Determination of Paraoxonase 1 Status Without the Use of Toxic Organophosphate Substrates

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Paraoxonase 1 (*PON1*) is a member of a tandem 3-gene family localized on human chromosome 7q21-22.[1] High-density lipoprotein-associated *PON1*[2,3] and PON3[4,5] are synthesized primarily in the liver, whereas PON2 is ubiquitously expressed.[1] *PON1* was initially characterized and named for its ability to hydrolyze paraoxon, the toxic oxon metabolite of parathion.[6] Although Aldridge[6] proposed in 1953 that serum paraoxonase (POase) and arylesterase (AREase) activities were carried out by the same enzyme, controversy about 1 versus 2 enzymes persisted for many years, resulting in a reclassification of POase/AREase from EC 3.1.1.2 to EC 3.1.8.1 for *PON1* as an example of an organophosphorus (OP) hydrolase.[7] The controversy was finally settled when Sorenson et al[8] demonstrated both activities in recombinant *PON1*. However, the revised nomenclature remains in place. Early studies of plasma *PON1* found a large variability of POase activity among different species and in different tissues.[6] Serum POase activity distribution studies in human populations revealed an activity polymorphism of high versus low POase activity. Studies on the polymorphic distribution of *PON1* in human populations using a variety of different assays revealed bi or trimodal distributions of plasma POase activity (reviewed in Ref.[9]).

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Our initial characterization of the human *PON1* cDNA clones revealed 2 coding region polymorphisms Q192R and L55 M.[10] Subsequently, it was shown that the Q192R polymorphism determined high versus low rates of paraoxon hydrolysis by the enzyme, with the $PON1_{R192}$ alloform specifying high activity.[11,12] After the demonstration that high-density lipoprotein-associated *PON1* was implicated in reducing low-density lipoprotein[13] and high-density lipoprotein[14] oxidation, epidemiological studies were undertaken to explore the possible role of genetic variability of *PON1* in cardiovascular disease[15] (reviewed in Ref.[16]). Several meta-analyses of studies that examined only the association of *PON1* genotypes with risk of vascular disease have been published in recent years. The first meta-analysis in 2001 by Mackness et al[17] examined the 19 studies carried out up to that time as part of a study of *PON1* status in 417 coronary heart disease subjects and 282 controls. A second meta-analysis examined 38 studies in addition to their own,[18] whereas a third analyzed 43 previous studies.[19] Unfortunately, the majority of the epidemiological studies examined only *PON1* genotypes using DNA methodologies and ignored the large interindividual variability in plasma *PON1* activity levels. Fundamental biochemical and physiological principles dictate that rates of detoxication or metabolism depend on the quantity of enzyme present. Thus, it is not surprising that many analyses examining disease or exposure risk using only single-nucleotide polymorphism (SNP) analysis and not enzyme activity levels have been inconclusive. Several of the most experienced investigators in *PON1* research have pointed out the inadequacy of examining *PON1* genotype alone as a risk factor for disease or exposure.[15,20–27] We introduced the term *PON1* status to include both plasma *PON1* activity levels and $PON1_{192}$ genotype.[23] The few studies that have examined *PON1* status have found that plasma *PON1* activity level is indeed a risk factor for vascular disease,[17,25,26,28–31] whereas there was no association observed with *PON1* genotypes.[17,25,26]

The importance of plasma *PON1* activity level in protecting against OP exposure has been clearly demonstrated in the mouse and genetically modified mouse model systems.[23,32–36] Resistance to diazoxon exposure is modulated primarily by *PON1* plasma activity level, whereas both *PON1* activity level and *PON1* genotype are important in modulating exposures to chlorpyrifos oxon, due to substrate-specific differences in catalytic efficiency between the $PON1_{Q192}$ and the $PON1_{R192}$ alloforms.[34]

The most convenient protocol for determining *PON1* status—plasma activity levels as well as functional position 192 genotype—makes use of a 2-substrate assay, 2-dimensional enzyme activity plot that displays rates of diazoxonase activity versus POase activity under high salt conditions.[24,27,37,38] The high salt conditions are used to separate the $PON1_{192Q/R}$ data points from the $PON1_{192R/R}$ data points. Unfortunately, this protocol involves the use of 2 highly toxic OP substrates. We report here a 2-substrate assay/analysis protocol that makes use of non-OP substrates and is convenient for general laboratory use. A third assay that measures rates of phenyl acetate (PA) hydrolysis (AREase activity) at low salt concentration reveals plasma *PON1* activity levels for all 3 $PON1_{192}$ genotypes.

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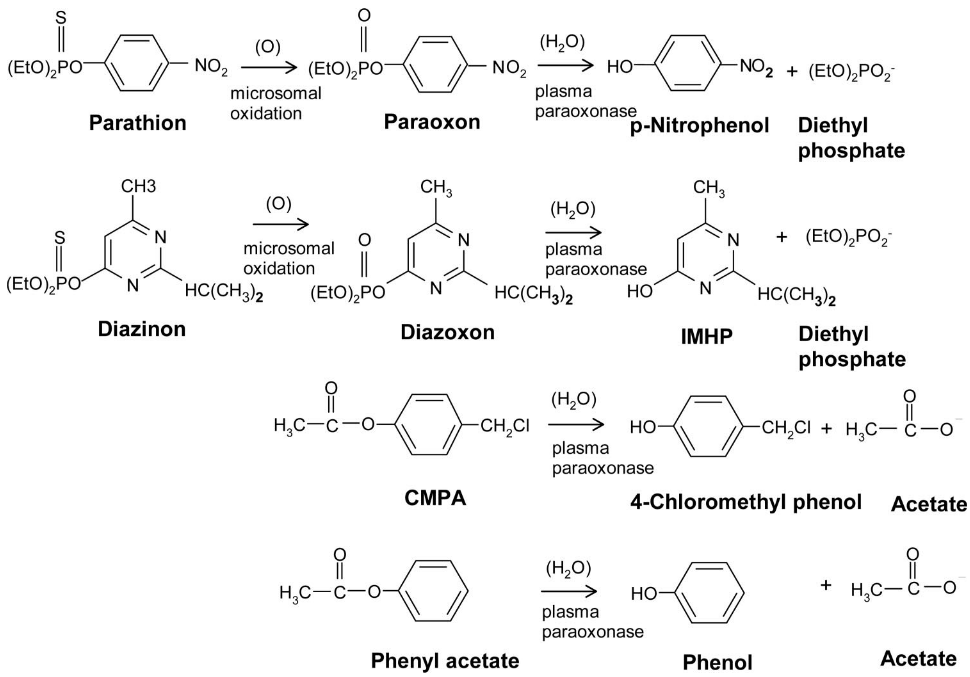

Figure 1. Structures of the substrates used for determining *PON1* status.

Factors are provided to allow the conversion of rates of hydrolysis of one substrate to another for each $PON1_{192}$ phenotype.

Methods

Subjects

The plasma samples used for this study came from an institutional review board-approved project investigating the role of *PON1* in vascular disease. Plasma samples were drawn into lithium-heparin tubes, and the cells were separated from the plasma by centrifugation for 15 minutes at 1800*g*.

4-(Chloromethyl)phenyl Acetate Assay

CMPA [4-(chloromethyl)phenyl acetate] was obtained from Sigma Chemical Co (St Louis, Mo). Rates of CMPA hydrolysis were determined in a SPECTRAmax PLUS Microplate Spectrophotometer (Molecular Devices, Sunnyvale, Calif) using ultraviolet transparent 96-well microplates from Costar (Cambridge, Mass.). Rates of hydrolysis were measured at 280 nm for 4 minutes at 25°C. Only initial linear rates were used for calculations, and results were normalized using the path-length correction software supplied by the system manufacturer. Replicate assays that varied by >10% were repeated. Plasma samples were diluted 1:40 in dilution buffer [20 mmol/L Tris-HCl (pH 8.0), 1.0 mmol/L $CaCl_2$] and 20 μL was added per microplate well. The data points were run in triplicate. The substrate solution for CMPA determinations was 20 mmol/L Tris-HCl (pH 8.0), 1.0 mmol/L $CaCl_2$ to which CMPA was added to a final concentration of 3 mmol/L. The substrate solution was shaken vigorously for 30 seconds in a screw-capped polypropylene tube before use. Substrate solution (200 μL) was added to initiate the assay. Activities were expressed in Units/mL, based on the molar extinction coefficient of 1.30 mmol/$L^{-1}cm^{-1}$ for the CMPA hydrolysis product, 4-(chloromethyl)phenol.

Arylesterase Assays (High Salt/No Salt)

Rates of PA hydrolysis were determined in the SPECTRAmax PLUS Microplate Spectrophotometer using ultraviolet transparent 96-well microplates from Costar. Rates of hydrolysis of PA were measured for 4 minutes at 270 nm, with only initial linear rates used for calculations, with results normalized using the path-length correction software provided by the manufacturer. Replicates that varied by >10% were repeated. AREase assays used plasma dilutions (in dilution buffer) of 1:40 for assays run at high salt concentration and 1:80 for assays run at low salt concentration. The assay used 20 μL of diluted plasma per well to which 200 μL of 3.26 mmol/L PA substrate was added in either high salt assay buffer, or no salt assay buffer. High salt PA buffer contained 2 mol/L NaCl, 20 mmol/L Tris-HCl (pH 8.0), 1.0 mmol/L $CaCl_2$, and low salt assay buffer contained 20 mmol/L Tris-HCl (pH 8.0), 1.0 mmol/L $CaCl_2$. Activities were expressed in Units/mL, based on the molar extinction coefficient of 1.31 mmol/$L^{-1}cm^{-1}$ for phenol.

Paraoxonase and Diazoxonase Assays

Plasma *PON1* activities toward paraoxon (PO) and diazoxon (DZO) and were determined as described previously.[24,38] Paraoxon and diazoxon were obtained from Chem Service (West Chester, Pa.). Rates of paraoxon and diazoxon hydrolysis were determined in the SPECTRAmax PLUS Microplate Spectrophotometer using either ultraviolet transparent 96-well microplates from Costar for UV diazoxonase readings (270 nm) or standard flat bottom 96-well microplates from Greiner One (Monroe, N.C.) for visible wavelength POase readings (405 nm). All assays were carried out in triplicate using a multi-channel pipette (Matrix, Hudson, N.H.). Outlier samples were reassayed. Rates of hydrolysis were measured for 4 minutes, with only initial linear rates used for calculations and results normalized using path-length correction. PO hydrolysis rates (POase) were expressed in Units/liter (U/L), based on the molar extinction coefficient of 18 mmol/$L^{-1}cm^{-1}$ for p-nitrophenol. DZO hydrolysis (DZOase) activities were expressed in Units/liter (U/L), based on the molar extinction coefficient of 3 mmol/$L^{-1}cm^{-1}$ for the diazoxon hydrolysis product, 2-isopropyl-4-methyl-6-hydroxypyrimidine.

Results

Identification and Characterization of Nontoxic Discriminatory Substrates

The aim of this study was to develop assays for the determination of *PON1* status (plasma *PON1* activity levels and functional position 192 genotype) that do not use the highly toxic organophosphate substrates paraoxon and diazoxon. More than 70 compounds with many assay conditions (vari-

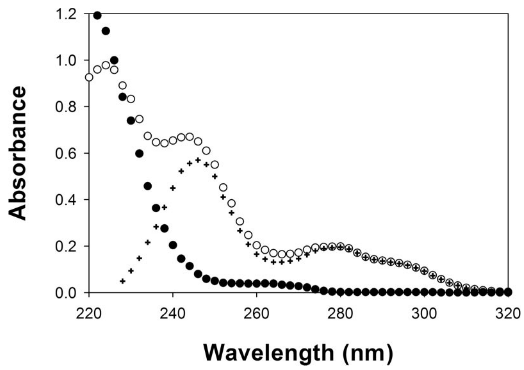

Figure 2. Spectra of CMPA (●–●) and 4-(chloromethyl)phenol (○–○) and the difference spectrum (+–+).

ation of salt concentration and pH) were examined in attempts to find 2 substrates and assay conditions that would provide the same resolution of the $PON1_{192}$ phenotypes as the toxic DZO/PO substrate pair.[24] Of the many substrates and conditions tried, hydrolysis of PA at 2 mol/L salt and CMPA at low salt provided the best resolution of functional $PON1_{192}$ phenotypes. The primary requirement for useful substrates is that the substrate and assay conditions reveal different rates of hydrolysis between the $PON1_{Q192}$ and the $PON1_{R192}$ alloforms.[24]

Figure 1 shows the structures of the 4 substrates used to determine *PON1* status. The first step in the design of a spectrophotometric assay for substrate hydrolysis is to examine the spectra of the unhydrolyzed ester and the released alcohol. Aromatic alcohols in general provide useful spectral shifts on hydrolysis. Figure 2 shows the spectra of CMPA and 4-(chloromethyl)phenol. The wavelength of 280 nm was chosen for continuous monitoring of the hydrolysis of CMPA.

In designing assays that best discriminate between the 2 $PON1_{192}$ alloforms, it was necessary to find conditions where the kinetic properties of the 2 alloforms differed sufficiently to separate the 3 $PON1_{192}$ phenotypes or "functional genotypes" (QQ, QR, and RR). Previous experience with *PON1* assays has shown that either pH and/or salt conditions are most conveniently used to generate optimal assay conditions that will resolve the 3 $PON1_{192}$ phenotypes. Our earlier work also showed that it is necessary to measure *PON1* activity at or below pH 8.5 to avoid interference from the esterase activity of albumin, which catalyzes a higher rate of OP hydrolysis than *PON1* at high pH values in plasma samples with low *PON1* activity levels.[9] A pH value of 8.0 proved to be optimal for measuring rates of hydrolysis of both PA and CMPA (data not shown).

The effects of varying NaCl concentration on rates of PA hydrolysis are shown in Figure 3A. As with diazoxon hydrolysis,[24] $PON1_{R192}$ was more sensitive to inhibition by NaCl than was $PON1_{Q192}$. Because most of the currently used assays are run at 2 mol/L NaCl, and this level of salt provided a good differentiation of the activity of the 2 $PON1_{192}$ alloforms, 2 mol/L NaCl was selected for optimizing the spread of the data points for the "*y* axis substrate" PA. Because we have previously shown that rates of PA hydrolysis in absence of NaCl may be used to compare levels of plasma *PON1* across $PON1_{192}$ genotypes, rates of PA hydrolysis were also determined in the absence of salt.

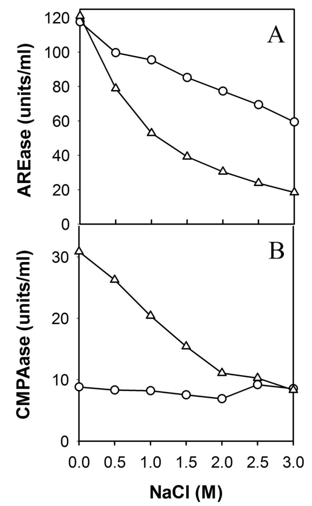

Figure 3. Rates of hydrolysis of (A) PA by $PON1_{Q192}$ (○–○) and $PON1_{R192}$ (Δ–Δ); and (B) CMPA by $PON1_{Q192}$ (○–○) and $PON1_{R192}$ (Δ–Δ) as a function of sodium chloride concentration.

Figure 3B shows the dependence of rates of hydrolysis of CMPA by the 2 $PON1_{192}$ alloforms as a function of salt. Interestingly, $PON1_{R192}$ had higher rates of CMPA hydrolysis than $PON1_{Q192}$ in the absence of salt, indicating that it would be a useful "*x* axis substrate." Figure 4A shows the substrate dependence of PA hydrolysis by $PON1_{Q192}$ (K_m=443 μmol/L), and Figure 4B by $PON1_{R192}$ (K_m=279 μmol/L) at pH 8.0 in the presence of 2 mol/L NaCl. Figure 4C shows the substrate dependence of rates of hydrolysis of CMPA for $PON1_{Q192}$ (K_m=341 μmol/L), and Figure 4D by $PON1_{R192}$ (K_m=454 μmol/L) at pH 8.0 in the absence of NaCl. K_m values were determined from plots of substrate concentration/velocity versus substrate concentration.[39]

Comparison of the 2 Protocols for Determining *PON1* Status

Figure 5 compares the population distribution of rates of hydrolysis of the substrate pair DZO/PO (Figure 5A) with those of the substrate pair PA/CMPA (Figure 5B) for 183 individuals (*PON1* status). Both distributions clearly resolve the 3 functional $PON1_{192}$ phenotypes (QQ, QR, and RR). These plots reveal not only the $PON1_{192}$ alloform(s) present in an individual's plasma but also the relative levels of each individual's plasma *PON1* activity within each $PON1_{192}$ phenotype, data that is for most considerations much more relevant for estimating risk than the *PON1* SNP data. All $PON1_{192}$ genotypes were correctly inferred by both 2-substrate analyses as verified by polymerase chain reaction assays.[12] We have reported previously, mutations discovered by discrepancies observed between the *PON1* status determinations and polymerase chain reaction analyses.[27]

Generation of Assay Conversion Factors

To facilitate comparison of data obtained with this new protocol for establishing *PON1* status with data generated with the DZO/PO substrate pair, plots of rates of hydrolysis of a given substrate versus rates of a second substrate for each $PON1_{192}$ phenotype were prepared (data not shown) to obtain

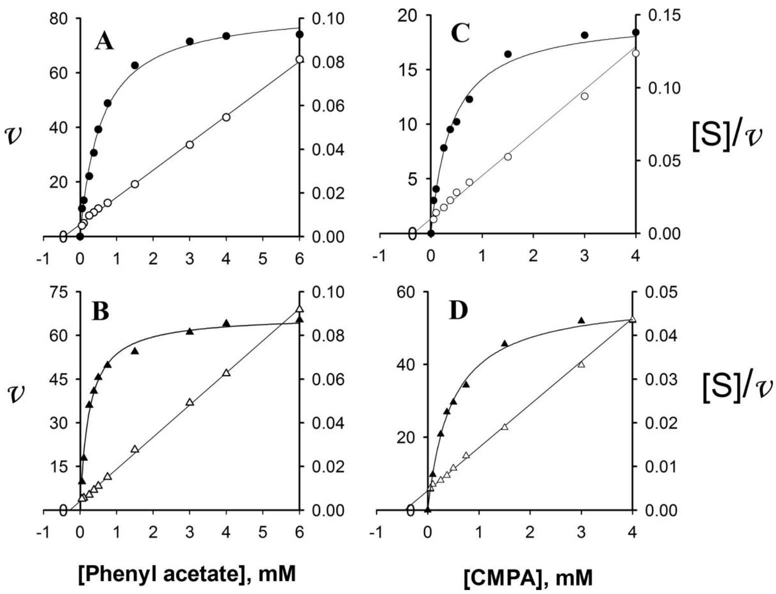

Figure 4. Substrate dependent rates of hydrolysis of (A) PA by $PON1_{Q192}$, (B) PA by $PON1_{R192}$, (C) CMPA by $PON1_{Q192}$, and (D) CMPA by $PON1_{R192}$. The assays for PA hydrolysis were run at high salt and those for CMPA at low salt as described in Methods section. Closed symbols indicate plots of velocity (*v*) versus substrate concentration [S]; open symbols, [S]/v versus [S].

the conversion factors shown in the Table. It was necessary to determine the conversion factors for each $PON1_{192}$ phenotype separately because the catalytic efficiency of substrate hydrolysis differs for each phenotype. Because the rates of PA hydrolysis at low salt are not affected by $PON1_{192}$ phenotype (Figure 6), they can be used to compare *PON1* plasma activity levels across genotypes.[40] Factors for converting rates of PA hydrolysis at high salt to low salt values were also determined so that it would not be necessary to run 3 different substrate assays for a study of *PON1* status and plasma *PON1* level determination (Table). If a laboratory environment has high ambient temperatures, it may be necessary to generate temperature correction factors.

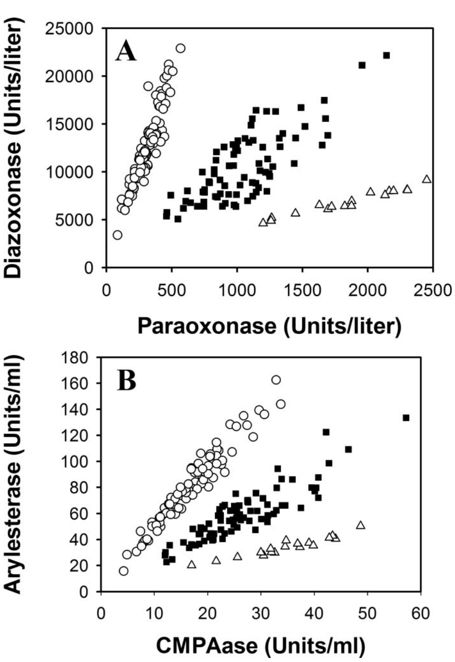

Figure 5. Comparison of the 2 protocols for determining *PON1* status. A, Assays using the highly toxic OP substrates DZO and PO. B, Assays using the non-OP substrates PA and CMPA. (○) indicates $PON1_{Q/Q192}$; (■), $PON1_{Q/R192}$; (Δ), $PON1_{R/R192}$.

Discussion

The studies on the relationship of genetic variability of *PON1* to risk of disease or exposure now number into the hundreds. Unfortunately, most of these studies have looked for association of *PON1* SNPs with susceptibility and have ignored the more important factor, plasma *PON1* activity levels.[15–19,25,26,28–31] In our initial characterization of human *PON1* cDNA sequences, we identified 2 coding region SNPs (L55 M and Q192R).[10] It was subsequently shown that it was the Q192R polymorphism that determined the catalytic efficiency of *PON1*,[11,12] with $PON1_{R192}$ having approximately 9-times the catalytic efficiency for hydrolysis of paraoxon compared with $PON1_{Q192}$.[34] The effects of the Q192R polymorphism are substrate dependent, with $PON1_{Q192}$ having higher activity against some of the nerve agents and $PON1_{R192}$ having higher activity against PO and chlorpyrifos oxon.[37] Both alloforms hydrolyze DZO[34] and PA[40] with approximately the same efficiency. Further research from our laboratory and 2 others examined the effects of 5 *PON1* promoter region polymorphisms on plasma *PON1* levels.[41–44] The promoter region polymorphism that had the largest effect on *PON1* activity levels was the C-108T polymorphism that occurs

Table. Factors for Converting Rates of Hydrolysis of Substrates

Phenotype	Conversion Factors	r^2
QQ	$AREase_{HS}$ (U/mL)×185=DZOase (U/L)	0.81
QR	$AREase_{HS}$ (U/mL)×205=DZOase (U/L)	0.90
RR	$AREase_{HS}$ (U/mL)×236=DZOase (U/L)	0.88
QQ	CMPAase (U/mL)×18.9=POase (U/L)	0.92
QR	CMPAase (U/mL)×36.3=POase (U/L)	0.90
RR	CMPAase (U/mL)×54.3=POase (U/L)	0.95
QQ	$AREase_{HS}$ (U/mL)×1.6 = $AREase_{LS}$ (U/mL)	0.95
QR	$AREase_{HS}$ (U/mL)×2.0=$AREase_{LS}$ (U/mL)	0.66
RR	$AREase_{HS}$ (U/mL)×3.5=$AREase_{LS}$ (U/mL)	0.83

r^2 indicates correlation coefficient squared; $AREase_{HS}$, arylesterase at high salt; $AREase_{LS}$, arylesterase at low salt.

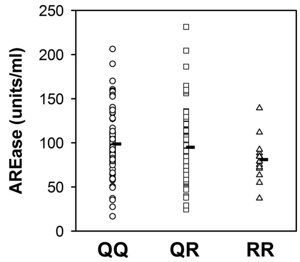

Figure 6. Rates of PA hydrolysis for each $PON1_{192}$ phenotype/genotype at low salt concentration: $PON1_{Q/Q192}$ (○–○), $PON1_{Q/R192}$ (□–□), and $PON1_{R/R192}$ (Δ–Δ). Horizontal bars represent the mean AREase activity values for each phenotype.

in an Sp1 transcription factor binding site.[45] Homozygotes for $PON1_{C\text{-}108}$ had on average twice the plasma level of *PON1* activity compared with $PON1_{T\text{-}108}$ homozygotes.[42]

There have been a number of reports linking low *PON1* activity levels to the L55 M polymorphism with the $PON1_{M55}$ allele being associated with low activity levels. However, most of this effect seems to be related to linkage disequilibrium of $PON1_{M55}$ with the inefficient $PON1_{T\text{-}108}$ allele.[42] Leviev et al have reported that message levels[46] and stability[47] of the $PON1_{M55}$ alloform may also contribute to the lower levels of *PON1* activity associated with the $PON1_{M55}$ genotype. AREase levels in a study of 1527 postmenopausal women reported by Roest et al[48] were lower among $PON1_{M55}$ homozygotes when compared with heterozygotes and $PON1_{L55}$ homozygotes across all 3 $PON1_{C\text{-}108T}$ genotypes, lending support to an independent effect of the $PON1_{M55}$ allele.

Of the 70 substrates tested, only PA (at high salt) and CMPA (in absence of salt) provided resolution of the 3 *PON1* phenotypes comparable with that provided by the paraoxon/diazoxon substrate pair. Another advantage of this substrate pair is that rates of hydrolysis can be determined at saturating substrate concentration. This was not feasible for diazoxon, where a nonsaturating concentration of 1 mmol/L was chosen for convenience and substrate solubility.[24,38]

Conclusions

The protocols described here will allow most laboratories to determine individuals' *PON1* status without the use of toxic substrates. The conversion factors presented here will also allow for the comparison of newly generated data with data reported from earlier studies. Again, it is important to note that epidemiological studies that examine only *PON1* SNPs will be missing data on *PON1* activity levels, which are more important than genotype in estimating an individual's risk of disease or exposure. For some exposures, genotype can also be important,[34] but in no case are *PON1* activity levels unimportant for estimating risk. Analysis of all ≈200 *PON1* DNA polymorphisms will not provide the critical information generated by the 2-substrate *PON1* status analysis protocol (functional $PON1_{192}$ genotype and plasma activity levels).[48]

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Disclosures

None.

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