematic presentation of the suggested antioxidative mechanism of PON2 and PON3. A current model for the role of PON2/3 in the development of atherosclerosis. Ubisemiquinone is released from ETC in the mitochondria during Q cycle. *Right.* In the absence of PON2/3, ubisemiquinone donates electron to molecular oxygen to form superoxide; superoxide generates other reactive oxygen/nitrogen species (RONS), which oxidize LDL to form oxLDL; macrophages engulf oxLDL to form foam cells; foam cells attach to the arterial wall and subsequently develop into atherosclerotic lesions. *Left.* In the presence of PON2/3 (in wild type mice), ubisemiquinone binds to PON2/3. The binding of PON2/3 and ubisemiquinone prevents superoxide generation thereby preventing the development of atherosclerosis. Note: it is currently unknown if PON2/3 face the matrix side of the inner mitochondrial membrane or the one directed towards the innermembrane space; also, the stoichiometry of PON2/3 versus Q10 is unknown. Abbreviations: I-NADH: ubiquinone oxidoreductase, II: succinate coenzyme Q reductase, III: ubiquinol cytochrome coxidoreductase, IV: cytochrome c oxidase, V-ATP synthase, Cyto c cytochrome c, Q10-coenzyme Q10.

proteins has been reported. On the other hand, oxidative stress is mutually linked with autophagy, and there it plays an important role in cancer therapy resistance and tumor progression. The connection between ROS and autophagy is illustrated, for instance, by TNFα-induced signaling in sarcoma cells [56], or by the autophagy-relevant factor Atg4 whose delipidating activity is sensitive to mitochondrial H_2O_2 production [57] (see [58] for a detailed overview of this topic). Paraoxonases hence could have a profound impact on autophagy due to their central redox effects. Because autophagy by ROS can serve as rescue pathway but may also initiate autophagic cell death, it requires more in-depth evaluation including the origin and targets of ROS. In a similar manner, this may also be true for necroptosis (or necrotic cell death), which contrasts with the chemical- or injury-triggered necrosis and represents another, RIP1 kinase-dependent programmed cell death pathway. Necroptosis is of relevance, for example, for damages resulting from ischemia-reperfusion, such as stroke or myocardial infarction. Moreover, necrotic cell death may paradoxically be even beneficial to neoplasias as this form of cell death attracts tumor-promoting inflammatory cells [30]. TNF-induced necroptosis has been shown to generate complex-I-mediated ROS in mitochondria, which is crucial to this process and accounts for ultrastructural changes observed in such cells [59]. Because PON2 as well as PON3 were able to reduce superoxide released from mitochondrial complex-I [12, 24, 38], it would hence be a promising endeavor to test PONs in TNF-induced necroptosis.

Another hallmark of tumor cells is the reprogramming of glucose metabolism in order to provide efficient fueling of the high energy demand associated with rapid cancer growth. For the most part, this is manifested as a switch to (aerobic) glycolysis but also involves two different cancer cell subpopulations—one using glucose and a second set consuming lactate produced by the former (see [30] and references therein). How overexpressed PONs could play a role in this system has not been explored, and speculations can

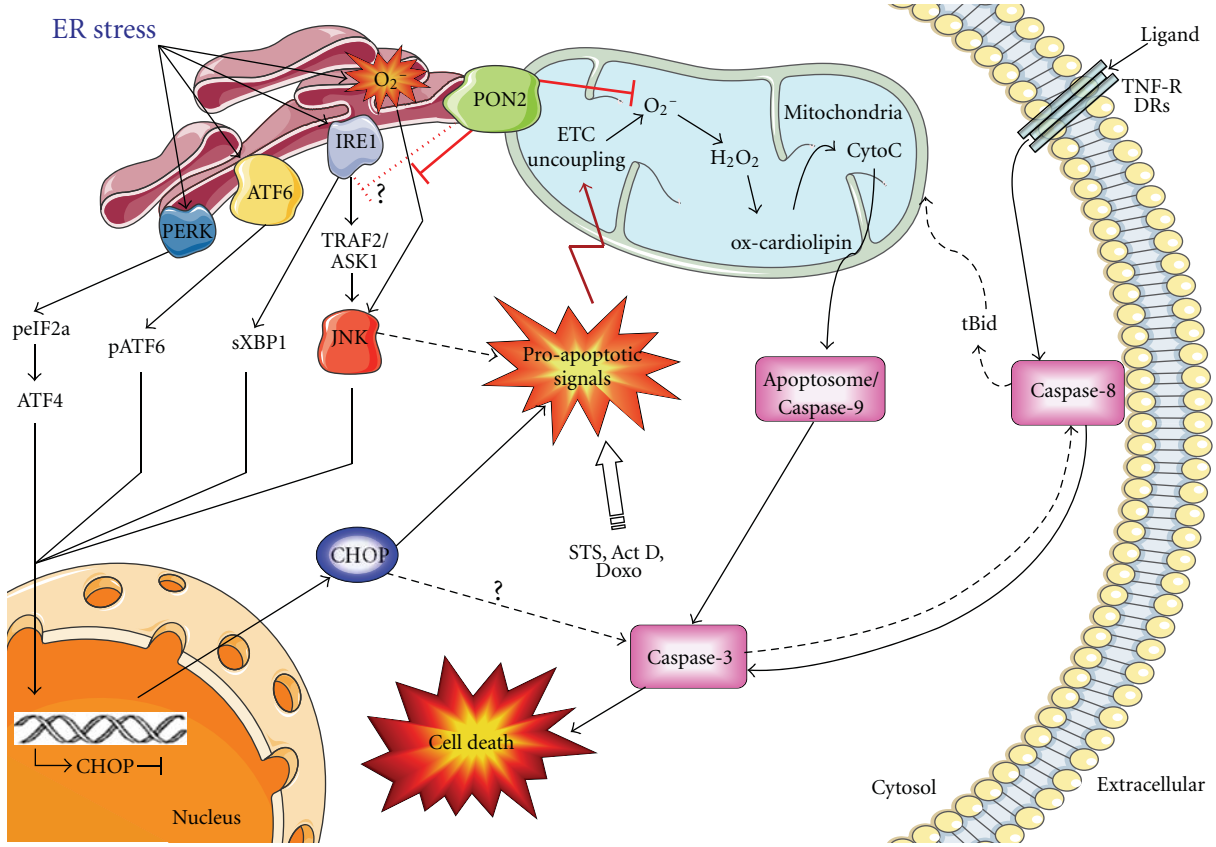

FIGURE 3: Schematic presentation of the suggested antiapoptotic mechanism of PON2 and PON3. Its ability to prevent mitochondrial O_2^- formation impacts on both ER stress-induced pathways (via acting on JNK and CHOP) as well as mitochondrial proapoptotic signaling such as cardiolipin peroxidation and cytochrome C release. See text for details. From our current understanding, PON2 is functionally interchangeable with PON3.

only be extrapolated from the PON2-deficient mice. Intriguingly, mitochondria from PON2 deficient mice produced less ATP, had impaired complex-I and -III activities, and showed enhanced oxidative stress and consumed less oxygen, resulting in an overall exhausted mitochondrial function [38]. Thus, if the opposite was true for PON2 overexpressing cells, this would ensure mitochondrial functionality and could support the energy efficacy of tumor cells.

Despite a role of paraoxonases directly in cancer cells, it could also be interesting to scan for other near-by functions. Cancer progression is determined by intracellular changes in the malignant cell itself, but also modulated by surrounding stromal cells in the tumor microenvironment. It is composed of leukocyte infiltrates consisting, for example, of endothelial cells, mast cells, T cells, and tumor-associated macrophages (TAMs). The protumorigenic TAMs are involved in critical features of neoplastic cells (such as migration & metastasis), in the inflammatory tumor microenvironment, angiogenesis, survival under hypoxia, and immune evasion [60]. Although most research groups that work on PONs employ macrophages, no study addressed TAMs to our knowledge. PON2 expression is enhanced during monocyte to macrophage differentiation in a ROS-dependent manner [28], but it is uncertain how the established anti-inflammatory and antioxidative effect of PON2 could fit particularly into TAM functions. Thus, it may be worthwhile to assess PON2 levels in M1 versus M2 macrophages. One may speculate that TAMs have low levels of PON2, which would favor ROS formation and inflammatory responses [25, 38] and may also increase production of the metastasis-augmenting IL-1β (at least, the latter has been shown for PON2 knockdown in endothelial cells [61]). Alternatively, given that TAMs represent an interesting therapeutic target [60], monitoring their recruitment in tumors of wild-type mice compared to those deficient in PON2 or PON3 mice may also uncover new aspects of both paraoxonases and TAM infiltration with potential therapeutic implications. A direct role of PON3 in TAMs may be unlikely considering that PON3 appeared undetectable in human macrophages [27].

5. Future Perspectives

5.1. Manipulation of PON Expression: A Double-Edged Sword? The relevance of PON2 and PON3 to the cancer field has been demonstrated only recently. Few studies addressed their direct role, and our current knowledge appears somewhat fragmented. However, much of their antiapoptotic mechanism has been revealed. This allows tentative evaluation of their pharmacological usefulness. We presented multiple

lines of evidence demonstrating that PON2 and PON3 are frequently found upregulated in cancer samples. Specific regulatory mechanisms are mostly unknown. The altered expression appears similar but also distinct for each of the two enzymes, varying with the tissue itself, with the specific kind of tumor and its stage, progression, differentiation, and/or metastasizing potency (see above). Up to now, two studies directly analyzed PON2 and PON3 levels in a variety of different tumors or representative cell lines [23, 24]. Addressing this question from the opposite direction, many independent laboratories used microarrays to investigate gene expressions in different tumors and often (though not always) found enhanced levels of these enzymes (see above).

Our current understanding allows to conclude that overexpression of PON2/PON3 diminishes the execution of the apoptotic program. Most likely, the antioxidative function of these enzymes represents the antiapoptotic trigger, whereas the contribution of their enzymatic activities remains unknown, if at all significant. How this works in the ER and relates to the UPR that is unknown. In the simplest model, however, this refers to electron transport within the inner mitochondrial membrane, which is in close proximity to and control of powerful apoptotic modifiers. From such perspective, upregulation of PON2 or PON3 in cells destined for apoptotic evasion appears consequential. As a logical deduction, the controlled reduction of overexpressed PON2 and/or PON3 in a given tumor may represent a novel approach to enhance its susceptibility to chemotherapeutics and to improve the therapy's effectiveness. Such hypothesis is encouraged by the observation that PON2 knockdown induced spontaneous apoptosis of several human tumor cell lines and because overexpression of either PON2 or PON3 granted robust chemotherapeutic resistance [23, 24]. PON2/PON3 expression varied substantially between different cell types, and high levels did not automatically correlate with cellular responsiveness to its knockdown. This outlines that individual approaches must be identified because PON2 and PON3, similar to already established targets, are unlikely to be beneficial in every setting. Therefore, future studies need to identify a rapid, reliable, and simple read-out system to monitor if a given tumor relies on high PON levels. This should be worthwhile, for example, in leukemic transformation in pediatric B-precursor ALL, where PON2 was among a very small group of factors highly expressed in patients with worst outcome, high risk, and affected relapse-free survival [18]. Other rewarding projects may be deduced from Table 1, where we summarized the combination (if available) of studies reporting PON2 overexpression in a given tumor. There is limited evidence for PON3 since we are just beginning to understand its function and because PON3, compared to PON2, is expressed to much lower levels and in fewer tissues. Given our recent data [24], we nevertheless conclude PON3 also represents a molecule actively involved in cell death regulation.

Can we then simply strive for a systemic downregulation of PON2/PON3 in selected cancer therapies and if so, which specific risks could be expected? PON2-deficient mice were in a pronounced inflammatory status [25] and suffered from a series of other defects linked to severe malfunctions (Witte & Horke; unpublished). Furthermore, (i) reduced PON2 levels enhanced atherogenesis in mice [25, 38], modulated monocyte chemotaxis and cell-mediated LDL oxidation [25, 32], and correlated with atherosclerosis progression in humans [62]; (ii) PON2 may have a neuroprotective role [63]; (iii) genetic associations linked PON2 with amyotrophic lateral sclerosis [64], Alzheimer disease [65], microvascular complications in diabetes [66], coronary heart disease [67], or perhaps also obesity [68]; (iv) PON2 plays a dominant role in the hydrolysis of bacterial virulence regulators [69–71] such that its knockdown may favor certain infections. In a similar manner, human PON3 also has a protective role against atherosclerosis and obesity [1, 33–35], but interpretation is complicated by the fact that there are conflicting reports on its expression pattern, which also varies with the species. Marsillach et al. found PON3 by immunohistochemistry in human aortic walls and macrophages [72], while we did not detect human PON3 message or protein in immortalized EA.hy 926 macrovascular endothelial cells, in primary HUVECs (human umbilical vein endothelial cells), SMCs (human coronary artery smooth muscle cells), or AoAFs (aortic adventitial fibroblasts; [24] and data not shown); this makes it difficult to reveal the mechanistic site of action. Moreover, human PON3 is present on HDL particles and absent in macrophages while the opposite is true for mice [1, 27]. In general, it has been postulated that human PON3 exerts its (antiatherogenic) function rather inside than outside the cells [35, 73], similar to PON2 and likely different from PON1. Studies performed by Shih et al. also revealed a role for PON3 in lipid metabolism that links to adiposity; intriguingly, this was gender-specific for yet unknown reasons [35]. Collectively, PON2 and PON3 have protective functions in cardiovascular diseases, and PON2 plays a dominant role in antibacterial defense, such that an untargeted knockdown may favor these illnesses. As a consequence, a systemic downregulation of PON2 or PON3 does not seem advantageous, as it likely causes a range of serious side effects.

Given that PON2/PON3 protect against atherosclerosis and stabilize atherosclerotic plaques (see above and [33, 36, 74]), may their upregulation then be beneficial to combat atherosclerosis? This is a relevant aspect given the overall number of deaths caused by cardiovascular diseases, which outnumber all cancers [75]. We would first need to determine if enforced PON2/PON3 expression blocks progression or, under optimal conditions, causes regression of established atherosclerotic plaques. Overexpression has been shown to prevent atherogenesis in murine models [25, 33, 35, 36, 74]; however, in clinical reality, patients show up with fully established plaques and need alleviating care, as it is too late for prophylactic approaches. Yet, there is little [33] or no evidence if PONs block progression of established plaques or even cause regression, perhaps due to their anti-inflammatory effects. Should such studies be positive, how can we exploit the beneficial effects of PON2/PON3 against atherosclerosis while concomitantly avoiding their pro-oncogenic function? The first step is the identification of pathways regulating PON expression and the identification

of lead substances increasing or decreasing endogenous levels. Then, one solution may come from drug-eluting stents implanted into the atherosclerotic vascular wall—an already established clinical application. This would allow an upregulation of PON2/PON3 directly in the diseased vessel without promoting tumor formation in distant organs. Another solution may come from the specific targeting of effector molecules or pathways (once they are identified) for example, via surface receptors—a likely realistic mission given the accessibility of the vascular wall. In turn, similar approaches could be useful to downregulate PONs in cancer tissues. It would also be valuable to inhibit the interaction of PONs with coQ10 as this could block their antioxidative effect and render these enzymes useless for cancer cells. Finally, the time line may be advantageous in consideration of slow-progressing atherosclerosis and fast-progressing tumors; in some cases this may allow transient downregulation of paraoxonases to boost efficacy of anticancer therapies while not immediately causing plaque formation.

In summary, there exists a remarkable twist in the paraoxonase field since we know that PON2 and PON3 protect against cardiovascular diseases but favor tumor formation. It will be exciting to await further developments and the usefulness of these enzymes in the fight against two of the most significant human diseases.

Abbreviations

ALL: Acute lymphoblastic leukemia
CHOP: C/EBP homologous protein (growth arrest/DNA-damage inducible gene 153, GADD153)
CML: Chronic myeloid leukemia
ER: Endoplasmic reticulum
HDL: High-density lipoprotein particle
LDL: Low-density lipoprotein particle
PON: Paraoxonase
ROS: Reactive oxygen species
SOD: Superoxide dismutase
TNF-α: Tumor necrosis factor-α
TRAIL: Tumor necrosis factor-related apoptosis inducing ligand
UPR: Unfolded protein response.

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Research Article

Novel Associations of Nonstructural Loci with Paraoxonase Activity

Ellen E. Quillen, David L. Rainwater, Thomas D. Dyer, Melanie A. Carless, Joanne E. Curran, Matthew P. Johnson, Harald H. H. Göring, Shelley A. Cole, Sue Rutherford, Jean W. MacCluer, Eric K. Moses, John Blangero, Laura Almasy, and Michael C. Mahaney

Department of Genetics, Texas Biomedical Research Institute, P.O. Box 760549, San Antonio, TX 78245-0549, USA

Correspondence should be addressed to Ellen E. Quillen, equillen@txbiomedgenetics.org

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The high-density-lipoprotein-(HDL-) associated esterase paraoxonase 1 (PON1) is a likely contributor to the antioxidant and antiatherosclerotic capabilities of HDL. Two nonsynonymous mutations in the structural gene, *PON1*, have been associated with variation in activity levels, but substantial interindividual differences remain unexplained and are greatest for substrates other than the eponymous paraoxon. PON1 activity levels were measured for three substrates—organophosphate paraoxon, arylester phenyl acetate, and lactone dihydrocoumarin—in 767 Mexican American individuals from San Antonio, Texas. Genetic influences on activity levels for each substrate were evaluated by association with approximately one million single nucleotide polymorphism (SNPs) while conditioning on *PON1* genotypes. Significant associations were detected at five loci including regions on chromosomes 4 and 17 known to be associated with atherosclerosis and lipoprotein regulation and loci on chromosome 3 that regulate ubiquitous transcription factors. These loci explain 7.8% of variation in PON1 activity with lactone as a substrate, 5.6% with the arylester, and 3.0% with paraoxon. In light of the potential importance of *PON1* in preventing cardiovascular disease/events, these novel loci merit further investigation.

1. Introduction

More than 2,200 Americans die from cardiovascular disease each day with 75% of those deaths attributable to atherosclerosis [1]. Atherosclerosis is characterized by the buildup of fatty lesions, inflammation, and scarring of arterial walls with oxidative stress as a primary contributing factor. Paraoxonase 1 (PON1) is a high-density-lipoprotein-(HDL-) associated esterase which appears to contribute to the antioxidant and antiatherosclerotic capabilities of HDL. PON1 is synthesized in the liver and secreted into the bloodstream where it is capable of breaking down both man-made and naturally occurring compounds. Named for its ability to hydrolyze organophosphates like paraoxon [2, 3] found in insecticides, PON1 is also able to hydrolyze *N*-acyl-homoserine, a lactone used by pathogenic bacteria [4], and lipid peroxides, thereby inhibiting the formation of foam cells known to contribute to atherosclerosis [5, 6].

PON1 has been widely studied following evidence that high activity levels decrease systemic oxidative stress and are associated with a lower incidence of cardiovascular events [7]. PON1 levels have been tied to a number of other disorders including type 1 and 2 diabetes [8, 9], thyroid dysfunction [10], uremia [11], renal failure [12], and inflammatory response [13].

The structural gene *PON1* is by far the largest contributor to variation in serum PON1 activity levels with four known single nucleotide polymorphisms (SNPs) in the promoter region [14–16] and two nonsynonymous substitutions in the coding region of the gene [17, 18] shown to significantly influence activity levels. Amino acid substitution 192Q > R (rs662) specifies 2 allozymes [17] whose differences in activity are substrate dependent. The R allozyme shows greater activity for the organophosphates paraoxon and fenitroxon while the Q form more efficiently hydrolyzes other organophosphates including diazoxon, soman, and sarin.

Phenyl acetate is hydrolyzed at the same rate by both forms. [19–21] The 192Q > R substitution is associated with up to 13-fold interindividual differences in PON1 activity [22] and an adjusted hazard ratio for major cardiac events of 1.5 [23]. The 55L > M (rs854560) substitution has also been associated with variation in serum PON1 activity levels, but has a smaller effect size. These polymorphisms have also been linked to Parkinson's disease [24], inflammatory bowel diseases [25], and, controversially, to Alzheimer's disease and vascular dementia [26].

PON1 is part of a family of genes including *PON2* and *PON3* located within a 140 kb region at 7q21.3. Although *PON2* and *PON3* also synthesize paraoxonase proteins, PON2 is not excreted into the blood and any effect by either protein on atherosclerosis or cardiovascular disease is small [27]. Although the *PON* region explains a large degree of the variation in PON1 activity, PON1 activity levels are still better predictors of disease than *PON1* genotypes alone [22, 28]. This supports the existence of additional, unidentified polymorphisms associated with PON1 activity as well as potential epigenetic contributors.

Despite the large body of literature on the *PON* loci, this study is the first to take a genome-wide association approach to identify additional genomic regions contributing to interindividual variation in PON1 activity. Previous studies of this sample identified QTLs for PON1 activity with paraoxon as a substrate on chromosomes 7 (*PON1*), 12, 17, and 19 using whole-genome multipoint linkage analysis [29]. Further investigation, which included alternate substrates and conditioned on the major QTL at chromosome 7, located additional QTLs on chromosomes 1, 3, and 14 [30].

2. Methods

2.1. Subjects. Samples were drawn from the San Antonio Family Heart Study (SAFHS) which is composed of 1414 individuals (837 females, 577 males) belonging to 42 extended pedigrees originating with probands randomly ascertained with respect to disease status and phenotype. All probands were Mexican-American individuals between the ages of 40 and 60 at the time of ascertainment, living in San Antonio, TX, with a minimum of six offspring and/or siblings who were at least sixteen years of age and also living in the area. After giving their informed consent, participants underwent a physical examination, demographic and lifestyle interview, and provided blood samples for genotyping and blood chemistry analysis. The study protocol was approved by the Institutional Review Board at the University of Texas Health Science Center in San Antonio and is described in more detail in a previous publication [31].

2.2. Paraoxonase Activity. Based on previous evidence of genetic variation giving rise to different activity levels in a substrate-dependent fashion, PON1 activity was assessed on an organophosphate, an arylester, and a lactone. Activity was calculated for 767 individuals based on standard spectrophotometric assays described previously [32]. Briefly, PON1-para activity was determined from the rate of conversion of paraoxon to *p*-nitrophenol, PON1-aryl activity was calculated from the conversion of phenyl acetate to phenol, and PON1-lact activity was based on the conversion of dihydrocoumarin to 3-(2-hydroxyphenyl)propionate. The underlying shared genetic relationship between the activity levels was estimated by calculating the shared genetic covariance (ρ_G) for each pair of activity levels. PON-aryl showed significant evidence of a shared genetic contribution with both PON-lact ($\rho_G = 0.54$, $P = 1.9e-19$) and PON-para ($\rho_G = 0.65$, $P = 5.5e-23$); however, the genetic correlation between the activity levels for PON-lact and PON-para was essentially zero. This suggests that there are likely to be independent variants influencing activity levels for the different substrates.

2.3. Statistical Genetic Analyses. DNA was extracted from buffy coats and used for genotyping on a series of Illumina microarrays (Illumia, Inc., San Diego, CA). 931,219 SNPs passed quality control and were included in the genome-wide association (GWA) analysis. Where it could be done with a high degree of certainty, known pedigree relationships were used to infer missing genotype data using the PEDSYS routine INFER [33]. For ambiguous genotypes, a weighted average of the possible genotypes was used.

Association was assessed for each measurement of paraoxonase activity using the measured genotype test implemented in the program SOLAR (Sequential Oligogenic Linkage Analysis Routines) [34] which takes into account relationships among family members. For all analyses, paraoxonase activity and age were normalized and sex, age, age^2, and the interaction of sex by age were used as covariates. Additional covariates considered but not found to be associated with PON1 activity in this sample ($P > 0.1$) include dietary measures (proportion of saturated fats, monounsaturated fats, polyunsaturated fats, and fat calories reported in diet), alcohol and cigarette consumption, body mass index (BMI), and total metabolic equivalents as a measure of activity level. To minimize the risk of false associations due to stratification in this admixed sample, principal component analysis was performed on the full set of approximately one million genotypes to capture the total genetic variation in the sample and the first four principal components (accounting for only 2.8% of the variation in the sample) were included as covariates. The efficacy of this correction for stratification was examined by calculating λ from the distribution of the lower 90% of P values for each GWA. There was no evidence of overdistribution due to stratification as all λ values were less than 1.02. Manhattan and Q-Q plots for each GWA can be found in Supplementary File 1 available online at doi:10.1155/2012/189681.

To identify genes contributing to the residual variation, genome-wide association was assessed while including genotypes at the two major *PON1* substitutions 192QR and 55LM (rs662 and rs854560) as covariates. Despite the inclusion of these known variants, other SNPs in the *PON* region of chromosome 7 still showed association with PON1 activity for all three substrates. This suggests that additional variation in *PON1* or nearby genes is contributing to the

variation in PON1 activity levels. To remove all effects of the *PON* loci, four additional SNPs—rs854522, rs854534, rs757158, and rs7803148—each tagging a haploblock in the region surrounding the three *PON* genes—were included as covariates for the GWA analysis. With the inclusion of these covariates, there is 95% power to detect variants with an effect size (R_G^2) of at least 0.041 for PON1-aryl, 0.041 for PON1-lact, and 0.024 for PON1-para.

2.4. Candidate Gene Identification. The program SUSPECTS was used to identify candidate genes in the region surrounding each SNP showing significant association with PON1 activity [35]. SUSPECTS determined candidate genes on the basis of similarities in structure, gene ontology, InterPro domains, and/or gene expression with genes known to be related to atherosclerosis, cholesterol regulation, or heart disease. Additionally, all genes within 250 kb of the associated SNP were investigated for potential functional relationships to PON1 activity. A 250 kb region is sufficient to encompass more than 97% of haploblocks in Mexican populations [36]. To further contextualize the results of the GWA analysis, gene set and pathway enrichment analysis was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) [37, 38]. All markers were annotated and the genes found in the top 1% of associations were clustered based on similarity of gene ontology (GO) terms and compared to the genes represented by the remaining 99% of markers.

Additionally, the relationship between gene expression levels measured on an Illumina Sentrix Human Whole Genome (WG-6) Series I BeadChip array [39] and associated SNPs was calculated from the genetic covariance in a polygenic model including sex, age, age^2, sex*age, 192QR, 55LM, and the additional associated loci in the *PON* region.

3. Results

The heritability of paraoxonase activity varies with substrate, $h^2 = 0.65$ for phenyl acetate (PON1-aryl), $h^2 = 0.73$ for paraoxon (PON1-para), and $h^2 = 0.79$ for dihydrocoumarin (PON1-lact) after the inclusion of sex and age in the model. Similarly, the phenotypic variation explained by the covariates and the *PON1* alleles differs among the substrates (Table 1). Cumulatively, age, sex, and their interaction effect explain less than 4% of the variation in PON1 activity for all substrates. In this sample, known *PON1* variants (192QR and 55LM) explain 50.4% of the variation in PON1-para activity but only 9.6% of the variation in PON1-aryl and 12.4% of the variation in PON1-lact activities. Previous research in individuals of Korean ancestry reported that 192QR explained 65.8% of variation in PON1-para activity [40]. A similar study in individuals of European ancestry estimates that 192QR explained 46% and 55LM explained 16% of the PON1-para variation [41]. Although the haplotype blocks in the Mexican American sample are similar in location to those seen in the CEPH Europeans downloaded from HapMap, the linkage disequilibrium (LD) is more extreme, likely due to admixture. This increase in LD would be expected to inflate the explanatory power of the known polymorphisms, so it is unclear why 192QR and 55LM explain less variation in this sample.

Table 1: Proportion of variance explained by covariates. The proportion of variation explained by each significant variable was calculated for the three substrates by adding each to a polygenic model in SOLAR.

	Variation explained		
Covariate	PON1-aryl	PON1-lact	PON1-para
Sex	0.4%	0.0%	0.4%
Age	1.1%	1.4%	0.4%
Age^2	1.1%	1.9%	0.1%
Sex*age	0.7%	0.4%	0.7%
192QR	3.9%	8.3%	36.5%
55LM	5.7%	4.1%	13.9%
rs854522	2.7%	1.8%	1.1%
rs854534	3.4%	3.8%	2.1%
rs757158	11.5%	9.2%	4.1%
rs7803148	0.9%	1.1%	0.6%

To assess the residual variation in the *PON* region of chromosome 7, haplotype blocks were identified using the solid spine of linkage disequilibrium method implemented in Haploview [42]. The SNP rs757158 tags a haploblock including the 5′ promoter region of *PON1* and explains the greatest proportion of remaining variation in PON1 activity for all substrates. This is in line with previous reports of promoter polymorphisms. As seen for 192QR and 55LM, the promoter polymorphisms have different effects depending on the substrate—this region explains a larger proportion of the variation in PON1-aryl and PON1-lact than in PON1-para. Polymorphisms in the remaining haploblocks are also associated with variation in PON1 activity. rs854534 tags a haplotype block including the majority of the genic region of *PON1*, rs854522 tags a region downstream of all the *PON* genes, and rs7803148 lies in the genic region of *PON2* and tags a haplotype block that includes the entirety of that gene and the majority of *PON3*. Variation in these three haploblocks cumulatively explains less variation in PON1 activity than the haploblock tagged by rs757158. Following the inclusion of these markers as covariates, there are no significant associations in the *PON* regions for PON1-para or PON1-lact; however, PON1-aryl activity is suggestively associated with rs2299262, an intronic SNP in *PON1* that explains 2.8% of the variation in PON1-aryl activity. It should be noted that while the proportion of variation described here and subsequently is useful for comparing the relative contributions of the loci to the variation in activity levels, there can be a substantial upward bias in these estimates unless replicated in an independent sample [43].

The variation in effect size of the polymorphism by substrate bolsters the previously identified substrate-specific effect and indicates additional genetic variants must be contributing to differences in activity levels for PON1-aryl and PON1-lact in particular. The relatively large amount of variation captured by including additional SNPs in the

Table 2: Summary of Significant and Suggestive Associations. SNP associations varied among substrates with overlap only at rs1078701 on chromosome 4. For each SNP, the chromosomal location, proportion of variation in PON1 activity explained, measured genotype test *P*-value, and minor allele frequency are listed. Shoulder SNPs are SNPs ranking in the top 5% of associations located within 500 kb of the significantly associated SNP. Genes identified by SUSPECTS are within 7.5 Mb of the candidate SNP and have a similarity score greater than 15. Bolded genes are discussed in the texts as the most likely contributors to PON1 activity based on known function, however, other genes may have unknown functions important in the regulation of PON1. The two associated SNPs on chromosome 3 are in perfect linkage disequilibrium and explain 2.7% of the variation in PON1-lact cumulatively.

Substrate	SNP	Chr	Position	Variation Explained	MG *p*	MAF	Shoulder SNPs	SUSPECTS Genes
para	rs12083993	1	64,691,396	1.2%	$1.8E-07$	0.3%	34	*ALG6*, ***ANGPTL3***, ***CYP2J2***, *IL12RB2*, *INSL5*, ***LEPROT***, *OMA1*, *PTGER3*
lact	rs13322362	3	76,613,497	2.7%	$4.8E-07$	5.4%	6	*PROK2*, *ROBO1*, *ROBO2*
lact	rs11915977	3	76,613,530		$4.8E-07$	5.5%		
para				1.8%	$4.7E-09$		32	
aryl	rs1078701	4	8,241,119	2.8%	$3.0E-08$	2.2%	19	***ADD1***, *CPZ*, *DGKQ*, *FGFBP1*, *FGFR3*, *FGFRL1*, *HGFAC*, *HS3ST1*, *LRPAP1*, *MXD4*
lact				2.3%	$3.8E-07$		15	
aryl	rs2299262	7	94,787,864	2.8%	$1.3E-07$	43.9%	24	*PON1*
lact	rs7225624	17	47,811,373	2.9%	$4.7E-09$	0.3%	4	*ABCD4*, *ADAM11*, *DGKE*, *GRN*, *HOXB2*, *NGFR*, *NMT1*, *OSBPL7*, *PLCD3*, ***PCTP***, *SCPEP1*, *SLC35B1*

region once the known *PON1* variants are included in the model suggests finer-scale analysis of this region may identify additional contributing polymorphisms.

Association was assessed for each SNP using a genome-wide significance threshold of $P < 5E-8$ with $P < 5E-7$ considered a suggestive association. As this study assesses associations in Mexican-American families, it should be noted that there is wider linkage disequilibrium than would be found in a population of randomly mating, unadmixed individuals, making this value of α conservative. Using these thresholds, two SNPs are associated with PON1-para activity, four with PON1-lact activity (two of which are in perfect LD), and two with PON1-aryl activity after conditioning on the associated SNPs in the *PON* region (Table 2, Supplemental File 1). Only a single SNP (rs1078701) on chromosome 4 is significantly associated with activity on all two substrates and suggestively associated for the third. The proportion of variation explained by rs1078701 varies among the different substrates, ranging from 1.8–2.8%.

Located less than 200 kb from rs1078701, *ACOX3* is a strong positional candidate gene as well as a potential contributor to cholesterol regulation. This gene encodes a peroxisomal pristanoyl-CoA oxidase essential for the catabolism of branched fatty acids into precursors for cholesterol biosynthesis [44]. However, the degree to which peroxisomal fatty acid metabolism contributes to circulating cholesterol is unclear [45]. Also found near rs1078701 is *LRPAP1* which produces a glycoprotein that has been linked with gallstone disease caused by an excess of cholesterol [46] and with cholesterol-related brain disorders such as dementia [47] and Alzheimer's disease [48]. *LRPAP1* regulates the amount of LDL-receptor-related protein expressed in the liver and brain and may also act as a chaperone for lipoprotein lipase [49]. A final candidate gene from this region, *ADD1*, is localized to the erythrocyte membrane and is involved in renal sodium handling and hypertension [50]. It has been implicated in blood pressure, adipogenesis, and coronary heart disease [51, 52]. The other significantly associated SNP is rs7225624 on chromosome 17 which explains 2.9% of the observed variation in PON1-lact. *PCTP* is 6Mbp away from rs7225624, but has a strong SUSPECTS score due the involvement of this gene in cholesterol metabolism and transport as well as lipid binding. PCTP is a transfer protein found in macrophages, which are pervasive in atherosclerotic legions, and work in model organisms indicates *PCTP* regulates lipid efflux into the blood stream [53, 54].

Two additional loci show suggestive associations with PON1 activity. Located on chromosome 1, rs12083993 is associated with variation in PON1-para and replicates a previous linkage result for PON1-aryl [32]. This polymorphism explains 1.2% of the variation in PON1-para and SUSPECTS prioritizes three candidate genes involved in lipid metabolism. *ANGPTL3* is predominantly expressed in the liver, but suppresses lipoprotein lipase in the blood stream which in turn hydrolyzes HDL [55]. Polymorphisms in this gene are also associated with increased arterial wall thickness

[56]. *LEPROT* is involved in the cell-surface expression of the leptin receptor, regulation of growth hormones linked to obesity in mice, and cell signaling in response to circulating nutrient levels [57, 58]. Finally, *CYP2J2* is a member of the cytochrome P450 gene family which is widely involved in the oxidation of organic substances and metabolism. *CYP2J2* is primarily expressed in the aorta and coronary artery and has been linked to hypertension risk [59, 60]. The most likely mechanism for this relationship is the metabolism of arachidonic acids to epoxyeicosatrienoic acids (EETs) which are vasodilating agents capable of inhibiting inflammatory response and promoting fibrinolysis [61, 62]. Because PON1 can be inactivated under oxidative conditions [63] similar to those present in the absence of functional *CYP2J2* [64, 65], this association may be related to the realized activity of inactivated PON1 enzyme rather than the basal concentration of PON1 or the activity level under normal plasma conditions.

A suggestive association was also found for PON1-lact with two SNPs in perfect LD (rs13322362 and rs11915977) on chromosome 3. Jointly, these explain 2.7% of the overall variation in PON1-lact. Although a region of chromosome 3 was identified in previous linkage analyses [32], that QTL is more than 44 Mbp from rs13322362. This region of the genome contains few genes but may contain one or more transcription factors indicated by the significant association between rs13322362 and transcript levels of three genes including *SLC25A26*, a mitochondrial transport gene [66], *PROK2*, which regulates circadian rhythms [67], and *RYBP*, a broadly expressed binding protein essential for development [68].

The candidate genes identified in this analysis are consistent with the evidence of enrichment of the top 1% of associations for genes analyzed in DAVID. These results suggest the involvement of pathways related to vasculature development and angiogenesis, cell junctions and signaling, cell adhesion, transmembrane glycoproteins and ion transport, and immunoglobulin.

4. Discussion

The use of known polymorphisms in *PON1* as covariates in this genome-wide association analysis is an unusual but essential method for identifying regions of the genome with smaller effects. By accounting for more than 40% of the variation in PON1 activity in this way, four additional regions of the genome showing an association with residual PON1 activity were identified that could not otherwise be isolated.

The pathways through which candidate genes influence PON1 activity are, for many of the genes, unclear. Frequently, this is due to a lack of full, functional understanding of the genes themselves. Genes that are associated with cholesterol levels and associated syndromes, but have no clear mechanism, may be contributing to the PON1 pathway but further research would be required to demonstrate this. Because PON1 binds to, and is carried by, nonoxidized HDL molecules, these regions may play a role in plasma HDL concentration which would indirectly increase PON1 concentration and activity. Additionally, it is important to recognize that PON activity is likely influenced by the tertiary structure of the PON1 protein itself or the circulating levels of PON in the blood, which may be influenced by the lipoprotein milieu.

It is necessary, therefore, to consider these results within the broader network of genes and proteins involved in the regulation of lipid metabolism in the blood stream. However, lipid metabolic pathways are not the only ones implicated in these association results. Ubiquitous transcription factors and genes related to oxidative stress were also identified and could play substantial roles in the regulation of PON1 concentration in the blood or the inactivation of PON1 which would decrease activity even in the presence of high levels of the enzyme. Considering the number of factors associated with the activity of single enzyme, future work on the genetic underpinnings and biochemical regulation of atherosclerosis, hypertension, cholesterolemia, and inflammatory diseases more broadly must be understood in the broadest biochemical context.

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Research Article

Paraoxonases-2 and -3 Are Important Defense Enzymes against *Pseudomonas aeruginosa* Virulence Factors due to Their Anti-Oxidative and Anti-Inflammatory Properties

Eva-Maria Schweikert,[1] Julianna Amort,[1] Petra Wilgenbus,[1] Ulrich Förstermann,[1] John F. Teiber,[2] and Sven Horke[1]

[1] *Institute of Pharmacology, University Medical Center of the Johannes Gutenberg-University Mainz, Obere Zahlbacher Straße 67, 55131 Mainz, Germany*
[2] *Division of Epidemiology, Department of Internal Medicine, The University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390, USA*

Correspondence should be addressed to Sven Horke, horke@uni-mainz.de

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The pathogen *Pseudomonas aeruginosa* causes serious damage in immunocompromised patients by secretion of various virulence factors, among them the quorum sensing N-(3-oxododecanoyl)-L-homoserine lactone (3OC12) and the redox-active pyocyanin (PCN). Paraoxonase-2 (PON2) may protect against *P. aeruginosa* infections, as it efficiently inactivates 3OC12 and diminishes PCN-induced oxidative stress. This defense could be circumvented because 3OC12 mediates intracellular Ca^{2+}-rise in host cells, which causes rapid inactivation and degradation of PON2. Importantly, we recently found that the PON2 paralogue PON3 prevents mitochondrial radical formation. Here we investigated its role as additional potential defense mechanism against *P. aeruginosa* infections. Our studies demonstrate that PON3 diminished PCN-induced oxidative stress. Moreover, it showed clear anti-inflammatory potential by protecting against NF-κB activation and IL-8 release. The latter similarly applied to PON2. Furthermore, we observed a Ca^{2+}-mediated inactivation and degradation of PON3, again in accordance with previous findings for PON2. Our results suggest that the anti-oxidative and anti-inflammatory functions of PON2 and PON3 are an important part of our innate defense system against *P. aeruginosa* infections. Furthermore, we conclude that *P. aeruginosa* circumvents PON3 protection by the same pathway as for PON2. This may help identifying underlying mechanisms in order to sustain the protection afforded by these enzymes.

1. Introduction

The bacterium *Pseudomonas aeruginosa* is an opportunistic nosocomial pathogen, which infects the pulmonary tract of, for example, immunocompromised patients or those suffering from cystic fibrosis, pneumonia, burn wounds, HIV, or cancer chemotherapy [1]. The infection causes serious damage in the host, complicated by an often hindered antibiotic treatment due to multiresistances and biofilm formation that provides physical protection. Furthermore, *P. aeruginosa* secrets a variety of virulence factors to regulate bacterial communication and weaken the defense mechanisms of the infected host. Two important factors are the quorum sensing signal *N*-(3-oxododecanoyl)-L-homoserine lactone (3OC12) and the redox-active pyocyanin (PCN). 3OC12 is a mediator of the cell-density-dependent signaling system known as quorum sensing, by which the bacteria coordinate their gene expression. If bacterial density and 3OC12 concentration exceed a certain threshold, the bacteria become virulent by expression of virulence factors (immunogenic exoenzymes and toxins) and by inducing inflammation. The lactone 3OC12 has numerous immunomodulatory and inflammatory properties, such as an inhibitory effect on dendritic cells and T-cell activation [2], proinflammatory

induction of IL-6 and IL-8 in airway epithelial cells and lung fibroblasts [3], and promoting apoptosis [4, 5]. Studies in mice have shown that 3OC12 is a critical determinant for bacterial colocalization and the establishment of chronic lung infections [6]. Therefore, the development of quorum sensing inhibitors would be a major advance in the ability to combat *P. aeruginosa* infections [7, 8].

The production of the virulence factor PCN is positively regulated by quorum sensing signals including 3OC12 [9]. PCN causes oxidative stress and has a broad range of effects on airway epithelial cells such as cellular senescence and ciliary dyskinesia, induction of IL-8 secretion, decrease of glutathione levels, and inhibition of catalase activity [9, 10]. The redox-activity of PCN is central to the damage observed in exposed host cells. The zwitterionic PCN transfers electrons from reduced NADH or NADPH in the cytosol to molecular oxygen leading to production of superoxide (O_2^-), which is converted to H_2O_2 [11]. Additionally, PCN causes a disturbance of the antimicrobial Duox/SCN^-/LPO-system, by consuming the same substrates (molecular oxygen and NADPH) [12]. The requirement for PCN in lung infection was demonstrated in an acute pneumonia mice model: *P. aeruginosa* strains lacking the ability to produce PCN are much more rapidly cleared from lungs and showed less virulence than the wild-type strain [13].

The paraoxonase family consists of the three members PON1, PON2, and PON3, which exhibit about 70% similarity at the amino acid level [14]. PON1 is associated with HDL in serum, whereas PON2 and PON3 are intracellular proteins. In contrast to PON2, which is ubiquitously expressed, PON3 appears restricted to fewer tissues/cells; its expression in cells relevant to cardiovascular diseases is contradictory, because Marsillach et al. [15] found PON3 by immunohistochemistry in human vascular walls and macrophages, while our studies revealed absence in macrophages, endothelial, smooth muscle, and many other cell types [16]. All three PONs share a lactonase activity with distinct and overlapping substrate specificities [17–19]. PON2 dominantly hydrolyzes 3OC12 presumably resulting in the ability to interfere with quorum sensing, which may significantly attenuate bacterial virulence of *P. aeruginosa*. In support of this concept, epithelial tracheal cells from PON2 deficient mice showed a reduced ability to inactivate 3OC12 [20]. In addition to its lactonase activity, PON2 is a major anti-oxidative protein that diminishes mitochondrial superoxide production and thus considerably determines cell survival [21–23]. In particular, it has also been shown that PON2 diminishes PCN-induced ROS production in human epithelial cells [24]. Intriguingly, 3OC12 causes a rapid Ca^{2+}-mediated PON2 inactivation and degradation in cultured cells by a yet unknown mechanism, which enables 3OC12 to protect itself from its hydrolysis by PON2 [25]. As a consequence, 3OC12 potentiates the formation of ROS induced by PCN, revealing a potential mechanism by which the bacterium may circumvent the protection afforded by PON2 [25].

Our recent studies demonstrated that PON3, much like PON2, reduced the generation of mitochondrial superoxide. We also revealed that PON3 was frequently found overexpressed in tumors. There, it reduced susceptibility to chemotherapeutics and reduced apoptosis, in line with the central involvement of mitochondrial ROS to cell death [16]. Hence, we hypothesized that PON3 attenuated PCN-induced oxidative stress, which was tested here for the first time. Our studies also included inflammatory pathways subsequent to PCN stimulation, that is, NF-κB activation and secretion of various cytokines. Our results support the concept of marked anti-inflammatory roles for PON2 and PON3. Finally, we found that both enzymes, PON2 and PON3, were inactivated and degraded in response to Ca^{2+}-disturbances caused by 3OC12. Identifying the underlying pathway(s) by which the PONs are downregulated may reveal a therapeutic target, which could be exploited to help sustain or enhance the host's PON2 and PON3 activities. Such a clinical intervention could be of great benefit in the defense against *P. aeruginosa* infections.

2. Material and Methods

2.1. Cell Culture and Material. Human endothelial EA.hy 926 cells obtained from the ATCC were cultured in Dulbecco's modified Eagle's medium without Phenol Red (Sigma, St. Louis, MO, USA) containing sodium pyruvate (PAA Laboratories, Pasching, Austria), antibiotics penicillin/streptomycin, hypoxanthine/aminopterin/thymidine supplement, L-Glutamine (Invitrogen, Carlsbad, CA, USA), and 10% (v/v) fetal calf serum (PAA). Human PON2 or PON3 cDNA was subcloned into pDsRed-Express-N1 or pEGFP-N1 plasmids (Clontech). Stable cell lines, plasmids, and transfection procedures were described before [25, 26]. HEK293 and A549 cells were from the German Collection of Microorganisms and Cell Cultures. HEK293 received the same medium as EA.hy 926, but without hypoxanthine/aminopterin/thymidine supplement. A549 cells received the same medium as HEK293, but 5% serum. Cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 (10% for EA.hy 926). Pyocyanin was purchased from Cayman Chemical Company (Ann Arbor, MI, USA); Mito-HE and thapsigargin were from Molecular Probes; L-012 was from Wako Chemicals (Neuss, Germany), and all other reagents were from Sigma.

2.2. ELISA. EA.hy 926 cells were seeded one day prior to stimulation in 6-well dishes at 80% confluency (5×10^5/well). Treatment occurred in 1.5 mL medium without FCS for 16 h with or without pyocyanin (10 μM). Cell supernatants were taken for Multi-Analyte ELISArray Kit for human inflammatory cytokines (SA Biosciences, Frederick, MD, USA) according to the supplier's instructions. Absorbance was determined using a FluoStar Optima microplate reader (BMG Labtechnologies). Corrected OD was calculated by subtracting the A450 reading by the A570 reading to clear any minor optical imperfections in the ELISA plate.

2.3. Reporter Gene Assays. Cells at 80% confluency were cotransfected with pcDNA3-HA or pcDNA3-PON2-HA or pEGFP-N1 or pEGFP-N1-PON3 and a plasmid allowing

for constitutive renilla luciferase expression (a kind gift of H. Kleinert, University Medical Centre, Mainz) and the NF-κB reporter plasmid (pGL4.32[luc2P/NF-κB-RE/Hygro] from Promega, Madison, WI, USA). We used Nanofect-inTM (PAA) for transfection according to the supplier's instructions. Cells were treated 24 h after transfection with pyocyanin (100 μM) for 4 h. Subsequently the NF-κB activity was measured by Dual-Luciferase Reporter Assay System (Promega) according to the supplier's instructions.

2.4. qRT-PCR. RNA isolation and cDNA generation was performed as reported previously [21]. PON3 expression level was determined by quantitative real-time PCR normalized to GAPDH as described before [21]. The following Taqman primers (Eurofins, MWG Operon) were used: PON3: sense 5′-TGGGATCACAGTCTCAGCAG-3′; antisense 5′-TCCACTAAGGTGCCCAACTG-3′; probe 5′-TGGAAAAACATGATAACTGGGA-3′; GAPDH: sense 5′-CAACAGCCTCAAGATCATCAGC-3′; antisense 5′-TGGCATGGACTGTGGTCATGAG-3′; probe 5′-CCTGGCCAAGGTCATCCATGACAAC-3′.

2.5. Western Blotting. Preparation of lysates, SDS-PAGE, and Western blotting was performed as reported previously [25]. Rabbit-anti-PON3 polyclonal antibody was used at 1 : 750 (Sigma, St. Louis, MO, USA), rabbit-anti-PON2 [26] used at 1 : 2000. Mouse-anti-GAPDH 6C5 (Santa Cruz, Santa Cruz, CA, USA) and HRP-conjugated secondary antibodies were from Sigma or Cell Signaling Technology. Immunodetected proteins were visualized and quantitatively evaluated as described before [24].

ROS detection and determination of lactonase and lovastatinase activities were performed as described before [17, 24, 27].

2.6. Software, Statistics, and Image Acquisition. GraphPad Prism-5 was used for calculations, statistical evaluation using 1-/2-way ANOVA with Bonferroni's multiple comparisons posttest (see Figures 1–6). $P < 0.05$ was considered significant. Adobe Photoshop software was used for image acquisition. If necessary, only brightness and/or contrast were changed simultaneously for all areas of any blot.

3. Results

3.1. PON2 and PON3 Protect Cells from PCN-Induced ROS and Inflammatory Responses. PCN is essential for *P. aeruginosa* infections *in vivo* and causes oxidative stress, which leads to serious damage of airway epithelial cells [12, 13]. We recently found that PON2 decreases ROS production in EA.hy 926 cells in response to treatment with PCN [24]. Given the high degree of homology between the paraoxonases and previous descriptions of PON3 antioxidative effects, we wanted to test whether PON3 also attenuated PCN-induced ROS production. This was measured by loading naïve or PON3 overexpressing cells with the fluorescent ROS indicator carboxy-H_2DCFDA followed by stimulation with PCN (2.4 μM); PON2 overexpressing cells were used in these (and subsequent) studies for comparison. As with PON2, PON3 overexpression afforded a marked protection against PCN-induced oxidative stress (Figure 1(a)). To control for a potential direct oxidation of carboxy-H_2DCFDA by PCN, an unwanted effect by the GFP tag and for effects specific to HEK293 cells, we also used the luminol derivate L-012 to report ROS in EA.hy 926 cells overexpressing a PON3-dsRed construct. As with HEK293 PON3-GFP cells, the EA.hy 926 PON3-GFP and PON3-dsRed cells show decreased ROS production after PCN treatment (Figure 1(b)).

PCN also leads to a proinflammatory response by causing the release of interleukins, which might be triggered by activation of NF-κB [12, 28]. To test for anti-inflammatory effects of PON2 and PON3, we next addressed activation of the NF-κB pathway in lung epithelial A549 cells in response to PCN treatment. Employing gene reporter studies, we cotransfected A549 cells with (i) an NF-κB firefly luciferase reporter plasmid, (ii) a renilla luciferase expression vector for normalization purposes, and (iii) a PON2 or PON3 expression plasmid. Controls received the same vectors, but without PON2/PON3 inserts. As expected, NF-κB promoter activity was significantly increased 4- to 6-fold above control cells following PCN treatment (Figure 2). Importantly, PON2 and PON3 overexpression caused a dramatic decrease in activation of this major pathway involved in inflammatory response.

Finally, because NF-κB has both pro- and anti-inflammatory properties, we wished to determine which cytokine is secreted from endothelial cells exposed to PCN and if this was altered by paraoxonase overexpression. For this purpose, we performed an ELISA with a panel of inflammatory cytokines by using cell supernatants from PCN-treated naïve EA.hy 926 cells or cells overexpressing PON2-GFP or PON3-GFP. By using this approach, we covered several major inflammatory mediators, like IL-1A, IL-1B, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17A, IFN-γ, TNF-α, and GM-CSF. Remarkably, only one single cytokine was induced by PCN in endothelial cells, namely, IL-8 (>1000 pg/mL); the IL-8 level was about 6-fold higher in PCN treated compared to untreated EA.hy 926 cells (Figure 3(a)). IL-8 release was markedly lowered by PON2 or by PON3 overexpression (about 2-fold to 4-fold, respectively; Figure 3(b)). Taken together, PON2 and PON3 act as potent anti-inflammatory enzymes, which is shown by their reducing effects on ROS production, NF-κB activation and IL-8 release in response to *P. aeruginosa* virulence factor PCN.

3.2. 3OC12 Downregulates PON3 Hydrolytic Activity and Protein. Besides the virulence factor PCN, *P. aeruginosa* secrets the quorum sensing signal 3OC12, which can be hydrolyzed and thus inactivated by PON2. In fact, it appears that PON2 has a dominant role in 3OC12 hydrolysis [27]. Our further studies showed that PON2 hydrolytic activity, mRNA, and protein are actively downregulated by 3OC12, which disrupts PON2's protection against both 3OC12 levels and PCN-induced ROS production [24]. Given that PON3 diminished PCN-triggered ROS, it is worthwhile to address effects of 3OC12 on PON3 in a similar manner. To this end, we addressed the effect of 3OC12 on PON3 hydrolytic

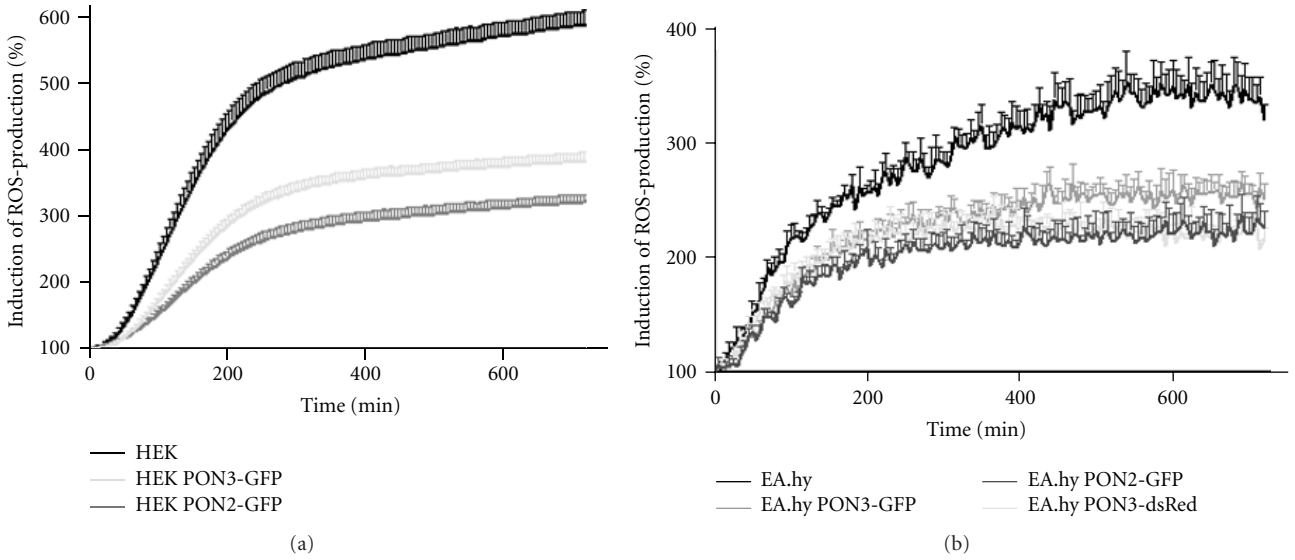

Figure 1: PON2 or PON3 overexpression diminishes ROS production induced by *P. aeruginosa* signaling molecule pyocyanin (PCN). (a) Naïve, PON2-GFP, or PON3-GFP overexpressing HEK293 cells were loaded with carboxy-H_2DCFDA and stimulated with PCN (2.4 μM). Carboxy-H_2DCFDA fluorescence as means of ROS was recorded over several hours. (b) Similar to A. Naïve, PON2-GFP, PON3-GFP, or PON3-dsRed overexpressing EA.hy 926 cells were loaded with L-012 and stimulated with PCN (2.4 μM). Curve maxima calculated by nonlinear regression showed statistically significant differences ($P < 0.001$) between naïve and PON2 or PON3 overexpressing cells.

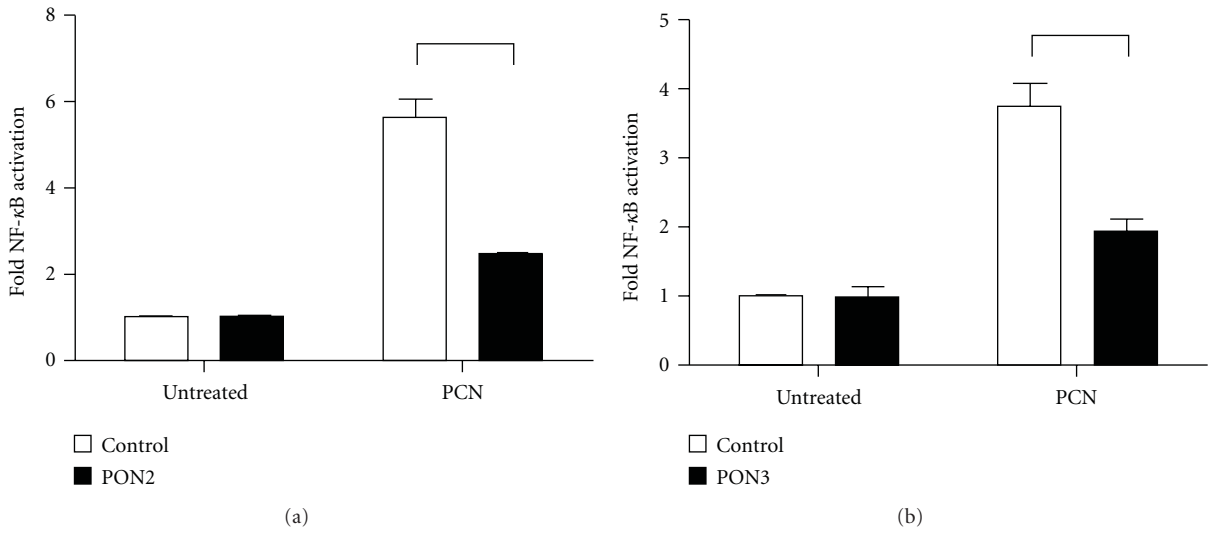

Figure 2: PON2 or PON3 overexpression diminishes NF-κB activation induced by *P. aeruginosa* signaling molecule PCN. A549 cells transiently overexpressing HA or PON2-HA (a) and GFP or PON3-GFP (b) were stimulated with PCN (100 μM, 4 h) and analyzed for NF-κB activation. Symbols represent ± S.E.M. $n = 6$–9; $^{***}P < 0.001$.

activity, mRNA, and protein. We used PON2-GFP or PON3-GFP overexpressing HEK293 cells for measuring lactonase or lovastatinase activity (for PON2 and PON3, resp.). In agreement with previous observations in other cell lines, PON2 activity was rapidly decreased after 3OC12 treatment. The PON2 hydrolytic activity was reduced by ~50% in 10 min and >80% in 30 min (Figure 4(a)). Our previous studies revealed that PON2 activity was inversely related to intracellular Ca^{2+}-homeostasis, with 3OC12 hydrolysis being depleted in response to Ca^{2+}-release as it was inhibitable by Ca^{2+}-chelator BAPTA [24]. Interestingly, PON3 lovastatinase activity was also rapidly inactivated by 3OC12 to an almost identical level as observed for PON2 (Figure 4(b)).

Our previous data demonstrated that 3OC12 caused a pronounced calcium influx in A549 and EA.hy 926 cells in a very short time-interval. This formed the basis for an active, calcium-dependent inactivation and subsequent degradation of PON2. Therefore, we wanted to explore if PON3 is also degraded in a calcium-sensitive manner, as this could point to regulatory pathways shared by these two different

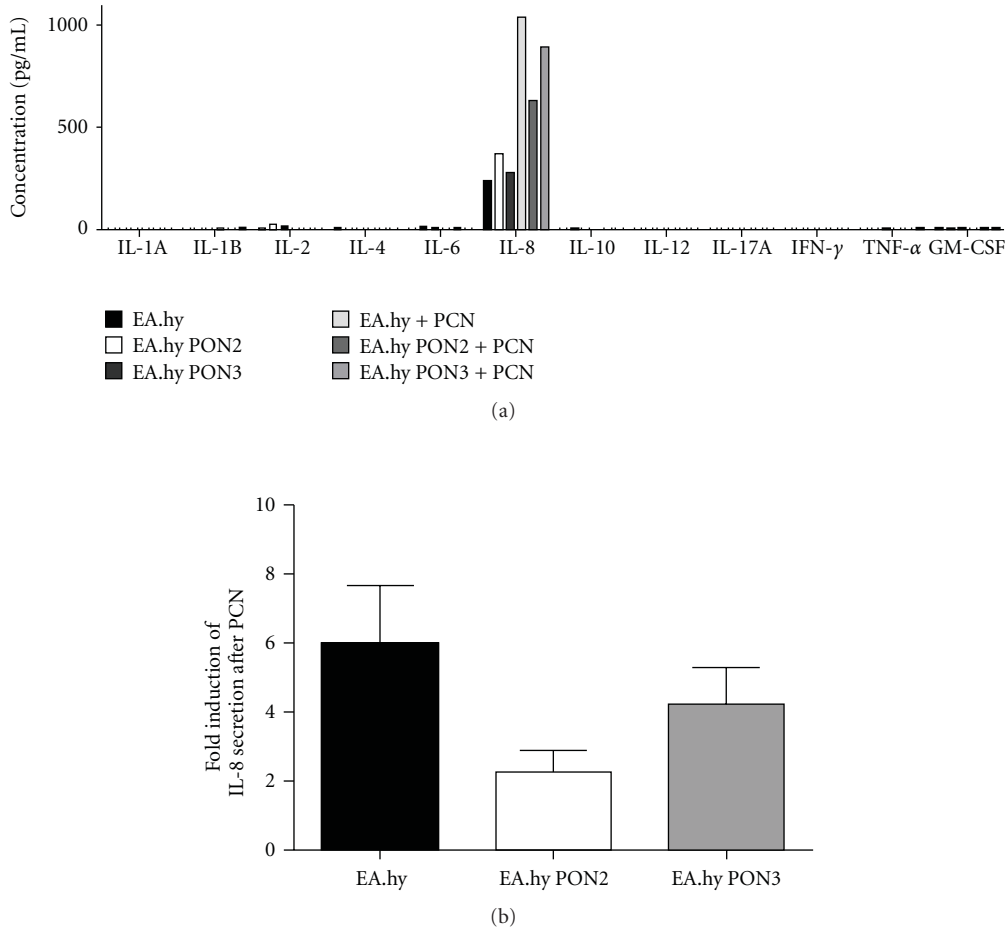

Figure 3: PCN induces IL-8 secretion, which can be lowered by PON2 or PON3 overexpression. (a) Naïve, PON2-GFP or PON3-GFP overexpressing EA.hy 926 cells were treated with PCN (10 μM, 16 h). Cell supernatants were analyzed for the secretion of the listed cytokines and chemokines by ELISA. (b) Quantitative evaluation of results from panel (a) Fold induction of IL-8 release was calculated between untreated and PCN-treated samples.

enzymes [24]. To this end, we treated A549 cells with 3OC12 and analyzed PON3 mRNA levels by qRT-PCR at different time-points. Figure 5(a) shows a decrease of PON3 mRNA after 16 h 3OC12 treatment to ~70%. Next we verified, if PON3 mRNA is actively degraded or if the decrease results from a discontinued transcription or reflection of normal mRNA turnover. We treated A549 cells with 3OC12 or the RNA synthesis inhibitor 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside (DRB), or combinations thereof. Unlike PON2, PON3 mRNA was not actively degraded in response to 3OC12 (Figure 5(b)).

Finally we performed Western blot analyses to monitor PON3 protein levels after major Ca^{2+}-disturbances caused by treatment with 3OC12. For reasons of comparison, we used the SERCA inhibitor thapsigargin, which also causes serious Ca^{2+}-disturbances. Treatment of A549 cells with 3OC12 for different durations showed that PON3 protein level decreased time dependently and vanished nearly completely after 16 h; while PON2 protein was also degraded by ~50% after 16 h (Figure 6(a)). In accordance, A549 cells treated with different thapsigargin concentrations showed a significant dose-dependent degradation of PON3 and PON2 (Figure 6(b)).

4. Discussion

P. aeruginosa infections are difficult to treat since the bacteria often develop multiple antibiotic resistance and form a biofilm, which hinders the access of the antibiotics to the bacteria. Additionally, *P. aeruginosa* secrets different virulence factors, which regulate the bacterial communication and damage the infected host. Therefore, it is important to understand *P. aeruginosa* host-pathogen interactions to identify new potential therapeutic targets. Combined with the knowledge gained in previous studies, our results suggest that human paraoxonases PON2 and PON3 comprise a major defense against virulence factor-induced oxidative stress, inflammatory response, and cytokine release.

In this study, we revealed, for the first time, the protective effect of PON3 against PCN-induced host cell damage. By

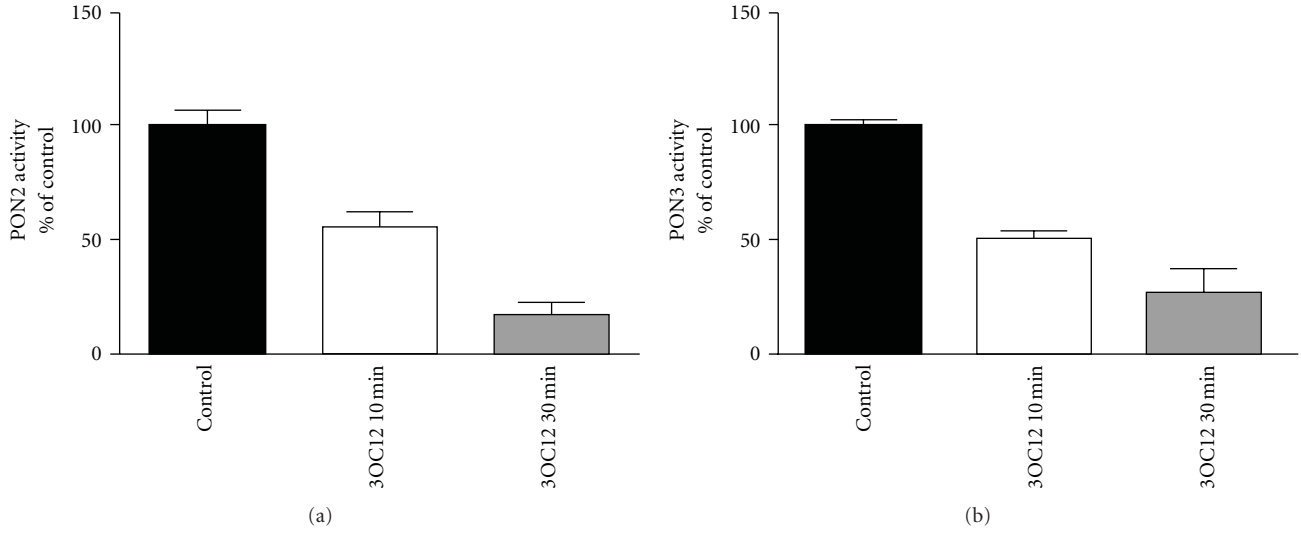

Figure 4: PON2 and PON3 activity decrease after 3OC12 treatment. (a) PON2-GFP overexpressing HEK293 cells were treated with 3OC12 (100 μM) for the indicated durations and tested for 3OC12-HSL hydrolytic activity. (b) PON3-GFP overexpressing HEK293 cells were treated with 3OC12 (100 μM) for the indicated durations and tested for lovastatinase hydrolytic activity. Symbols represent ± S.E.M. $n = 3$; $^{***}P < 0.001$.

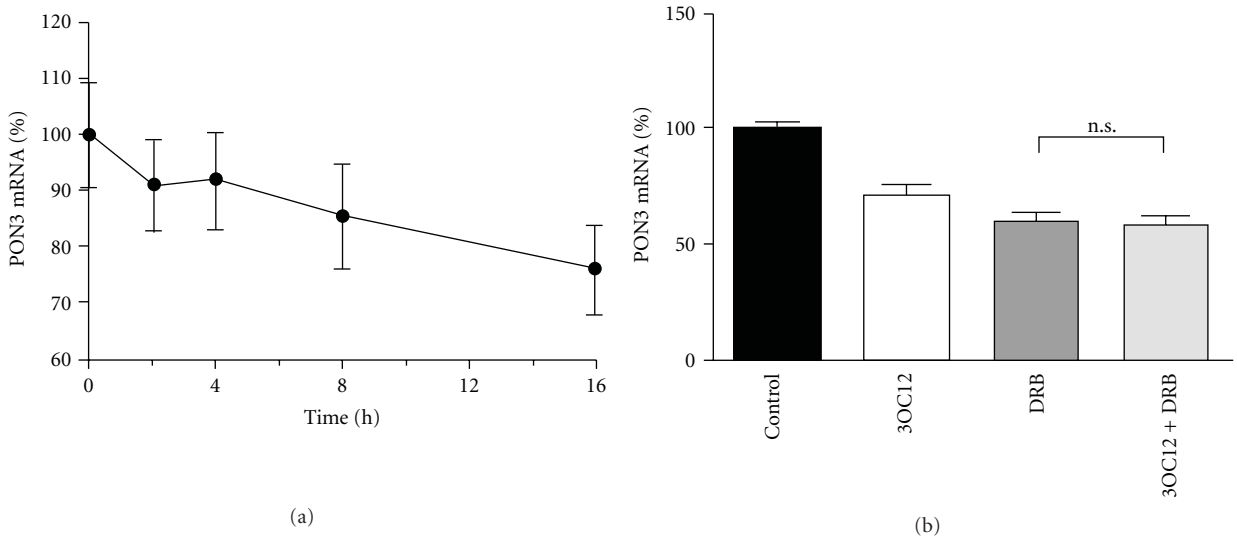

Figure 5: PON3 mRNA is not actively degraded in response to 3OC12 treatment. (a) A549 cells were treated with 3OC12 (100 μM) for the indicated durations and analyzed for PON3 mRNA levels by qRT-PCR. (b) A549 cells were treated with 3OC12 (100 μM, 24 h) or with DRB (100 μM, 24 h) or combinations thereof. There was no statistically significant difference in PON3 mRNA levels after DRB or DRB/3OC12 treatment. Symbols represent ± S.E.M. $n = 3$; $^{***}P < 0.001$ versus control.

various technical approaches and using different cell lines, we showed that PCN leads to ROS production, NF-κB-activation, and IL-8 secretion, which can be prevented by PON2 or PON3 overexpression. PCN induces the production of O_2^- and H_2O_2, which causes damage in various cell types [10]. Overexpression of PON2 or PON3 in relevant systems leads to a significant reduction of ROS production, reflecting the protective effect of both enzymes against PCN-induced oxidative damage. Importantly, PON2 and PON3 differ in their substrate specificities, as PON2 has a dominant lactonase activity, whereas PON3 has much better activity with some large lactones or arylesters (i.e., statins; estradiol acetates). Thus, we conclude that PON2 and PON3 act by a common anti-oxidative mechanism. Intriguingly, our data also suggest that the anti-oxidative effect of PON3 is independent from its enzymatic activity. This would be in agreement with previous results demonstrating an independent anti-oxidative and hydrolytic activity of PON2 [22]. Addressing the underlying anti-oxidative mechanism of PON2 and PON3, we and others recently showed that PON2 and PON3 localize to the inner mitochondrial membrane where they interact with coenzyme Q10 (coQ10) resulting in

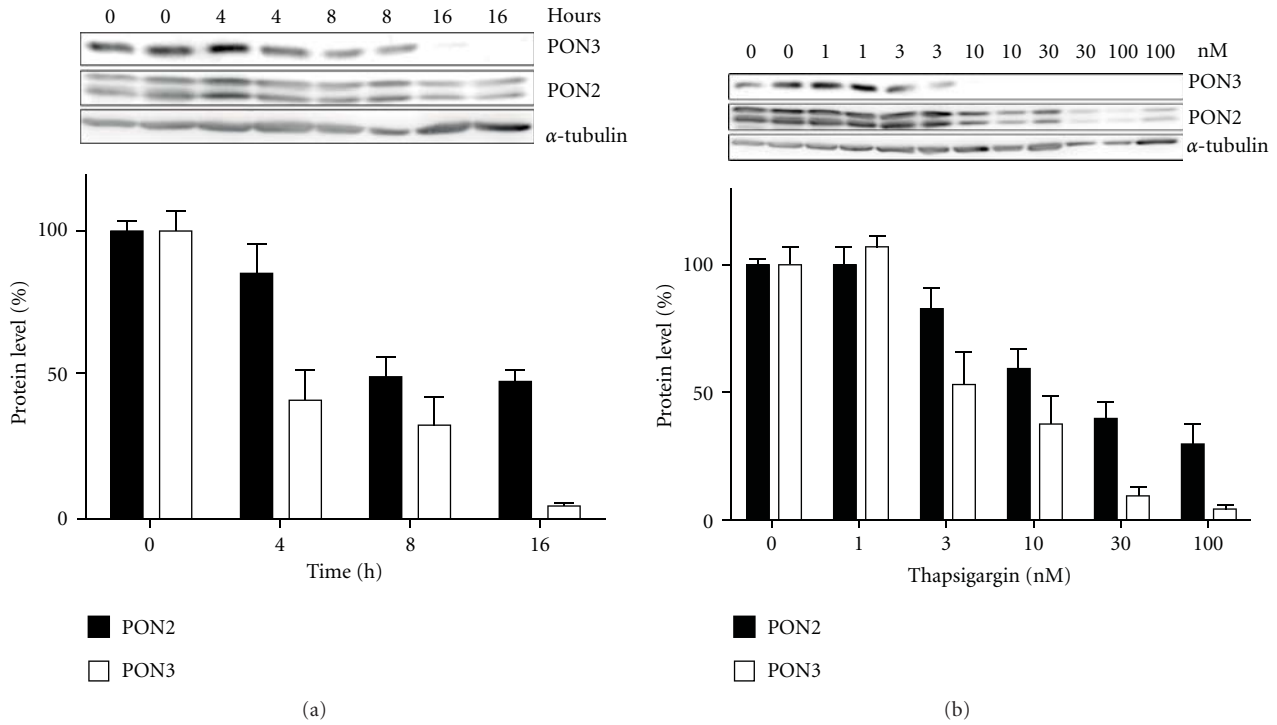

Figure 6: Both PON2 and PON3 are degraded after 3OC12 treatment. (a) A549 cells were treated with 3OC12 (100 μM) for the indicated durations or (b) with thapsigargin (24 h) with the indicated concentrations. Lysates (50 μg of protein) were analyzed by Western blotting using anti-PON2, anti-PON3, or anti-α-tubulin antibodies. One representative blot is shown. Results (right) are the means ± S.E.M. of three replicate analyses; $^{**}P < 0.01$; $^{***}P < 0.001$.

abrogated superoxide production [16, 22, 23]. According to the current model (reviewed in this issue; [29]), it is assumed that PON2 and PON3 protect against ROS formation by acting as an insulator for coQ10 to prevent coincidental superoxide production at the mitochondrial membrane.

It has also been reported that PCN increases IL-8 expression in human airway epithelial cells [28]. Similarly, we observed an increase in IL-8 secretion by PCN in endothelial EA.hy 926 cells. Interestingly, this can be lowered by PON2 or PON3. Additionally, we monitored the release of numerous proinflammatory cytokines and chemokines after PCN treatment. None of the tested factors was induced except for IL-8, suggesting that IL-8 acts as a central mediator of endothelial inflammation triggered by PCN. It is known that IL-8 promoter activity and expression can be induced by NF-κB [30, 31]. Our data also imply a role for NF-κB as mediator between PCN-induced effects on ROS formation and proinflammatory immune response by release of IL-8. In particular, PCN was able to activate NF-κB, which could be reduced by PON2 or PON3. It is fully established that NF-κB is regulated by redox signaling. Taken together, our data suggest that PON2 and PON3 anti-inflammatory activities result from their ability to prevent ROS formation, as less oxidative stress likely diminishes NF-κB activation and subsequent IL-8 release.

Furthermore, we revealed the effect of 3OC12 on PON3 hydrolytic activity, mRNA, and protein. PON3 mRNA, in contrast to PON2 mRNA [22], was not actively degraded, indicative of independent mechanisms that regulate stability of these two mRNAs. PON3 protein levels were dramatically decreased by 3OC12 treatment. A severely altered calcium homeostasis as underlying mechanism appears highly likely, as this has previously been demonstrated for PON2 [24]. Our data may also suggest that PON2 and PON3 degradation occurs through the same Ca^{2+}-mediated pathway, as it acts in a similar time (in case of 3OC12) or dose-dependent (in case of thapsigargin) manner. Similar to PON2, PON3 hydrolytic activity was decreased much more extensively and rapidly than the protein, indicating a likely posttranslational event blocking PON2's and PON3's enzymatic function.

Our findings emphasize roles for PON2 and PON3 in the defense against *P. aeruginosa* virulence but show also that the bacterium may circumvent the protection by PON2 and PON3. Identification of the posttranslational modification of PON2, which causes its inactivation and induces the signaling pathway that mediates PON2 and PON3 downregulation, may lead to the identification of an important therapeutic approach. It may be beneficial to block the 3OC12-mediated decrease of PON2 activity, sustaining PON2's protective effect of inactivating 3OC12. Given the high similarity of PON2 and PON3 and considering the fact that both proteins are inactivated by 3OC12-mediated Ca^{2+}-disturbances, the same regulatory posttranslational modification may reside in conserved position(s). The distinct modification remains to be determined, as protein function can be altered by many modifications,

like methylation, acetylation, ubiquitinylation, and glycosylation/deglycosylation. Our previous data showed that PON2 inactivation is rapid and reversible, which would be consistent with phosphorylation/dephosphorylation by protein kinases/phosphatases [24]. If true, about 50 potential serine, threonine, and tyrosine phosphorylation residues could be responsible for the (in-)activation of PON2. Hence, future studies are needed to identify the precise mechanism that regulates enzymatic activity of PON2 and PON3. Given that inactivation occurs within minutes and the knowledge that activity does not require cofactors (except for calcium), the presence of a Ca^{2+}-triggered, regulatory posttranslational modification appears highly likely. Revealing this mechanism is of great interest, not only for protection against *P. aeruginosa* virulence factors but also for activities of paraoxonases beyond this specific interaction.

Abbreviations

carboxy-H_2DCFDA:	5-(and-6)-carboxy-2′, 7′-dichlorodihydrofluorescein diacetate
coQ10:	coenzyme Q10
IL-8:	Interleukin-8
PCN:	Pyocyanin
PON:	Paraoxonase
ROS:	Reactive oxygen species
3OC12:	*N*-(3-oxododecanoyl)-L-homoserine lactone.

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Review Article

Regulation of Hepatic Paraoxonase-1 Expression

Bianca Fuhrman

The Lipid Research Laboratory, The Ruth and Bruce Rappaport Faculty of Medicine, Technion—Israel Institute of Technology and Rambam Medical Center, 31096 Haifa, Israel

Correspondence should be addressed to Bianca Fuhrman, fuhrman@tx.technion.ac.il

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Serum paraoxonase-1 (PON1) is a member of the paraoxonases family (PON1, PON2, and PON3). PON1 is synthesized and secreted by the liver, and in circulation it is associated with HDL. PON1 has antioxidative properties, which are associated with the enzyme's capability to decrease oxidative stress in atherosclerotic lesions and to attenuate atherosclerosis development. Epidemiological evidence demonstrates that low PON1 activity is associated with increased risk of cardiovascular events and cardiovascular disease and is an independent risk factor for coronary artery disease. Therefore, pharmacological modulation of PON1 activity or PON1 gene expression could constitute a useful approach for preventing atherosclerosis. A primary determinant of serum PON1 levels is the availability of the enzyme for release by the liver, the principal site of PON1 production. Together with the enzyme secretion rate, enzymatic turnover, and protein stability, the level of PON1 gene expression is a major determinant of PON1 status. This paper summarizes recent progress in understanding the regulation of PON1 expression in hepatocytes.

1. Introduction

The atherosclerotic lesion is dominated by accumulation of lipid peroxides along with the progression of early plaque development [1]. Serum paraoxonase-1 (PON1) is an HDL-associated lipolactonase, which is synthesized and secreted by the liver [2]. PON1 has antioxidative properties, which are associated with the enzyme's capability to protect LDL [3], as well as HDL [4] from oxidation, to decrease macrophage oxidative status [5], to stimulate cholesterol efflux from macrophages [6], to decrease oxidative status in atherosclerotic lesions [7], and to attenuate atherosclerosis development. Immunohistochemical analysis has revealed accumulation of PON1 in the human atherosclerotic lesion as it progresses from fatty streak to advanced lesion [8]. Recently it was demonstrated that PON1 acts to reduce the oxidizing potency of lipids in atherosclerotic lesions, thus providing protection against oxidation [9]. Epidemiological evidence demonstrates that low PON1 activity is associated with increased risk of cardiovascular events [10] and is an independent risk factor for cardiovascular disease [11].

A variety of nongenetic factors have been shown to influence serum PON1 levels and activity. PON1 undergoes inactivation under oxidative stress and its activity is preserved by dietary antioxidants [12]. Moderate daily consumption of alcohol [13], vitamin C and E [14], wine [15], or pomegranate juice [16], increased serum levels of PON1 in animals and in humans. The level of PON1 in serum is determined mainly by the status of PON1 gene expression in the liver. However, the molecular mechanisms involved in the regulation of hepatic PON1 gene expression were less explored. This paper focuses on nongenetic factors that influence PON1 gene expression in hepatocytes, revealing thus the molecular regulatory mechanisms modulating hepatic PON1 gene expression.

2. PON1 Gene Structure

The paraoxonase (PON) gene family consists of three members, PON1, PON2, and PON3, aligned next to each other on chromosome 7 in human and on chromosome 6 in

the mouse [17]. In both species the three PONs contain nine exons of approximately the same length. The human PON1 and PON2 genes both have eight introns, and the exon/intron junctions occur at equivalent positions. All PON1s have an extracodon at position 106 (lysine in human PON1). The approximate length of the human PON1 is about 27 kb. In previous studies, Deakin et al. identified a single nucleotide polymorphism (SNP) in the proximal region of the PON1 promoter (C-107T) with an important impact on serum concentrations and activities of the enzyme in different populations [18]. A role for Sp1 and sterol regulatory element-binding protein-2 (SREBP-2) was proposed. A number of potential sterol regulatory element (SRE) sequences exist within the proximal PON1 promoter region that has been shown to be sufficient to respond to statin treatment. The data designate the region around the C-107T polymorphism as being the focus for transcription factor actions and suggest a synergistic effect of Sp1 and SREBP-2 on promoter activity.

3. Inflammation

The liver plays a central role in the host response to inflammation, which is associated with a wide array of metabolic changes. These metabolic changes can be induced by the administration of endotoxin (LPS) and by cytokines which mediate the acute phase response, such as TNF and IL-1. LPS and inflammatory cytokines induce composition changes in HDL, due to alterations in hepatic mRNA levels for HDL-related proteins. Administration of LPS and of cytokines in Syrian hamsters resulted in a rapid and marked reduction in PON1 mRNA in the liver, which was sustained as long as 48 hours [19] implicating PON1 as a negative acute phase protein. PON1 was also a negative, acute phase gene in male mice. Within 24 hours of LPS administration, PON-1 mRNA level was reduced by 50% in male mice and increased moderately in female mice, thus showing to be gender dependent. Anti-inflammatory dexamethasone enhanced PON-1 mRNA level by 2-fold in male and female LPS-treated mice and increased PON-1 expression by 8-fold in Hepa cell, a mouse hepatoma cell line [20]. PON1 mRNA expression in hepatocytes was reduced also by oxidized phospholipids found in mildly oxidized LDL through the inflammatory cytokine IL-6, and IL-6 alone produced the same pattern of PON1 mRNA changes [21]. Liver damage induced by CCl_4 resulted in decreased PON1 gene transcription but increased hepatic PON1 concentration that was related to inhibited protein degradation [22]. Decreased PON1 gene transcription was associated with PPARδ expression. These changes were accompanied by increased hepatic MCP-1 concentration and gene expression, suggesting that PON1 has a hepatoprotective role against inflammation, fibrosis, and liver disease mediated by MCP-1. The interrelationships between PON1 and MCP-1 in the regulation of hepatic inflammation were recently reviewed by Camps et al., [23]. Proinflammatory cytokines, including tumor necrosis factor-(TNF-)α, interleukin-1β, and interleukin-6 decreased the expression of PON-1 and of apoA-I in hepatocytes by inhibiting PPARα activation [24] and coordinately increased the expression of serum Amyloid A (SAA) via nuclear factor κB (NF-κB) in a manner dependent on both these key transcriptional mediators.

4. Oxidation

The atherosclerotic lesion is dominated by accumulation of lipid peroxides along with the progression of early plaque development [25]. Oxidative stress is implicated in atherosclerosis and cardiovascular diseases. Recent lines of evidence appear to support the notion that serum PON1 undergoes inactivation under oxidative stress [12, 26–32]. However, the status of PON1 in the liver and its response to oxidative stress were very poorly investigated. Lipid peroxidation induced by iron-ascorbate decreased PON1 protein in hepatic microsomes derived from humans and rats [33], and this effect was attributable to oxidative stress, because the addition of the BHT antioxidant simultaneously prevented the occurrence of lipid peroxidation and improved the level of PON1 protein.

5. Hypolipidemic Drugs

Probucol, a cholesterol-lowering drug with strong antioxidative property, significantly increased serum PON1 concentration and upregulated PON1 mRNA expression in hepatocytes of hypercholesterolemic rabbits [34].

Thus, PON1 gene transcription is modulated by various factors related to inflammation, oxidative stress, or cholesterol. However, the mechanisms of regulation of PON1 gene expression itself remained elusive. Recent studies investigated the molecular mechanisms regulating PON1 gene expression. By comparing the effects of fenofibrate to those statins on PON1 gene expression in hepatocytes, Gouédard et al. characterized the promoter region of the PON1 gene and identified at least one inducer and one class of repressors of the PON1 gene [35]. They have shown that fibrates induced PON1 gene expression and this effect was repressed by PPARα activation, whereas statins inhibited PON1 gene expression via antagonizing the liver X receptor (LXR). On the contrary, Deakin et al. [36] demonstrated that simvastatin upregulated dose-dependently PON1 gene promoter activity, via increasing the nuclear factor sterol regulatory element-binding protein-2 (SREBP-2), which is capable to bind to the PON1 promoter. Clinical studies confirmed these in vitro findings, showing that during statin treatment serum PON1 concentration and activity increased. Complementary studies of the same group revealed that SREBP-2 binds to the PON1 promoter in an interactive manner with Sp1 [36]. Another study presented evidence that Sp1 acts as a positive regulator of PON1 transcription, and that an interaction between Sp1 and protein kinase C (PKC) is a key mechanism for the effect of Sp1 on PON1 gene transcription [37]. The effect of statins on PON1 gene expression was further investigated using a reporter gene assay by measuring luciferase activity of plasmids with a PON1 promoter region transfected into human hepatoma

HepG2 cells [38]. Pitavastatin, simvastatin and atorvastatin each significantly increased PON1 promoter activity. Transactivation by pitavastatin was completely abrogated by mithramycin, an inhibitor of Sp1. More recently, the same group of investigators demonstrated that pitavastatin activates the transcription of PON1 gene via phosphorylation of SREBP-2 and stimulation of Sp1 binding to PON1 DNA through the activation of p44/42 MAP kinase signaling cascade [39]. These effects were mediated via PKC activation [40]. Another class of hypolipidemic drugs are the bile acids sequestrates, such as cholestyramine. Based on the findings reported by Gutierrez et al. that bile acids repress PON1 mRNA expression via FXR activation of ileal FGF15 [41], bile acids sequestrates may have beneficial effect on PON1 regulation.

High glucose was also shown to transactivate PON1 promoter through Sp1 activation by PKC in cultured hepatocytes [42].

6. Polyphenols

Polyphenols constitute one of the largest category of phytochemicals, most widely distributed among the plant kingdom, and an integral part of the human diet. Dietary consumption of some polyphenols present in wine [43] or in fruit juices increase serum PON1 activity in humans and in mice [44–47]. The mechanisms of action of polyphenols in the upregulation of PON1 were recently investigated by several groups and these studies leaded to elucidation of cellular signal transduction pathways and transcription factors involved in hepatocyte PON1 gene regulation. Quercetin is an ubiquitous flavonoid present in all fruits and vegetables. Dietary quercetin administration to rats was shown to markedly upregulate hepatic PON1 expression at the molecular level [48]. Other dietary polyphenols, such as naringenin, catechin, and quercetin, increased PON1 gene expression by an aryl-hydrocarbon-receptor-(AhR-) dependent mechanism [49]. Resveratrol, a polyphenolic phytoalexin found in grapes and wine, increased PON1 gene expression in human hepatocytes primary cultures and in the HuH7 hepatocytes cell line, and this effect involved a transcriptional mechanism mediated by the unconventional AhR responsive element in the PON1 gene promoter [50]. Berberine, a botanical alkaloid that has been isolated from a number of medicinal plants, has major applications in Chinese medicine. Treatment of HepG2 and HuH7 hepatocytes with berberine increased PON1 expression at the transcriptional level, via a JNK/c-Jun signaling pathway [51]. Pomegranate juice contains polymolecular ellagitannin compounds, such as punicalagins, which are potent antioxidant and antiatherogenic agents [52]. We have recently shown that pomegranate polyphenols mediated stimulation of PON1 gene expression in hepatocytes via cAMP-PKA signaling cascade [53]. Based on previous analysis of the promoter sequence of PON1 gene indicating that it could possibly be regulated by nuclear receptors [35], we have expanded these findings to elucidate a multisteps pathway of the proximal signaling by which pomegranate juice polyphenols can regulate PON1 gene transcription in hepatocytes. Our data show that PPARγ acts as the transcription factor downstream of cAMP-PKA signaling cascade that upregulates PON1 gene transcriptional activity and increases PON1 mRNA expression in hepatocytes [53, 54].

7. Urokinase-Type Plasminogen Activator

Direct evidence for PON1 being a target gene of PPARγ evolved from studies investigating atherogenic roles of the urokinase-type plasminogen activator (uPA). uPA is a serine protease enzyme of the fibrinolytic system, and uPA binding to its receptor, uPAR, is implicated in plasmin generation and also in nonproteolytic processes that extend beyond its role in fibrinolysis. We have recently shown that uPA enhanced macrophage atherogenicity by increasing cellular cholesterol accumulation [55] and by promotion of oxidative stress [56]. Very recently we demonstrated that uPA reduces hepatic PON1 gene transcription via its interaction with uPAR on hepatocytes surface. Studies on the mechanism responsible for this effect showed that uPA binding to uPAR stimulates MEK interaction with PPARγ in the nucleus, leading to export of the nuclear PPARγ to the cytosol. By using the chromatin immunoprecipitation (ChIP) assay we have evidenced for the first time that PPARγ binds to DNA sequences in the PON1 promoter region and that uPA reduces the association of PPARγ to PON1 promoter, reducing thus PON1 gene transcription [57].

8. Conclusions and Perspectives

There is increasing epidemiological evidence that PON1 protects against development of atherosclerosis. The emphasis in this review was on nongenetic factors that modulate molecular processes related to PON1 gene expression in hepatocytes, reflecting PON1 level and activity in serum. Research in this area has provided mechanistic insight into how PON1 gene transcription can be increased by dietary nutrients. However, as reviewed herein, many of the studies use a single mechanistical approach in a specific model, and thus progression to more pathophysiologically relevant in vitro and animal models is essential. Understanding of these molecular mechanisms is of fundamental importance for atherosclerosis and cardiovascular disorders and could lead to development of novel therapeutical avenues in treatment of these diseases.

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Research Article

Paraoxonase-1 55 LL Genotype Is Associated with No ST-Elevation Myocardial Infarction and with High Levels of Myoglobin

Francesca Marchegiani,[1] Liana Spazzafumo,[2] Maurizio Cardelli,[1] Mauro Provinciali,[1] Francesco Lescai,[3] Claudio Franceschi,[4,5] and Roberto Antonicelli[6]

[1] *Advanced Technology Centre for Aging Research, Italian National Research Centre on Aging (INRCA), INRCA-IRCCS, 60121 Ancona, Italy*
[2] *Centre of Biostatistics, Italian National Research Centre on Aging (INRCA), 60121 Ancona, Italy*
[3] *Institute of Child Health, University College London, London, UK*
[4] *Department of Experimental Pathology, University of Bologna, Bologna, Italy*
[5] *Interdipartimental Centre "L. Galvani", University of Bologna, Bologna, Italy*
[6] *Department of Cardiology (CCU), Italian National Research Centre on Aging (INRCA), 60121 Ancona, Italy*

Correspondence should be addressed to Francesca Marchegiani, fr.marchegiani@inrca.it

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It is well known that serum paraoxonase (PON1) plays an important role in the protection of LDL from oxidation. PON1 55 polymorphism is currently investigated for its possible involvement in cardiovascular diseases. The objective of our study is to verify if PON1 55 polymorphism is associated with risk of acute coronary syndrome (ACS) and with biochemical myocardial ischemia markers, such as troponin I, creatine kinase (CK)-MB, myoglobin, and C-reactive protein. We analysed PON1 55 polymorphism in a total of 440 elderly patients who underwent an ACS episode: 98 patients affected by unstable angina (UA), 207 AMI (acute myocardial infarction) patients affected by STEMI (ST elevation), and 135 AMI patients affected by NSTEMI (no ST elevation). We found that individuals carrying PON1 55 LL genotype are significantly more represented among AMI patients affected by NSTEMI; moreover, the patients carrying LL genotype showed significantly higher levels of myoglobin in comparison to LM + MM carriers patients. Our study suggests that PON1 55 polymorphism could play a role in the pathogenesis of cardiac ischemic damage. In particular, the significant association between PON1 55 LL genotype and the occurrence of a NSTEMI may contribute to improve the stratification of the cardiovascular risk within a population.

1. Introduction

In western developed countries the atherosclerosis-related diseases, such as cardiovascular disease (CVD), remains so far the major cause of morbidity and premature death [1]. For this reason, atherosclerotic cardiovascular disease represents a major public health concern, and many efforts are addressed to better understand the mechanisms underlying this important pathology. The oxidative modification of low-density lipoprotein (LDL) in the arterial wall is claimed to play a central role in the pathogenesis of atherosclerosis. The oxidative stress is demonstrated to increase the formation of oxidized macrophages which are, in turn, responsible for the oxidation of LDL. Early studies focused on atherosclerosis generally considered LDL as the main cause of this pathology, and the inclusion of statins in clinical practice has significantly improved the quality of life of patients. The hypothesis of the inflammation as an underlying cause of atherosclerosis has been proposed only recently. For a long time, these two aspects (lipids and inflammation) have been kept apart, but actually they are the two faces of the same medal and both should be considered when we

deal with atherosclerosis. Serum paraoxonase (PON1), an HDL-associated enzyme, plays an important role in the protection of LDL from oxidation, and it may attenuate the development of atherosclerosis [2, 3]. In fact, PON1 hydrolyzes and reduces lipid peroxides in lipoproteins and in arterial cells. The atheroprotective function of PON1 has been also demonstrated in PON1 knock-out mice, which exhibited an accelerated atherosclerosis in contrast to hPON1 transgenic mice where the lesion size was decreased [4]. PON1 exerts its anti-inflammatory properties mainly hydrolyzing hydrogen peroxide, a major reactive oxygen species produced under conditions of inflammation. The most studied PON1 gene polymorphisms are due to amino acid substitutions at position 192 (Gln-Arg) and at position 55 (Leu-Met) in the coding region of the gene. Alleles at codon 192 (Q and R alleles) and 55 (L and M alleles) in PON1 locus have been associated with enzymatic activity and concentration, respectively [5–7]. PON1 192 and 55 polymorphisms have been widely investigated especially for their possible involvement in the onset or severity of cardiovascular disease (CVD) [8]. Some studies have found these two polymorphisms associated with the risk of cardiovascular diseases, but others have reported no association [8]. Until now, a definitive response about the role of PON1 genetic polymorphisms carried out in CVD does not exist, and further studies are necessary to better clarify the real involvement of this gene in cardiovascular pathology. The purpose of this study is to better explore this issue analysing the relationship between PON1 55 polymorphism and ACS (acute coronary syndrome). Today, more than ever, the use of genetics in the formulation of a diagnosis is a daily need. In fact, it is widely demonstrated that the traditional resources available to the clinician, that is, international guidelines and cardiac enzymes, are insufficient, and this is especially true in elderly patients where, frequently, the cardiovascular disease has an unusual onset, often leading to misdiagnosis. Therefore, studies carried out to find new molecular markers are warranted.

2. Materials and Methods

2.1. Sample. The sample enrolled for this study was composed of 440 elderly patients affected by ACS (acute coronary syndrome) (246 males and 194 females, mean age: 79.28 ± 9.94 years) consecutively admitted to the Coronary Care Unit (CCU) of the Italian National Research Center on Aging (INRCA) in a consequence of an ACS. Ninety-eight patients were affected by unstable angina (UA), two hundred and seven patients were affected by STEMI (ST-elevation myocardial infarction) and one hundred and thirty-five patients were affected by NSTEMI (no ST-elevation myocardial infarction). All patients were considered eligible if they fulfilled the diagnostic criteria for ACS [9]. Exclusion criteria were severe anemia, cancer, or life expectancy less than 12 months for other severe illness. Patients were then included in a one-year followup aimed at detecting the cardiovascular mortality rate (complete followup was available in 67.3% of the patients, $n = 296$).

During the hospitalisation the AMI diagnosis was confirmed with instrumental examination, such as coronary angiography and 2D-ecocardiography. The patients enrolled in the study were stratified as patients with a previous history of CHD, if the presence of CHD was previously documented, or patients without a history of CHD, if the current ACS episode was the first CHD manifestation. All subjects gave their informed consent to the study, which was approved by the Ethics committee of the INRCA. In all patients we evaluated arterial blood pressure, total and HDL-cholesterol, C-reactive protein (CRP), MB fraction of creatine kinase, measured by mass assay (CK-MB), troponin I (TnI), and myoglobin levels. Levels of HDL cholesterol were defined as low if <30 mg/dL (0.78 mmol/L), and hypercholesterolemia was defined by 200 mg/dL (5.18 mmol/L) cholesterol levels according to WHO guideline [10]. The diagnosis of arterial hypertension was made according to the 2003 European Society of Hypertension-European Society of Cardiology Guidelines for the management of arterial hypertension [10], if office blood pressure was repeatedly >140/90 mmHg or if patients were under antihypertensive treatment. Diagnosis of type 2 diabetes mellitus was based on revised American Diabetes Association diagnostic criteria [11].

2.2. Genotyping. DNA was extracted from peripheral blood mononuclear cells (PBMCs) using phenol/chloroform, according to standard procedures [12]. Polymerase chain reactions were performed using primer sequences derived from published data and specific for the amplification of the regions surrounding codon 55. The amplification reactions and methodologies have been previously described [13].

2.3. Statistical Analysis. Arterial hypertension, type 2 diabetes mellitus, documented history of CHD, hypercholesterolemia and low HDL cholesterol were coded as binary variables on the basis of the presence or absence of these conditions. Smoking habit was assessed through a questionnaire, and each subject was coded as current or noncurrent smoker (never and ex-smoker). C-reactive protein (CRP), MB fraction of creatine kinase (CK-MB), troponin, and myoglobin levels were natural log transformed before statistical analysis to achieve a normal distribution. Differences for categorical and continuous variables were assessed by χ^2 test and unpaired t-test, respectively. PON1 55 genotypes were grouped and analyzed as M+ (LM + MM genotypes) and M (LL genotype) carriers.

Because diagnoses are defined as 3-level categorical outcome (UA, STEMI, and NSTEMI), the multinomial logistic regression was performed to evaluate the relationships between the ACS and PON1 55 carriers. Odds ratio (OR) and 95% confidence interval (95% CI) were estimated for the STEMI and NSTEMI groups with respect to UA patients (reference group).

The hazard ratios (HRs) with corresponding 95% confidence interval (CI) for the occurrence of one-year follow-up mortality associated with the following covariates (PON1 55 carriers, age, documented history of CHD, arterial hypertension, smoking habit, hypercholesterolemia, type 2

Table 1: Baseline clinical and genetics characteristics of 440 elderly ACS patients.

Characteristics	ACS patients ($N = 440$)	
	N	%
ACS diagnosis: UA (unstable angina)	98	22.3
ACS diagnosis: NSTEMI (no ST-elevation myocardial infarction)	135	30.7
ACS Diagnosis: STEMI (ST-elevation myocardial infarction)	207	47.0
Low HDL-cholesterol	133	30.2
No low HDL-cholesterol	307	69.8
Smokers	66	14.9
noncurrent smoker	374	85.1
History of CHD	288	65.4
No history of CHD	152	34.6
Type 2 diabetes mellitus	156	35.5
No Type 2 diabetes mellitus	284	64.5
Arterial hypertension	315	71.6
No arterial hypertension	125	28.4
Hypercolesterolemia	276	62.7
No hypercolesterolemia	164	37.3
Dead	80	27.0
Alive	216	73.0

Table 2: PON1 55 carriers frequencies of 440 ACS patients.

ACS diagnosis	M− (LL) n (%)	M+ (LM + MM) n (%)
UA (unstable angina)	36 (36.7)	62 (63.3)
NSTEMI (No ST-elevation myocardial infarction)	70 (51.9)	65 (48.1)
STEMI (ST-elevation myocardial infarction)	85 (41.1)	122 (58.9)
Total	191 (43.4)	249 (56.6)

$\chi^2 = 6.158$, d.f. = 2, $P = 0.046$: for comparison between ACS diagnosis and PON55 carriers.

diabetes mellitus, low HDL cholesterol, ACS diagnoses, CRP, troponin, CK-MB, and myoglobin levels) were estimated with Cox proportional hazards analysis.

Throughout the study the level of statistical significance was defined by a two-tailed P value <0.05. All the analyses were performed by tests implemented with the SPSS package for Windows, version 18 (SPSS, Chicago, Illinois, USA).

3. Results

The baseline clinical characteristics of 440 CHD elderly patients affected by an ACS are reported in Table 1. PON1 55 carriers frequency distributions, separately considered on the basis of the diagnosis of UA, STEMI, and NSTEMI are reported in Table 2. Significant difference among the three groups of ACS was evident when carrier frequency distribution was compared ($\chi^2 = 6.158$, d.f. = 2 $P = 0.046$ for carrier frequency). In particular, by the multinomial logistic regression model, we found that subjects carrying LL genotype (M carriers) have a higher risk to develop a NSTEMI, in respect to the other ACS groups (OR: 1.855, 95% CI, 1.090 to 3.156; $P = 0.023$; UA patients were considered as the reference group). We then assessed the mean plasma levels of C-reactive protein and myocardial ischemia markers (troponin I, CK-MB, and myoglobin) according to the PON1 55 carriers. We found that among ACS patients, those who were carriers of the PON1 55 LL genotype displayed significantly higher plasma levels of myoglobin (Table 3). Interestingly, also for the other biochemical parameters considered (CRP, troponin I, and CK-MB), we found that LL patients displayed higher levels in respect to M+ carriers, although not significant (Table 3).

The one-year follow-up mortality rate was not significantly associated with PON1 55 carriers neither with any specific ACS diagnoses, such as STEMI, NSTEMI, and UA, nor with history of CHD, smoking habits, hypercholesterolemia, arterial hypertension, CK-MB levels, or troponin I levels (data not shown). Conversely, a significant association with mortality rate was found for the following variables: age (HR: 1.060, 95% CI, 1.020 to 1.102; $P = 0.003$), history of type 2 diabetes mellitus (HR: 1.874, 95% CI, 1.104 to 3.183; $P = 0.020$), HDL cholesterol levels (HR: 1.720, 95% CI, 1.003 to 2.948; $P = 0.049$), C-reactive protein (HR: 1.302, 95% CI, 1.073 to 1.580; $P = 0.008$), and myoglobin levels (HR: 1.511, 95% CI, 1.151 to 1.983; $P = 0.003$). Moreover, PON1 55 M+ and M carriers did not significantly correlate with history of CHD, diabetes mellitus, arterial hypertension, smoking habits, hypercholesterolemia, and low HDL-cholesterol (data not shown).

4. Discussion

Atherosclerosis is a multifactorial disease of great complexity, and we are only now beginning to sort out the many elements involved especially in the later stages of the disease. Accordingly, the main aim of our study was to investigate the relationship between the PON1 55 polymorphism and acute coronary syndrome in elderly patients. In the past, we have extensively analyzed the role of paraoxonase-1 in healthy elderly people, that is, centenarians, both as genetics and as activity and mass of the protein (as reviewed in [14]). The first result that we have obtained in this study is that PON1 55 M carriers have a significantly different distribution in the three groups of patients, that is, UA, STEMI, and NSTEMI patients. In particular, subjects carrying LL genotype have almost double the risk to develop an AMI of NSTEMI type. The other important finding is that individuals carrying LL genotype displayed significantly higher plasma levels of myoglobin in respect to M+ carriers. Although not significantly, also troponin I, CK-MB, and CRP levels were higher in LL subjects. All of them are important biomarkers evaluated in case of patients manifesting the classical symptoms of an ACS episode. The fact that these biomarkers were higher in LL patients compared to those with LM or MM genotype could suggest that they will have to face a more severe ACS episode. This finding, to the best

Table 3: Levels of biochemical parameters according to PON1 55 carriers in ACS patients.

Biochemical parameters	PON1 55 M− carriers (LL)	PON1 55 M+ carriers (LM + MM)	*P*
CK-MB (μg/L) mean ± SD	78, 54 ± 118, 98	64, 76 ± 106, 22	0.273
Myoglobin (nmol/L) mean ± SD	51.49 ± 108.57	33.76 ± 67.99	**0.044**
Troponin I (μg/L) mean ± SD	38, 38 ± 65, 36	26, 29 ± 61	0.118
C-reactive protein (nmol/L) mean ± S.D	62.19 ± 126.86	45.72 ± 58.10	0.728

of our knowledge, has not yet been reported in an other study. In this work the absence of a control group inhibits us to state if PON1 55 polymorphism could be responsible for the onset of cardiovascular disease. We can only suggest that this polymorphism could modulate the pathogenesis and the seriousness of cardiovascular disease.

Based on the above-mentioned assumptions, we would expect that the mortality rate would be higher in LL patients in respect to the other (LM or MM patients). Surprisingly, the one-year follow-up mortality rate was influenced by other variables, such as age, history of type 2 diabetes mellitus, HDL-cholesterol levels, C-reactive protein levels, and myoglobin levels. The fact that PON1 55 polymorphism does not directly influence the mortality rate could depend by the fact that subjects with the followup are a subgroup, and perhaps too few, or that the genotype alone could not explain the entire phenomenon. However, it is very interesting to note that among the variables affecting the mortality rate we have found myoglobin levels and HDL-cholesterol levels. The latter is particularly interesting as paraoxonase is a high-density lipoprotein- (HDL-) associated esterase that hydrolyses lipoperoxides, and so it could play a role, albeit in an indirect way.

In the literature, the possible role played by PON1 55 polymorphism, and in particular LL genotype, in the onset of cardiovascular heart disease (CHD) was extensively investigated and several authors have found a positive association between this variant and the cardiovascular pathologies (reviewed in [8]).

Conversely, the number of the studies that do not consider the onset of the disease but its severity are much less numerous, at least until now. Perhaps, it depends by the fact that the genetics of an individual is considered an important characteristic to determine if the individual could be at risk of developing a disease in order to make primary prevention. However, actually, it could also be important once the disease has occurred to stratify the subjects and treat them differently according to the genetic background, focusing on those who are at increased risk (secondary prevention).

In a recent paper, the authors to find the possible candidate genes involved in the pathogenesis of coronary atherosclerosis analyzed 1936 tag SNPs within 116 candidate genes. Interestingly, they found three SNPs associated with the disease, and one of them (rs854563) is included in an intron in the same region of PON1 55 polymorphism [15]. Another paper analyzed PON1 192 and 55 polymorphisms in the etiology of CAD. The authors failed to find an association between PON1 55 polymorphism and the onset of cardiovascular disease, but they found that LL genotype and L allele were significant risk factors in the nonsmoker group [16]. Accordingly to this study, Malin et al. [17] also have found that in nonsmoking men the LL genotype could represent a genetic risk factor for carotid artery atherosclerotic disease. Unlike previous studies, Robertson et al. [18] found an higher CHD risk for LL smokers compared to LL nonsmokers. Although in our study we have not found an association between smoking habit and PON1 55 polymorphism, in accordance with the former studies we have found that patients carrying LL genotype have to face with a more seriously ACS episode. A study in which the PON1 55/192 haplotype was correlated with the number of diseased vessels emerged that subjects with PON1 RR/LL genotype possess an higher number of diseased vessels in respect to the other genetic variants [19]. The presence of LL genotype was also demonstrated to be associated with a more severe degree of IR (insulin resistance), suggesting that the IR might be the possible missing link between Met-Leu 55 PON polymorphism and the increased cardiovascular risk [20]. Accordingly, the 55 Leu allele was found to be associated with coronary artery disease only in the subgroup with metabolic syndrome [21]. Moreover, another paper reported that the LL homozygous men had more atherosclerotic plaques and complicated lesions in the common iliac arteries than the M allele carriers [22]. Conversely, in an Italian study the PON1 55 polymorphism was not found to be associated with carotid abnormalities [23].

An important limitation of this study is the lack of paraoxonase activity, as this could be useful to clarify the role of this enzyme in CHD and to remove the existing bulk of conflicting results.

Finally, our study suggests that PON1 55 polymorphism could play a role in the pathogenesis of cardiac ischemic damage. Hence, the significant association between PON1 55 LL polymorphism and the occurrence of a NSTEMI may contribute to improve the stratification of the cardiovascular risk in UA, NSTEMI, and STEMI being useful in providing a more accurate assessment of a patient prognosis.

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Research Article

Lipid Peroxidation and Paraoxonase-1 Activity in Celiac Disease

Gianna Ferretti,[1] Tiziana Bacchetti,[2] Letizia Saturni,[1] Nicola Manzella,[1] Cinzia Candelaresi,[3] Antonio Benedetti,[3] and Antonio Di Sario[3]

[1] *Dipartimento di Scienze Cliniche Sperimentali e Odontostomatologiche, Università Politecnica delle Marche, 60131 Ancona, Italy*
[2] *Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, 60131 Ancona, Italy*
[3] *Clinica di Gastroenterologia, Università Politecnica delle Marche, 60131 Ancona, Italy*

Correspondence should be addressed to Tiziana Bacchetti, t.bacchetti@univpm.it

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Paraoxonase-1 (PON1) plays an antioxidant and anti-inflammatory role. Aim of the study was to investigate the alteration of paraoxonase-1 activity in celiac disease (CD), an intestinal disorder characterized by toxic injury exerted by gluten peptides. Activities of PON1, levels of biochemical markers of lipid peroxidation and total antioxidant capacity were evaluated in serum obtained from 27 celiac patients (11 at diagnosis, 16 treated with gluten free diet) and 25 healthy subjects. Moreover, the serum susceptibility of Cu^{2+}-induced lipid peroxidation was investigated in controls and patients. The results showed a lower PON1 activity in serum of both groups of celiac patients with respect to control subjects. PON1 activity in CD was related with markers of disease severity and was negatively correlated with the levels of lipid hydroperoxide and with the susceptibility of serum to lipid peroxidation induced in vitro by metal ions. The alteration of PON1 activity and markers of lipid peroxidation realized at lower extent in patients who were on a gluten-free diet.

1. Introduction

Environmental, genetic, and immunologic factors are involved in the pathogenesis of celiac disease (CD) [1], a chronic inflammatory disease characterised by flattened villi on the small bowel mucosa. CD is induced by the ingestion of cereals containing proline-rich and glutamine-rich proteins (such as wheat, rye, and barley) in genetically susceptible individuals [2, 3]. From a clinical point of view, CD is characterised by a clinical heterogeneity that ranges from asymptomatic to severely symptomatic and by increased morbidity and mortality [4]. At present, the only proven treatment for CD is strict and life-long adherence to a gluten-free diet (GFD) [1–4].

An increase of markers of oxidative damage of lipids (thiobarbituric acid-reactive substances and lipid hydroperoxides) and proteins (carbonyl groups) and a decrease of antioxidant enzymes have been demonstrated in plasma, in circulating cells and in intestinal cells of patients with CD with respect to controls [5–8]. Higher levels of nitric oxide (NO) and biochemical markers of oxidative damage of DNA has been demonstrated in leukocytes and in urine samples of celiac children [9, 10], suggesting that oxidative stress could be involved in the pathogenesis of CD.

Paraoxonase-1 (PON1) is an enzyme associated with the high-density lipoproteins (HDL) that plays both an antioxidant and an anti-inflammatory role [11–13]. A decrease in PON1 has been reported in several human diseases associated with inflammation and alteration of lipoprotein metabolism [14–17]. Interest in the study of PON1 in celiac disease is supported by recent studies that have suggested a possible role of PON1 in inflammatory intestinal diseases [18–20]. Furthermore it has been hypothesised that in the gastrointestinal tract, PON1 could behave as a local detoxifier, antioxidant, immunomodulator, and/or quorum-quenching factor [18].

To further investigate the relationship between oxidative damage and CD, we studied the levels of lipid peroxides, total antioxidant capacity, the susceptibility to copper-induced lipid peroxidation, and the activity of the enzyme paraoxonase-1 (PON1) in serum of three groups of subjects: untreated patients with a new diagnosis of celiac disease (CD patients), CD patients on a gluten-free diet (GFD patients), and healthy subjects.

2. Materials and Methods

2.1. Subjects Included in the Study. The study included 27 consecutive patients who were referred to the Celiac Disease Clinic of the Department of Gastroenterology of the Polytechnic University of Marche, and the study protocol was in accordance with the ethical standards formulated in Helsinki Declaration as revised in 2000. Eleven patients (3 males and 8 females, mean age 31.2 ± 11.7 years) had a new diagnosis of CD (CD patients), whereas 16 (GFD patients; 7 males and 9 females, mean age 35.8 ± 12.1 years) were celiac patients on a gluten-free diet (mean duration: 19.2 ± 5.7 months). Twenty-five subjects (12 males and 13 females, mean age 39.6 ± 8.1 years) who underwent esophagogastroduodenoscopy for the presence of dyspeptic syndrome and who did not result to be affected by CD were used as controls. Subjects with diabetes and clinical evidence of cardiovascular diseases or receiving antacids, lipid-lowering drugs, or antioxidant supplements were excluded from the study to avoid possible interferences on PON1 activity and plasma lipids.

All subjects included in the study underwent upper gastrointestinal endoscopy and at least 4–6 well-oriented duodenal specimens were taken for the histological evaluation.

The degree of intestinal mucosal damage was then classified according to the Marsh classification [21]. Thereafter, each stage was given a score from 0 (normal mucosa) to 5 (total villous atrophy) for statistical analysis.

Serum levels of antitransglutaminase(t-TG) antibodies were assessed by the laboratory of our institution by using a commercial FluoroEnzymeImmuno Assay kit (Phadia, Sweden) and the results were expressed as unit (U)/mL of serum. A value higher than 10 U/mL was considered positive [22].

In the same day of endoscopy, blood drawn by venous puncture by fasted subjects was collected into serum-separator tubes. After clotting, blood was centrifuged for 15 min at 3500 rpm to separate serum that was stored at −80°C until examination.

2.2. Serum PON1 Activities. PON1 activity has been evaluated using three different substrates [23]: paraoxon for paraoxonase activity, phenylacetate for arylesterase activity, and dihydrocoumarin for lactonase activity. All assays were performed in a 96-well plate, in a total reaction volume of 200 μL.

2.2.1. Paraoxonase Activity. Serum (10 μL, nondiluted samples) were analyzed. The basal assay mixture included 5 mM Tris-HCl, pH 7.4 containing 0.15 M NaCl, 4 mM $MgCl_2$, 2 mM $CaCl_2$, and 1.0 mmol/L paraoxon. Paraoxon hydrolysis was spectrophotometrically monitored for 8 min (every 15 s) at 412 nm. Nonenzymatic hydrolysis of paraoxon was subtracted from the total rate of hydrolysis. One unit of PON1 paraoxonase activity was equivalent to 1 nmol of paraoxon hydrolyzed/min/mL [23].

2.2.2. Arylesterase Activity. The serum samples were diluted 1 : 10 with 1 mmol/L $CaCl_2$ in 50 mmol/L Tris HCl, pH 8.0 and then, 5 μL was taken for a total reaction volume of 200 μL. After addition of the substrate phenyl acetate (1.0 mmol/L), the hydrolysis was monitored at 270 nm for 3 min (every 15 s). One unit of arylesterase activity was equivalent to 1 μmol of phenyl acetate hydrolyzed/min/mL [23].

2.2.3. Lactonase Activity toward Dihydrocoumarin (DHC). The serum samples were diluted 1 : 10 with 1 mmol/L $CaCl_2$ in 50 mmol/L Tris HCl, pH 8.0 and 3 μL was then taken for the assay. After addition of the substrate DHC (1.0 mmol/L), the hydrolysis was monitored at 270 nm for 10 min (every 15 s). Nonenzymatic hydrolysis of DHC was subtracted from the total rate of hydrolysis. One unit of lactonase activity was equivalent to 1 μmol of DHC hydrolyzed/min/mL [23].

2.3. Serum Oxidative Stress Parameters

2.3.1. Serum Basal Oxidative Stress Status. Basal serum oxidative status was determined by the evaluation of the levels of lipid hydroperoxides using FOX2 assay [15]. Briefly, an aliquot (200 μL) of serum was mixed with 1800 μL of FOX2-reagent (250 μmol/L ammonium ferrous sulphate, 100 μmol/L xylenol-orange, 25 mmol/L H_2SO_4 and 4 mmol/L BHT in 90% methanol (v/v) in 100 mL). After incubation at room temperature for 30 minutes, samples were centrifuged at 4000 rpm for 10 minutes. The supernatant was carefully decanted into a cuvette and the absorbance was determined at 560 nm. The levels of lipid hydroperoxides were quantified using a stock solution of t-butyl hydroperoxide. The results were expressed as μmol of lipid hydroperoxides for L of serum.

2.3.2. Copper-Induced Serum Lipid Peroxidation. Serum samples were diluted 4x with phosphate-buffered saline (PBS-citrate) and were incubated with 50 μmol/L $CuSO_4$. Serum lipid peroxidation was monitored by following the kinetic of conjugated dienes formation at 234 nm [24]. The increase of absorbance was recorded for 6 hours. A KC4 software was used to calculate the lag times of kinetic of conjugated dienes formation. The lag time (t-lag, min), defined as the intercept of the straight lines derived from the lag phase and the propagation phase, is an index for resistance of serum to lipid peroxidation [24]. As previously demonstrated longer lag time indicates a lower susceptibility of serum to lipid peroxidation [24].

2.4. Serum Total Antioxidant Capacity. Serum total antioxidant capacity (TAC) was assessed using the oxygen radical absorbance capacity (ORAC) assay described by Ou et al. [25]. The oxygen radical absorbance capacity of serum employs the oxidative loss of the intrinsic fluorescence of fluorescein induced by the free radical initiator 2,2′-azobis(2-amidinopropane)hydrochloride (AAPH). Trolox was used as a reference antioxidant for calculating the ORAC values. Results were expressed as μmol Trolox equivalents/L (μmol TE/L).

2.5. Statistical Analysis. Data were reported as mean ± standard deviation (SD). PON1 activities were presented as medians. For the comparison of normally distributed variables between groups, Student's *t*-test was used. Paraoxonase-1 activity showed a non-Gaussian distribution;

Table 1: Clinical characteristics and levels of serum lipids in healthy subjects, celiac patients at diagnosis (CD patients) and celiac patients treated with a gluten-free diet (GFD patients). Data are given as means ± S.D.

	Healthy subjects ($n = 25$)	Celiac patients ($n = 27$)	
		CD patients($n = 11$)	GFD patients ($n = 16$)
Serum t-TG[a] (U/mL)	0.4 ± 0.2	108.2 ± 35.9*	2.3 ± 1.9*#
Damage of intestinal mucosa[b]	0	4.4 ± 0.6*	1.4 ± 0.7*#
Serum triglycerides (mg/dL)	87.140.5±	61.3 ± 23.5	71.2 ± 48.2
Serum cholesterol (mg/dL)	186.6 ± 43.4	154.1 ± 27.6	177.1 ± 42.6
Serum HDL-cholesterol (mg/dL)	56.3 ± 13.4	51.1 ± 7.9	54.6 ± 8.7
Serum LDL-cholesterol (mg/dL)	113.2 ± 23.7	90.6 ± 16.1	108.8 ± 26.2

[a]t-TG: antitransglutaminaseantibodies. [b]Damage of intestinal mucosa was classified according to Marsh parameters and each stage was given a score from 0 (normal mucosa) to 5 (total villous atrophy).
*$P < 0.001$ versus healthy subjects; #$P < 0.05$ versus CD patients.

therefore we used a nonparametric test (Wilcoxon rank sum test). Spearman's correlation coefficient was used to test the strength of any association between different variables. All tests were 2-tailed and a $P < 0.05$ level of significance was used to assess statistical significance (Microcal Origin 5.0, OriginLab, Northampton, MA).

3. Results

3.1. Clinical Characteristics of the Study Population. As shown in Table 1, serum levels of anti-t-TG antibodies were within the normal range in healthy subjects (0.4 ± 0.2 U/mL). As expected, significant higher levels were observed in untreated celiac patients (108.2 ± 35.9 U/mL). In GFD patients the levels of anti-t-TG antibodies were significantly higher compared with controls but significantly lower with respect to untreated CD patients (Table 1). The degree of intestinal damage was in the order: controls < GFD patients < CD patients (Table 1). As summarized in Table 1, the differences were statistically significant.

3.2. Plasma Lipids in Controls and Celiac Patients. The mean levels of serum total cholesterol (TC), HDL-cholesterol and LDL-cholesterol, and triglycerides (TG) were lower in CD patients with respect to controls, even if the differences were not statistically significant (Table 1). The levels of plasma lipids in GFD patients were higher with respect to CD patients, although the differences were not statistically significant (Table 1).

3.3. Serum Paraoxonase-1 Activities in Controls and Celiac Patients. As summarized in Table 2, the median values of PON1 paraoxonase activity were in the order controls > GFD patients > CD patients. The differences between both groups of CD patients compared with controls were statistically significant (Table 2). The median values of lactonase and arylesterase activities of PON1 in serum of CD and GFD patients were significantly lower with respect to controls (Table 2). PON1 paraoxonase activity was significantly correlated with arylesterase ($r = 0.871$, $n = 52$, $P < 0.0001$) and lactonase ($r = 0.875$, $n = 52$, $P < 0.0001$) activities.

3.4. Serum Lipid Peroxidation Markers and Total Antioxidant Capacity in Controls and Celiac Patients. The levels of lipid hydroperoxides in control subjects were 2.02 ± 0.76 μmol/L. Higher levels of lipid hydroperoxides were observed in serum of CD and GFD patients with respect to controls. In serum of GFD patients, the values were significantly lower compared with untreated CD patients (Table 2).

To investigate serum susceptibility to lipid peroxidation in celiac patients, we evaluated the formation the conjugated dienes in serum oxidized "*in vitro*" with copper ions. The mean value of the lag time of the kinetic of lipid peroxidation in serum of controls was 66.4 ± 16.2 minutes (Table 2). The lag time was significantly lower in serum of CD and GFD patients. In GFD patients the lag time was significantly higher with respect to CD patients (Table 2).

As summarized in Table 2, in GFD patients serum TAC was lower than controls and significantly higher than that of CD patients.

3.5. Relationship between PON1 Paraoxonase Activity and Clinical Parameters in Celiac Patients. To study whether the activity of paraoxonase-1 and markers of oxidative stress were related to severity of CD, we compared the mean values of serum levels of anti-t-TG and the degree of mucosal damage (following Marsh index) in two subgroups of celiac patients. The first group included 14 CD patients having PON1 paraoxonase activity below the median value of PON1 observed in all celiac patients (124.6 U/mL) (10 untreated CD and 4 GFD patients). The second group included 13 patients with PON1 activity above the median value (1 untreated CD patient and 12 GFD patients).

As shown in Figure 1, the mean levels of serum anti-t-TG and the degree of mucosal damage were significantly higher in celiac patients with serum PON1 activity below the median value with respect to patients with PON1 activity above the median value. The differences were statistically significant.

Similar results were observed also for PON1 arylesterase and lactonase activities (data not shown).

3.6. Correlations between PON1 Paraoxonase Activity and the Other Markers of Oxidative Stress. The individual values

Table 2: PON1 activities, markers of lipid oxidative stress (levels of lipid hydroperoxides and lag time of conjugated dienes) and total antioxidant capacity (TAC) in serum of healthy subjects, celiac patients at diagnosis (CD patients), and celiac patients treated with a gluten-free diet (GFD patients). Data are given as median (range) for paraoxonase activities and means ± S.D. for other biochemical parameters.

	Healthy subjects ($n = 25$)	Celiac patients ($n = 27$)	
		CD patients ($n = 11$)	GFD patients ($n = 16$)
PON1 Activities			
Paraoxonase (U/mL)	306 (165.6–608.1)	101.5 (61.2–141.6)*	184.9 (75.3–347.8)*#
Lactonase (U/mL)	68 (32–184)	34 (19.7–68.5)*	54.7 (16.5–101.6)*#
Arylesterase (U/mL)	228 (90–612)	73.2 (41.2–109.7)*	105.5 (68.2–148.7)*#
Lipid hydroperoxides (μmol/L)	2.02 ± 0.76	5.31 ± 1.62*	4.14 ± 1.50*#
Lag time (min)	66.4 ± 16.2	39.4 ± 7.2*	46.7 ± 12.8*#
TAC (μM TE)	12048 ± 2678	8796 ± 976*	9977 ± 1021*#

*$P < 0.001$ versus controls; #$P < 0.05$ versus CD patients.

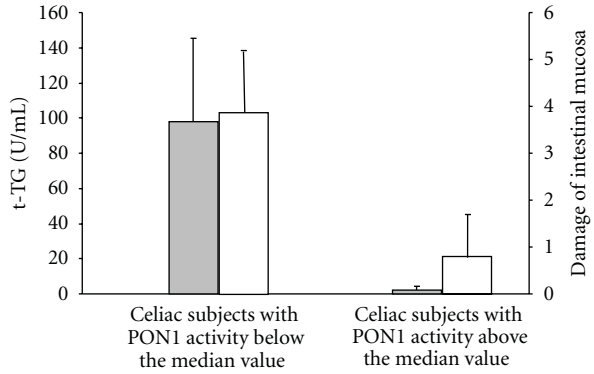

Figure 1: Mean levels of antitransglutaminase(t-TG) antibodies levels (grey square) and degree of damage of intestinal mucosa classified according to Marsh parameters (white square) in celiac patients with PON1 activity above or below the median value (124.6 U/mL). *$P < 0.001$ versus celiac patients with PON1 activity below median value.

of PON1-paraoxonase activity and the levels of serum hydroperoxides were negatively correlated ($r = -0.735$, $n = 52$, $P < 0.0001$), in agreement with our previous studies [15] (Figure 2(a)). In the whole group of subjects included in the study, the enzyme activity was positively correlated with the lag time of kinetic of lipid peroxidation ($r = 0.811$, $n = 52$, $P < 0.0001$) (Figure 2(b)) and with the values of total antioxidant capacity ($r = 0.74, n = 52, P < 0.0001$) (Figure 2(c)). Levels of lipid hydroperoxides were negatively correlated with total antioxidant capacity ($r = -0.52$, $n = 52$, $P < 0.0001$) and lag-time values ($r = -0.59$, $n = 52$, $P < 0.0001$).

No significant correlation was observed between PON1 activity and HDL cholesterol levels (data not shown).

4. Discussion

The clinical interest in human PON1 has been mainly focused on atherosclerosis and cardiovascular disease [14, 26], since PON1 exerts antioxidant and anti-inflammatory roles [14–16]. However, more recently a possible role of PON1 in the human small intestine in normal and pathological conditions has been suggested [18–20].

Although PON1 paraoxonase activity has been manly investigated in normal and pathological conditions using paraoxon as substrate [15–17], we evaluated also PON1 arylesterase and lactonase activities because previous studies have suggested that serum arylesterase activity could reflect serum enzyme concentration [27] and lactonase activity could represent a more physiological activity of the enzyme [28].

Our results demonstrated, for the first time, a significant decrease of PON1 activities (paraoxonase, arylesterase, and lactonase) in serum of celiac patients.

The modifications of PON1 activities in celiac patients were related with the severity of the disease and were associated with an increase of lipid hydroperoxide levels and with a higher susceptibility of serum to lipid peroxidation induced "in vitro." Moreover, a lower serum total antioxidant capacity (TAC) has been shown in celiac patients. The values of PON1 activity, markers of lipid peroxidation, and total antioxidant capacity in GFD patients were significantly different compared with healthy subjects and untreated CD patients.

Our results confirm that CD is associated with oxidative damage as suggested in previous studies mainly carried out in CD children [6, 8–10].

Some hypotheses can be made to explain the molecular mechanisms involved in the lower PON1 activity and the higher oxidative stress in CD patients (Figure 3).

Previous studies have demonstrated that, in CD patients, gluten ingestion induces an increased oxidative stress due to overproduction of free radicals (ROS and RNS) [29–31]. The higher levels of ROS and the decrease of dietary antioxidant can trigger lipid peroxidation (LOOH) in intestinal mucosa and blood lipoproteins.

PON1 is a lipid-dependent enzyme associated with high-density lipoprotein (HDL) [32]. Previous studies demonstrated that a decrease of serum PON1 activity could be related to a lower hepatic synthesis and secretion or to inhibition exerted by lipid hydroperoxides [27].

No significant correlation has been established between PON1 activity and levels of HDL cholesterol, suggesting that

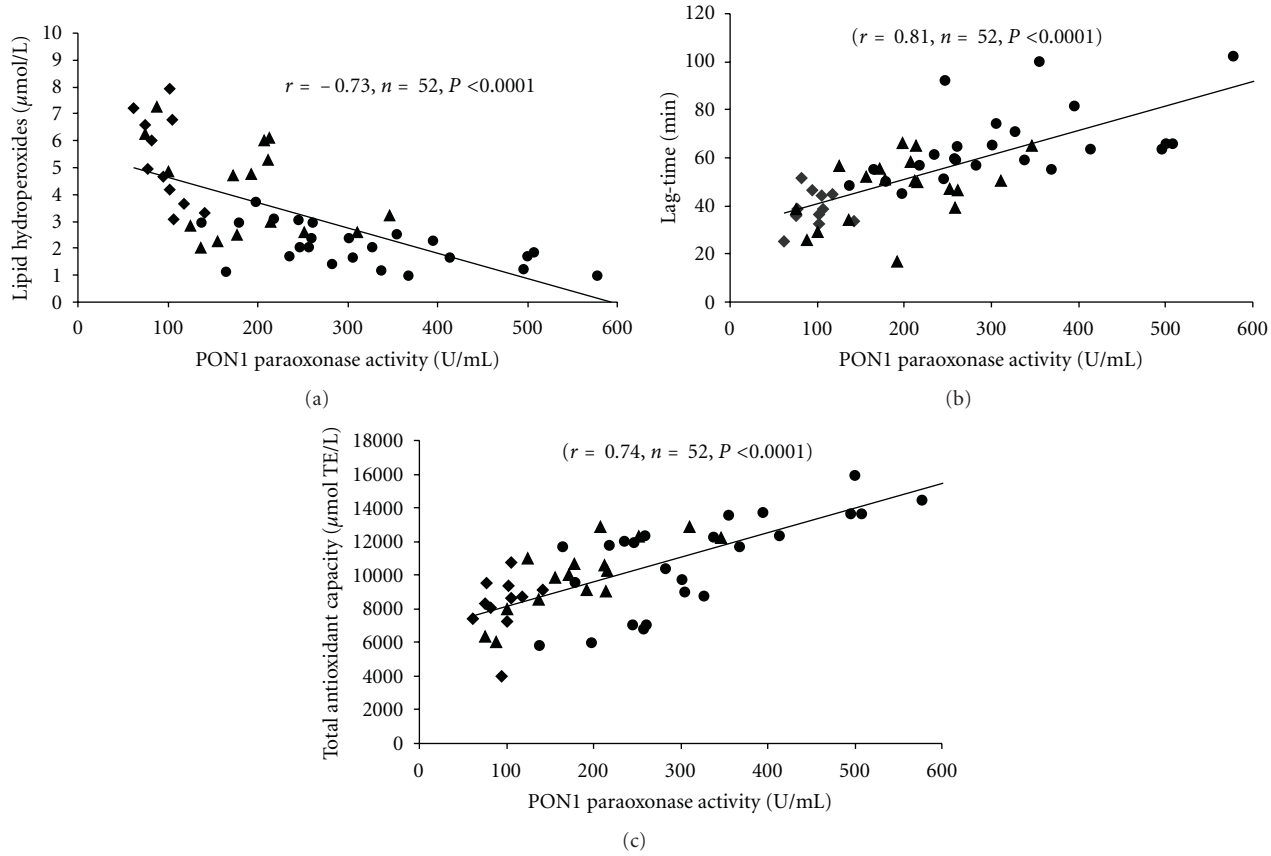

Figure 2: Correlations between PON1 paraoxonase activity versus the concentrations of serum lipid hydroperoxides (a), versus duration of the lag-time of Cu^{2+}-oxidized serum (b), versus serum total antioxidant capacity (c) of controls (•) and untreated (♦) or GFD-treated (▲) celiac patients.

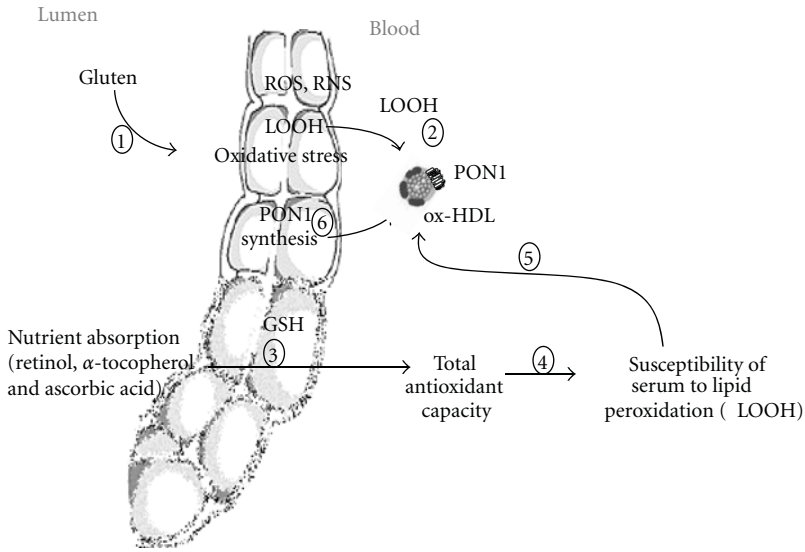

Figure 3: Possible molecular mechanisms involved in increased lipid peroxidation and decreased PON1 activity in celiac disease. Gluten ingestion induces an increased oxidative stress, due to overproduction of free radicals (ROS and RNS) (1). The higher levels of ROS can trigger lipid peroxidation (LOOH) in intestinal mucosa and blood (2). Oxidative stress could be favored by lower serum antioxidant capacity resulting from lower absorption of dietary antioxidants due to mucosal damage (3). All the aforementioned changes could explain the higher susceptibility of serum to lipid peroxidation (4) and the compositional alterations of HDL with consequent decrease of PON1 activities (5). In addition to the inhibition exerted by lipid peroxidation products, the lower PON1 activity could be mediated by inhibition of PON1 synthesis from gastrointestinal cells (6).

the lower enzyme activity in CD patients is not associated with alterations of HDL levels.

Although PON1 is mainly synthesised by the liver, it has been demonstrated that it is expressed also in human gastrointestinal tract and Caco-2 cell lines, suggesting that the intestine, as well as the liver, could represent a source for circulatory PON1 [33]. Since a decreased expression of PON1 mRNA has been reported by Rothem L et al. in intestinal biopsies of celiac patients [18], we could speculate that the lower PON1 activity could be related to changes of the enzyme synthesis from the gastrointestinal tract. However, contrasting results have been reported on the synthesis of PON1 in intestinal cells [18, 34–36].

The lower activity of PON1 in CD patients could be also mediated by inhibition exerted by lipid peroxidation products, since previous studies have shown that PON1 paraoxonase, arylesterase, and lactonase activities are inhibited by lipid peroxidation products [37, 38]. This hypothesis is supported by the significant negative correlation found between PON1 paraoxonase activity and levels of lipid hydroperoxides in serum of controls and celiac patients.

The higher susceptibility of serum of celiac patients to Cu^{2+}-triggered lipid peroxidation, demonstrated in our experimental conditions, may be related to the lower PON1 activity and/or to the lower serum total antioxidant capacity, as shown by the significant correlations observed between the lag time of kinetic of lipid peroxidation and PON1 paraoxonase values and TAC values.

The lower serum total antioxidant capacity in CD patients could be likely the result of a malabsorption of dietary antioxidants due to the mucosal damage. In agreement with this hypothesis other authors have reported lower levels of antioxidants (retinol, α-tocopherol, and ascorbic acid) in plasma of CD patients [5, 9]. The mean level of serum total antioxidant capacity in GFD patients was significantly higher compared with untreated CD patients, although it did not reach the values observed in controls; these results suggest that gluten-free diet is able to only partially improve the functionality of intestinal mucosa.

Whatever are the causes of the decrease in the activity of the antioxidant and anti-inflammatory enzyme PON1, our results might have a physiopathological relevance. In fact, the enzyme PON1 exerts several physiological roles. In the gastrointestinal tract PONs proteins and could behave as detoxifier against food antigens, antioxidant against exogenous and endogenous lipid peroxides, immunomodulators and/or inhibitors of bacterial quorum-quenching factor [18, 35]. Moreover, PON1 exerts a protective effect against lipid peroxidation of lipoproteins and biological membranes [11–13]. Oxidative stress is one of the main causes of damage to cell membranes and lipoproteins. End products of lipid peroxidation (aldehydes, lipid peroxides) react with biological macromolecules such as DNA and proteins and can cause changes in intestinal cell membrane structure and properties leading to loss of its integrity [39]. Moreover, lipid peroxidation products exert biological effects, modulate signal transduction and it has been proposed that they may have a role in inflammatory processes [39].

In conclusion, our results confirm that celiac disease is associated with oxidative damage and with significant decrease of PON1 activities. Although a decrease of paraoxonase-1 activity and an increase of lipid peroxidation products are not specific of celiac disease [14–17], they could exert a proinflammatory and cytotoxic effect and could therefore contribute to gastrointestinal cell injury in CD.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Review Article

Paraoxonase 1 in Chronic Kidney Failure

Alejandro Gugliucci,[1] Kazuhiko Kotani,[2] and Satoshi Kimura[3]

[1] *Glycation, Oxidation and Disease Laboratory, Touro University-California, Vallejo, CA 94592, USA*
[2] *Department of Clinical Laboratory Medicine, Jichi Medical University, Tochigi 329-0498, Japan*
[3] *Department of Laboratory Medicine and Central Clinical Laboratory, Showa University Northern Yokohama Hospital, Kanagawa 224-8503, Japan*

Correspondence should be addressed to Alejandro Gugliucci, alejandro.gugliucci@tu.edu

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In this review we summarize the findings from the literature and our own laboratory on the decreased PON1 activity in renal failure, the mechanisms proposed and the effect of interventions. In addition to profound alterations in lipoproteins, reduced serum PON1 activity has been clearly established in the past decade and could contribute to accelerated development of atherosclerosis in ESRD and in HD. PON1 lactonase activity is lower in ESRD patients. Hemodialysis partially restores PON1 lactonase and the other activities. PON1 activity recovery after dialysis suggests that uremic toxins may play a mechanistic role in PON1 inactivation. Lower PON1 activity in CRF patients is associated with low thiol concentration, high CRP, and is beneficially enhanced with vitamin C and flavonoids. Changes in HDL subclasses, namely lower HDL_3 in these patients may also play a role in PON1 lower activity. Future research should focus on: (1) mechanistic studies on causes for low PON1 activity and mass; (2) prospective studies focusing on whether there is an added predictive value in measuring PON1 activity (and PON1 activity in HDL_3) in this patient population; (3) intervention studies attempting to increase PON1 activity.

1. Introduction

The major cause of mortality in patients with end-stage renal disease (ESRD) receiving renal replacement therapy is cardiovascular disease. More than one million of these patients throughout the world are surviving with the assistance of renal replacement therapy [1–8]. More than 800,000 patients receive hemodialysis (HD), the most frequent modality. Survival on HD has improved, although vascular accidents, such as ischemic heart disease and hemorrhagic stroke, remain major problems [2, 7, 8]. All patients with chronic renal failure (CRF) have increased risk for death from cardiovascular disease, especially those undergoing HD [1, 2, 9]. They have numerous metabolic disorders that may hasten the development of plaques, such as insulin resistance, hypertension, and dyslipoproteinemia, along with other ESRD-related risk factors such as the classical calcium and phosphate metabolism disorders and secondary hyperparathyroidism [1–9]. CRF patients frequently have lipoprotein abnormalities such as low high-density lipoprotein (HDL)-cholesterol concentrations, increased remnant particles and hypertriglyceridemia. HDL-cholesterol concentrations are inversely correlated with atherogenic risk [3, 4, 6, 7].

HDL is not only a key player in reverse cholesterol transport but has the ability to protect low-density lipoprotein (LDL) against oxidation, is an anti-inflammatory mediator, protects the endothelium, and modulates coagulation [10–14]. There is mounting evidence that paraoxonase 1 (PON1) could be implicated in several of these processes, as shown in detail elsewhere in this special issue of this journal [15–26]. Human PON1 (aryldialkylphosphatase, EC 3.1.8.1) is an esterase associated with apolipoprotein AI (apoAI) and clusterin (apolipoprotein J) in HDL. PON1 displays paraoxonase and arylesterase activities since it hydrolyzes organophosphate compounds such as paraoxon, and aromatic carboxylic acid esters such as phenylacetate. PON1 possesses peroxidase-like activity that can contribute to its protective effect against lipoprotein oxidation [22, 27]. It

also displays homocysteine-thiolactonase activity that may be linked with its antiatherogenic properties [28, 29]. PON1 protects lipids in lipoproteins, macrophages and erythrocytes from oxidation [30–32]. Together with its antioxidative properties, PON1 has added antiatherogenic activities against macrophage foam cell formation: reduction of cholesterol and oxidized lipids influx, inhibition of macrophage cholesterol synthesis, and stimulation of macrophage cholesterol efflux [30–32]. However, the mechanism of PON1's protective action and its endogenous substrate remain elusive. Evidence is accumulating indicating that the lactonizing/lactonase activity of PON1 may be physiologically the most significant. Lactonase activity is exerted on oxidized phospholipids and on homocysteine-thiolactone [33–37] Hyperhomocysteinemia, encompassing also higher concentrations of homocysteine-thiolactone, is common in both ESRD and in patients on dialysis and may be an added risk factor for enhanced atherogenesis.

In the past decade, much progress has been made on PON1 status in patients with renal failure. Several case-control studies have addressed the changes in PON1 activity and mass as well as prevalence of polymorphisms. The effect of therapeutic modalities of intervention on PON1 activity has been explored: hemodialysis versus conservative treatment; hemodialysis and transplant; peritoneal dialysis; different types of dialysis membranes; erythropoietin; vitamin C and quercetin.

In this paper, we summarize the findings from the literature and our own laboratory on the decreased PON1 activity in renal failure, the mechanisms proposed, and the effect of interventions. A bird's eye view of the main findings is presented on Figure 1.

2. PON1 Activity is Lower in Chronic Renal Failure

In1998, two groups first studied PON1 activity in patients with CRF as compared to control subjects. In one of these studies, 119 hemodialyzed CRF patients, 107 patients with primary hyperlipoproteinemia, and 110 healthy control subjects were recruited and studied. PON1 activity was significantly lower both in hyperlipidemic and renal failure patients as compared with controls [38]. To assess whether the reduction in PON1 activity was due to changes in HDL and Apo-A1 levels, the authors standardized the enzyme activity for HDL and Apo-A1 concentrations. The standardized PON1 activity (paraoxonase/HDL ratio) was also lower in the renal failure patients as compared with hyperlipidemic patients and controls. The phenotypic distribution of paraoxonase was not different in the patient groups [38]. The same year, another group reported a study on 305 patients with CRF, 47 patients with non-ESRD, 22 patients treated with peritoneal dialysis 104 patients treated with hemodialysis, and 132 renal transplant patients [39, 40]. Patients were compared with two groups of aged-matched control subjects ($n = 195$). PON1 activity was lower in patients with renal insufficiency (chronic renal failure; chronic hemodialysis; chronic peritoneal dialysis) than in control subjects. Renal transplantation seems to restore PON1 activity. The same group then reported that PON1 in renal failure patients is better activated by salt than that of control subjects, suggesting qualitative changes in the molecule [39, 40]. These findings have since been confirmed by several groups in multiple studies [41–49].

3. PON1 Concentration is Lower in Chronic Renal Failure

The fact that PON1 activity is lower in the sera of CRF patients is well established as stated above. This led to multiple studies addressing the mechanism behind this change. The first issue to clarify is whether the PON1 protein is also low, or it is only a qualitative affect (inhibition or shift in HDL composition). Serum PON1 concentrations were measured in 81 patients undergoing hemodialysis and 103 age-matched healthy subjects using an enzyme immunoassay [50]. PON1 concentration was significantly lower in the patient group than the control group. There were no significant associations between serum PON1 concentrations and the PON1 genetic polymorphisms, 55Leu/Met (L/M), and 192Gln/Arg (Q/R). The concentration adjusted for HDL-cholesterol or apolipoprotein A-I was also lower in the patient group. Several other studies have since confirmed these findings in other series [50–52].

4. Hemodialysis versus Continuous Ambulatory Peritoneal Dialysis (CAPD), Conservative Treatment, Transplant

Several reports are consistent to show that PON1 activity and mass is lower at predialysis in CRF patients under HD or CAPD or other modalities, with no differences between these groups [42, 53]. Transplanted patients display no differences with controls [49].

5. PON1 Polymorphisms Prevalence Does Not Differ in Chronic Renal Failure Patients and Control Subjects

The PON1 genetic polymorphisms of 192 Gln/Arg and 55 Leu/Met in the amino acid sequence are partly involved in the PON1 enzyme activity. Namely, the 192 Gln/Arg QQ homocygotes have much lower PON1 activities when measured with paraoxon and not so significant with other substrates. Therefore, it is important to investigate whether the polymorphisms are associated with CRF, since a shift in the prevalence may provide an explanation for the lower activity found consistently in the aforementioned studies. Several papers have addressed this issue, some employing molecular biology techniques, some using functional phenotyping with 2 substrates, and finally some using combined approaches. In 2000, the two polymorphisms (192 Gln/Arg and 55 Leu/Met) were assessed in 96 patients undergoing hemodialysis and in 136 normal controls [52]. There was no difference in the distribution of the two polymorphisms between patients and controls, and in every subgroup classified by the polymorphisms, both paraoxonase and arylesterase activities

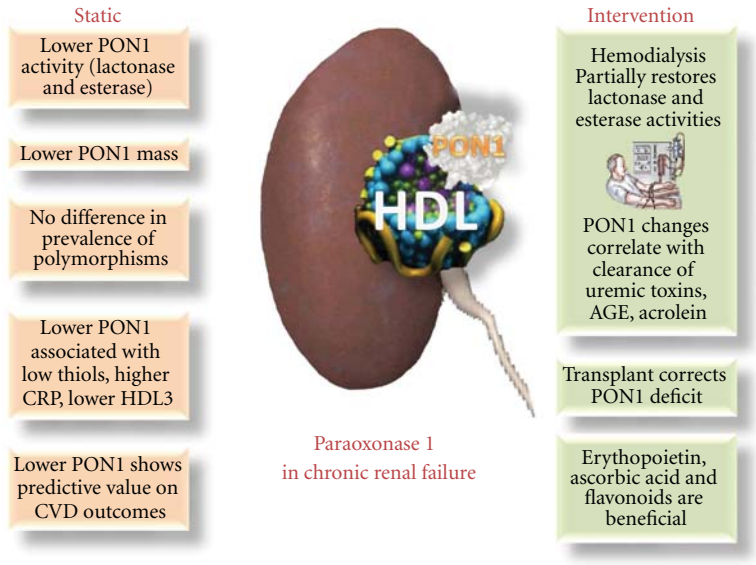

Figure 1: Paraoxonase 1 activity and function are compromised in chronic renal failure patients. This diagram summarizes the main findings discussed in the text as reported during the past decade in the literature.

were lower in patients than in controls. This suggested that the enzyme activities of PON1 decreased in hemodialysis patients, independent of the genetic polymorphism [52]. In another series, 74 HD patients and 92 control subjects were studied. PON1 activity, PON1 genotype (55 and 192 PON1 allelic polymorphisms), and the lipid profile, including HDL subfractions, were reported [54]. HD patients had decreased median PON1 activity, lower mean HDL, as well as decreased mean HDL_3 concentration. HDL retained about 70% of serum PON1 activity, almost completely carried (95%) by HDL_3 [54]. Finally, PON1 activity was significantly lower in HD versus control subjects even after matching for the allelic polymorphism, which did not differ from control subjects. In another recent study, researchers measured PON activity in 377 hemodialysis patients using the substrates 4-nitrophenylacetate and phenylacetate and its variation over time [41]. The PON ratio was calculated from 4-nitrophenylacetate-derived activity divided by phenylacetate-derived activity. Frequency distribution of the PON1 ratio showed three different PON phenotypes. 74% of hemodialysis patients showed PON1 phenotype 1, 21% PON1 phenotype 2, and 5% PON1 phenotype 3. They observed a significant reduction of PON1 ratio with increasing dialysis time [41]. This suggests that qualitative changes in PON1 occur in a time-dependent manner, by which changes in one activity are higher than in others. Finally, a very recent study included 238 control subjects and 263 hemodialyzed patients [55]. Genotype frequencies were different between two compared groups only for L55M polymorphism, with control group having higher frequency of MM genotype. Again, they confirm that Q192R, L55M, and −108C-T polymorphisms are not causal factors leading to the lower PON1 activity in hemodialyzed patients [55]. Several other studies provide confirmatory results [38, 56–59].

6. Intervention Studies

6.1. Hemodialysis and Other Modalities Have Beneficial Effects on PON1 Activity. Hemodialysis is the most frequent modality of treatment for ESRD patients. To a degree that depends on the nature of the membrane employed, HD, although life-saving, is prooxidative and has the potential of negatively affecting PON1 activity. Our laboratory undertook a series of studies to address the effect of HD on PON1 activity. We enrolled 22 ESRD patients undergoing hemodialysis in whom paired pre- and postdialysis samples were studied along with 30 age-matched control subjects [46]. ESRD patients showed a 76% decrease in PON1 activity confirming previous reports. HD results in a significant, consistent increase in the activity of the antioxidant enzyme PON1, which ranged from 4 to 40% of the predialysis value. HDL-cholesterol, apoAI, free cholesterol (as a LCAT surrogate), HDL-subclasses, and TG did not change significantly after dialysis [46]. Changes in PON1 activity display a good correlation ($r = 0.66$, $P < 0.001$) with rates in which creatinine and urea are cleared suggesting that another cause for the observed lower PON1 concentrations in CRF is the retention of low-middle molecules (see below) and demonstrate a positive effect of hemodialysis in the delicate oxidant-antioxidant state of these patients, that should be weighed against other pro-oxidant effects that have also been shown to occur previously [46].

6.2. Effect of Membranes. In a recent study, the question whether HD membrane permeability has any influence on

PON1 activity was addressed [60]. Forty-seven HD patients and 24 controls were enrolled. Blood samples were withdrawn after completion of 4-week treatment for a low-flux and a high-flux membrane. (TOS), total antioxidant status (TAS), and paraoxonase and arylesterase activities were measured in blood samples of the patients and the controls. Total oxidant status in HD patients (both types of membranes) were higher than controls while total antioxidant status and PON1 activity were lower in HD patients. There were no significant differences between "low-flux" and "high-flux" membranes in regard to oxidative stress parameters or PON1 enzyme activities. Membrane permeability seems to have no influence on oxidative stress parameters and PON1 enzyme activities [60].

6.3. HD and HDL Anti-Inflammatory Properties. By removing uremic toxins, dialysis may reduce LDL inflammatory and increase HDL anti-inflammatory properties. On the other hand, as stated above, exposure to dialyzer membrane tubing and impurities from dialysate could amplify LDL and HDL inflammatory activities. A recent study examined the effect of hemodialysis on LDL and HDL inflammatory activities [61]. ESRD patients had increased LDL chemotactic activity, reduced HDL anti-inflammatory activity, PON1 and glutathione peroxidase levels, and elevated plasma IL-6 before dialysis. Hemodialysis reduced LDL inflammatory and increased HDL anti-inflammatory activities. The beneficial effects of hemodialysis are in part mediated by heparin, which bears antioxidant lipolytic properties [61]. However, another study shows no difference in the ratio PON1/HDL in hemodialysis patients [62].

6.4. Vit C. In a study with 42 ESRD in HD and 50 control subjects, CRF patients treated with vitamin C showed an increase of PON1 activity and a decrease of AGE and lipid hydroperoxides levels [63].

6.5. Quercetin and Cathechins. In a study with using the model of renal failure induced by ethylene glycol, it was reported that treatment impaired kidney composition, increased oxidative damage, and decreased serum paraoxonase and arylesterase activities [64]. Quercetin and cathechins enhanced antioxidant defenses—superoxide dismutase and PON1 activities, reducing oxidative damage suggesting that PON1 mediates the protective effects of flavonoids against kidney damage by oxidative stress [64].

6.6. EPO. The effects of treatment of anemia with exogenous recombinant erythropoietin (EPO) beta and iron on levels of antibodies against oxidized low-density lipoproteins as well as on serum PON1 activity and concentration were studied in 49 predialysis patients with chronic renal disease [45]. After 6 months of treatment, compared with pretreatment values, the median levels of serum PON1 activity was slightly but significantly increased and the concentration of PON1 was significantly decreased. EPObeta and iron treatment of anemia of CRF promotes changes in serum PON1 activity and concentration that suggest a beneficial effect on oxidative stress.

7. Lactonase Activity of PON1 Is Lower in ESRD Patients and Is Restored by HD

As stated before, PON1catalyzes the hydrolysis of numerous substrates: lactones, thiolactones, esters, and phosphotriesters, including paraoxon, from which its misleading name is derived. Nevertheless, a consensus seems to be emerging that PON1 main physiological activity is acting as a lactonase [28, 65–72]. Findings reporting changes in the promiscuous esterase activities may not reflect changes in the physiological function. Indeed, the substrate docking moieties around the active site of PON1 differ for each major category of substrates, namely, the lactonase activity depends on a different region than the esterase activity [69, 73]. All of the studies reported above, including our own, had not employed lactones as substrates. In a study with 42 ESRD in HD and 49 control subjects, we found that our patients showed a significant 11% decrease in PON1 lactonase activity [74]. When we compared pre- and postdialysis samples, lactonase changed favorably as a result of dialysis, as did the other activities. This is worthy of note, given the likelihood that lactonase activity may be the most important physiological atheroprotective function of PON1. ESRD patients may be more susceptible to the harmful effects of lipid peroxidation than healthy subjects, lower serum lactonase activity would delay catabolism of oxidized phospholipids in LDL and oxidized macrophages, allowing more time for radical chain reactions to inactivate apolipoproteins and cell membrane proteins [74].

8. Mechanisms Proposed

8.1. Role of Uremic Toxins

8.1.1. AGEs. We have shown that HD results in a significant, consistent increase in the activity of the antioxidant enzyme PON1. The effect correlates with the effectiveness of dialysis to clear creatinine and urea. This strongly suggests that elimination of some inhibiting low molecular factor may be responsible in part for the recovery of PON1 activity. Uremic toxins, therefore, may be putative mediators of PON1-deficient activity. Kidney failure patients have very high levels of advanced glycation endproducts; even more than diabetic patients [75–78]. AGE residues are formed by the action of carbonyls (glucose, methylglyoxal, and other dicarbonyls) on long- and short-lived cellular and extracellular proteins. Cellular proteolysis forms AGE-peptide and AGE-free adducts from these proteins, which are released into plasma for urinary excretion [75–78]. When we studied AGEs in our HD patients, the clearance of low-molecular-weight AGEs after hemodialysis explained 79% of the changes in PON1 activity and are hence a much better predictor than creatinine changes [46]. *In vitro* incubation of paraoxonase with serum ultrafiltrates showed a time- and concentration-dependent inhibition of PON1 by the ultrafiltrates, an inhibition that is up to 3 times higher (from 8 to 24%) when CRF patients are the source of the ultrafiltrate [46]. We showed that HD results in a significant, consistent increase in the activity of the antioxidant enzyme PON1. The effect correlates with

the effectiveness of dialysis to clear creatinine and urea, and with the clearance of AGE adducts of low molecular weight. This effect was replicated *in vitro*, showing time and dose dependency. Our results suggest that another cause for the observed lower PON1 concentrations in CRF is the retention of low-middle molecules and demonstrate a positive effect of hemodialysis in the delicate oxidant-antioxidant state of these patients that should be weighed against other pro-oxidant effects that have also been shown to occur previously [46]. If the hypothesis that AGEs are the main culprits is proved in further research, this opens a putative therapeutic avenue for AGE blockers in ESRD.

8.1.2. Acrolein. Renal failure patients share lower PON1 levels and high serum acrolein levels [79–81]. Acrolein is a highly reactive air pollutant of human health concern, chiefly as it is major component of cigarette smoke and also has several endogenous sources [79–81]. Acrolein oxidizes cysteine and forms adducts with lysine and histidine through the Maillard reaction, with deleterious consequences on protein function. Since PON1 has a cysteine residue critical for its antioxidant activity, which is moreover modulated by apoA-I, we hypothesized that acrolein could also have deleterious effects on PON1 activity [82]. We have shown that acrolein inhibits PON1 activity in HDL at micromolar concentrations and this inhibition is cancelled by n-acetylcysteine [82]. PON1 has 1 critical cysteine residue in its catalytic hydrophobic pocket. The results suggest that in conditions where local acrolein concentrations are elevated (atheroma plaque, sites of lipoperoxidation), acrolein-mediated loss of PON1 activity could be a plausible phenomenon. In a study with 40 ESRD in HD and 40 control subjects we found that our patients had a 3-fold increase in free acrolein when compared to control subjects. When we compared pre- and posthemodialysis samples, PON1 activity changed favorably as a result of dialysis, confirming our previous data [46, 82–84]. On average, free acrolein was 32% lower in postdialysis samples [82]. When we correlated the increments in PON1 activity resulting from the hemodialysis intervention, with the corresponding decrements in free acrolein we found a significant correlation. Our results suggest that high acrolein levels may in part be responsible for the low PON1 levels found in ESRD [82].

8.2. HDL Composition and PON1 Association/Dissociation

8.2.1. HDL Composition. From the discussion above, it is clear that a certain consensus is emerging, favoring a multiprong explanation for PON1-deficient activity in CRF. Circulating inhibitors (uremic toxins and others) are likely candidates, together with decreased PON1 protein paired with qualitative changes in the molecule. Differences in allele distribution do not seem to play a role. PON1 activity largely depends on its association with apoAI and phospholipids in HDL, although a minor, free form can be found. In turn, HDL encompasses a wide range of particles with different sizes and diverse physiological functions. HDL subclasses are not homogeneous in PON1 content and activity. Small HDL_3 contains most of this activity and carries most of the antioxidant capacity [13]. As CRF is associated with changes in HDL subclasses distribution and function, this may be another factor that compounds the observed decreased PON1 activity in these patients. However, reduced HDL levels *in vivo* may result in reduced HDL antioxidant capacity in these patients. In a study with 74 patients in HD, it was shown that mean HDL_3 concentration is decreased [54]. Most of PON1 activity in HDL was carried (95%) by HDL_3. The authors suggest that a key determinant of PON1 activity reduction in HD is the depressed HDL_3 and not the genetic PON1 polymorphism [54].

8.2.2. Free PON1 Is Not Higher in CRF Patients. Aviram's group has shown that free PON1 increases in diabetes patients and that glycation of HDL promotes dissociation of the enzyme from the particle [85–87]. They suggest that this mechanism may contribute to the lower activity found in these patients and to a dysfunctional more atherogenic HDL particle. We tackled the question whether increased free PON1 in CRF patients could be a reason for the lower activity [83]. Other than the classical routine findings in these patients, we confirmed that both PON1 triesterase and lactonase activities are reduced in ESRD patients on hemodialysis. The free triesterase activity is larger than the free lactonase activity in both populations [83]. Free PON1, however, was not significantly different between the groups and that was true for the activity against paraoxon and the more physiological lactonase activity [83].

9. Association with Other Biomarkers

9.1. Thiols. PON1's only free sulfhydryl group is present at Cys284 and is associated with its activity even when it is not part of its active site; rather, it is part of a highly conserved stretch that includes active site histidine-285 [69, 73, 88–90]. Modification of the enzyme's free sulfhydryl group, such as via S-glutathionylation, leads to inactivation [91, 92]. In a study with 71 hemodialyzed patients and 87 healthy individuals, hemodialyzed patients had lower PON1 paraoxonase and arylesterase activity, concentrations of HDL, HDL_3, and HDL_2 and concentration of free thiol groups [55]. Decreased concentration of free thiol groups in sera suggest that the free thiol group (Cys284) in PON1 might also be oxidized, which can affect PON1 activity. In another study with 48 CRF patients on chronic maintenance hemodialysis, 41 patients on conservative management and 41 age-matched controls, serum PON1 activity correlated positively with protein thiols, and negatively with lipid hydroperoxides [93].

9.2. Ischemia-Modified Albumin (IMA). The amino-terminal end of albumin binds transitional metals such as cobalt. When a specific motif (DAKK motif) in the N-terminus is damaged, this results in a reduced binding capacity for transitional metals [94–98]. This ischemia-modified albumin is formed in ischemic capillary beds as found in CVD, especially in the acute coronary syndromes, and has also been recently documented in patients with diabetes mellitus, hyperlipidemia, and metabolic syndrome [94–98]. Recently,

prospective studies on CVD outcomes in ESRD using the new biomarkers of HDL dysfunction and/or oxidative stress, PON1 and IMA, have appeared in the literature [99–104]. In a pilot study, we explored this relationship in a small cohort of HD patients with ESRD [84]. We showed that PON1 levels were significantly and inversely correlated with IMA levels in these patients while such a clear correlation was not found in non-ESRD controls. An inverse correlation between PON1 and IMA levels indicate that low PON1 activity in these patients produces increased oxidative stress, leading to IMA formation. Monitoring serum PON1 and IMA simultaneously might thus provide another useful tool for the clinical pathologists as a prognostic biomarker of CVD in ESRD patients [84].

9.3. Cystatin C. Serum cystatin C is an alternative, more specific marker of glomerular filtration rate [57]. In a study with 74 hemodialized, 171 renal transplanted patients, and 110 healthy controls, a negative correlation was found between PON1 activity and cystatin C level [43]. Homocysteine level correlated negatively with PON1 activity, and positively with cystatin C level. OxLDL and lipoperoxide levels were significantly higher in the renal patients. Cystatin C may be a good predictive factor for homocysteine levels but for the antioxidant status in patients with renal failure [42, 43].

9.4. Cortical Thickness. In a study with 37 CRF patients and 18 controls, there was a positive correlation between renal cortical thickness and HDL levels and PON1 activity [105].

9.5. CRP. PON1 arylesterase activity and mass, C-reactive protein (CRP), cystatin C, were measured in 30 CRF patients and 30 control subjects [42]. PON1 activity and mass were inversely correlated with CRP in HD patients. The grouping of increased CRP and reduced PON1 may detect subjects at higher risk for cardiovascular complications [42, 106].

10. Predictive Value

Results from the multiple case-control studies reported in this paper provide substantial evidence that renal failure is associated with deficient PON1 activity and mass that are independent of changes in HDL-C. Evidence from prospective studies is more scarce but encouraging. One of these studies measured PON1 activity, concentration, and genetic polymorphisms with an interval of 6 years in 81 HD patients [51]. The relation between baseline cardiovascular risk factors and clinical events was investigated. During followup for 6 years, 42 deaths were recorded, including 24 fatal cardiovascular events. In univariate analyses, baseline PON1 concentration was associated with not only cardiovascular mortality, but also all-cause mortality during the period of follow-up, as were age, preexisting cardiovascular disease, and hemoglobin concentration [51]. In multivariate Cox regression analysis, PON1 concentration retained significant associations with cardiovascular mortality and all-cause mortality even after adjustment for other risk factors for CVD or mortality in HD patients. Significantly increased cardiovascular mortality and all-cause mortality were shown in the patients with low PON1 concentrations in Kaplan-Meier survival curves, suggesting that low PON1 concentration may be an independent predictor of cardiovascular mortality in maintenance HD patients [51]. In another study with 30 CRF patients undergoing HD, 30 patients with CRF under conservative treatment, and 30 healthy controls, basal PON1, salt-stimulated PON1 and arylesterase activities were lower in patients than controls [44]. Carotid intima-media thickness (IMT) was significantly higher in HD than in other CRF patients and both were significantly higher than controls. Linear regression showed that the most significant determinant of carotid IMT was PON1 arylesterase activity in HD. Modifying this risk factor could be salutatory in this patient population [44].

11. Conclusion

In addition to profound alterations in triglyceride, IDL- and HDL-cholesterol concentrations, classical hallmarks of renal failure, reduced serum paraoxonase activity has been clearly established in the past decade and could contribute to accelerated development of atherosclerosis in ESRD and in HD. PON1 activity and mass has been found consistently depressed in all studies papered. This is not due to associations of renal failure with specific phenotypes characterized by lower activity against paraoxon. PON1 lactonase and other activities all are the lower in ESRD patients. ESRD patients may be more susceptible to the harmful effects of lipid peroxidation than healthy subjects. Hemodialysis partially restores PON1 lactonase and the other activities. An association between PON1 activity recovery after dialysis has been found with creatinine changes, advanced glycation end products, and acrolein suggesting that uremic toxins may play a mechanistic role in PON1 inactivation. Lower PON1 activity in CRF patients is associated with low thiol concentration, high CRP, and is beneficially enhanced with vitamin C and flavonoids. Changes in HDL subclasses, namely, lower HDL_3 in these patients may also play a role in PON1 lower activity. Some studies indicate that PON1 has an independent predictive value on CVD risk. These studies lay the ground for future studies around three axes: (1) mechanistic studies addressing the intimate explanation for low PON1 activity and mass; (2) prospective studies focusing on whether there is an added predictive value in measuring PON1 activity (and PON1 activity in HDL_3) in this patient population; (3) intervention studies attempting to increase PON1 activity, studying outcomes providing information on effective delay in the progression of atherosclerosis.

Abbreviations

AGE: Advanced glycation enedproducts
ApoAI: Apolipoprotein AI
CRF: Chronic renal failure
CAPD: Continuous ambulatory peritoneal dialysis
CRP: C-reactive protein
ESRD: End-stage renal disease
HD: Hemodialysis
HDL: High density lipoprotein

IDL: Intermediate density lipoprotein
IMT: Intima-media thickness.

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