Accuracy and Biological Variation of Human Serum Paraoxonase 1 Activity and Polymorphism (Q192R) by Kinetic Enzyme Assay

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Background: Paraoxonase 1 (PON1) phenotype is a better predictor of atherosclerosis risk than are PON1 genetic polymorphisms alone. Larger studies are required to determine the role of PON1 and there is a need for standardized PON1 assays between laboratories.

Methods: We have adapted 5 enzyme kinetic assays for high-throughput automated analysis of PON1 activity. Using different substrates and reaction conditions, we measured PON1 activity and used activity ratios to identify the PON1 Q192R genetic polymorphisms and assessed the accuracy of the genotype assignments in 79 adult study participants by comparing them with genotypes determined by AlwI restriction enzyme digestion of a 176-bp PCR amplification product from genomic DNA. Imprecision was determined using pooled serum and purified enzyme preparations. Biological variability was estimated by analysis of serial samples from 17 individuals. Variability parameters were compared with total cholesterol as a point of reference to a recognized biomarker of coronary heart disease risk.

Results: Salt stimulation and inhibition ratios were 97.4% and 94.7% correct in assigning Q192R genotype, respectively. Analytical imprecision (CV) was 1.0%–3.0% for phenylacetate and paraoxon substrate assays and 3.0%–8.0% for the para-nitrophenylacetate substrate assays. Combination of the 2 ratios into a double ratio resulted in 100% correct genotype classification.

Conclusion: The described methods for measurement of PON1 activity and accurate genotype assignment are rapid and have potential to facilitate the efficient investigation of PON1 status in clinical and epidemiological studies.

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Human serum paraoxonase 1 (PON1; EC 3.1.1.2)4 plays a dual physiological role (1). PON1 was first studied in relation to its ability to metabolize organophosphate pesticides such as paraoxon. More recently, investigations have focused on the role of PON1 in the cardioprotective effects of HDL. HDL protects LDL from atherogenic, oxidative modification when LDL is incubated under oxidizing conditions in vitro (2). This effect is primarily attributed to PON1, which is exclusively associated with HDL (3,4) and has been shown to hydrolyze specific oxidized lipids in LDL (5,6).

PON1 has 2 exonic amino acid polymorphisms, 1 at position 192 [a glutamine (Q)/arginine (R) substitution], and 1 at position 55 [a methionine (M)/leucine (L) substitution] (7). The PON1-Q192R polymorphism has been the more extensively studied and has been shown to affect the activity of PON1 alloenzymes with respect to both organophosphate detoxification (8) and lipoprotein oxidation (5).

4 Nonstandard abbreviations: PON1, human serum paraoxonase 1; PA, phenylacetate; CHD, coronary heart disease; SALT, salt-stimulated paraoxonase activity; IA, phenylacetate-inhibited arylesterase activity; IAa, estimate of influence of nonspecific arylesterase activity of other carboxylic ester hydrolases; NIA, noninhibited arylesterase activity; PON 4SI ratio, PON salt-stimulation/similar-substrate inhibition, or (SALT/PA)/(IA-IAO)/NIA; PXON, the activity of PON to hydrolyze paraoxon in the absence of 1 mol/L NaCl; SS, total sum of squares; I, index of individuality; S2i, interindividual variance; S2j, intra-individual variance; %S2a, estimated percentage of variance attributable to analytical variation.
The PON1-Q192R (paraoxonase 1)\(^5\) genotype has been positively associated with coronary heart disease (CHD) in several case-control studies (9–16), but not in all (17–20), although a recent metaanalysis (21) indicates an increased frequency of the PON 192 R allele in CHD and that the R allele is associated with an increased risk of CHD. The discrepancy among studies is likely due to the high level of variation of gene expression coupled with the fact that the PON Q192R polymorphism is functional; meaning it affects PON1 activity. In this light, determination of PON1 activity in addition to genotype (referred to as PON1 status) has been advocated to be more important than PON1 genotype alone (21, 22). Larger, preferably prospective, studies are required to determine the relationship between PON1 status and CHD, and these studies will require high-throughput methods capable of measuring PON1 status.

PON1 phenotype can be determined by kinetic enzyme assays. The identification of PON1 phenotypes by their response to 1 mol/L NaCl (23) was refined into the paraoxonase:arylesterase ratio (24). A further modification of the salt-stimulation technique combined the salt-stimulation technique with the differential inhibition of the phenylacetate (PA) hydrolysis by 0.1 mmol/L chlorpromazine (25). Haagan and Brock (26) described the inhibited arylesterase:noninhibited arylesterase ratio, and Mueller (27) described an assay for PON1 phenotype that used the inhibitory effect of EDTA on paraoxonase activity (28). Paraoxon and diazoxon have been used as substrates with adaptation to a microtiter plate system (28). A semiautomated, microtiter plate-based assay has been described (29). These assays are not 100% accurate in assigning phenotype and may not be amenable to high-throughput automation. The activity ratios used in these studies overlap, especially between QR and RR genotypes at low enzyme activity, and may introduce misclassification into population-based studies. In the present study, we have automated 5 assays of PON1 activity, used them to assign genotype and may not be amenable to high-throughput methods capable of measuring PON1 status.

Materials and Methods

Patients and Specimens

As part of continuing, population based, case-control studies investigating oxidative stress and different diseases in men and women in western New York, we collected extra blood for ancillary methodological studies (in addition to main study requirements) from 67 healthy control individuals. Driver’s license bureau rolls were used as the sampling frame from which control participants were randomly selected from the population of Erie and Niagara counties in western New York. The method accuracy component consisted of genomic DNA and blood serum stored at −80 °C. Twelve additional samples obtained from faculty and staff volunteers were used to optimize analytical conditions and generate QC specimens. An additional sample set, designed for the estimation of biological variation, consisted of serial samples from 17 volunteers (7 men and 10 women) obtained at 0, 1, and 4 weeks. All study specimens (79 total) were collected, processed, and stored with standardized procedures that we have described previously (30). The experimental protocols were approved by the Institutional Review Board at the University of Buffalo and informed, written consent was obtained from all participants.

PCR Genotyping

Recombinant Taq DNA polymerase, forward and reverse custom oligonucleotide primers, and dNTPs were purchased from Invitrogen, Inc. Restriction endonuclease Alw I was purchased from New England Biolabs. Primers for amplification of a 176-bp sequence coding for position 192 of human PON1 were: 5’-GGG ACC TGA GCA CTT TTA TGG C-3’ and 5’-CAT CGG GTG AAA TGT TGA TTC C-3’. PCR product from each sample was restriction digested with Alw I for 2 h at 37 °C. The Alw I recognition site is not present in the PON1 192 A allele but is present in the B allele (7). One of 3 restriction fragment length polymorphism genotype patterns was possible: QQ 176-bp fragment, RR 118-bp and 58-bp fragments, and QR 176-bp, 118-bp, and 58-bp fragments.

Kinetic Enzyme Assays

Instrumentation included the Cobas Fara II automated chemistry analyzers (Roche Diagnostic Systems Inc.), and a Model 160U ultraviolet-visible recording spectrophotometer (Shimadzu Corporation). Unless otherwise indicated all reagents were obtained from Sigma Chemical Company. Diethyl p-nitrophenyl phosphate (paraoxon), 98.0%, was obtained from Chem Service. For all assays water blanks were used to correct for nonenzymatic hydrolysis.

Peroxonase Activity and Salt-Stimulated Peraxonase Activity

The rate of formation of p-nitrophenol was measured on the Cobas Fara II analyzer using 1 mmol/L paraoxon in 50 mmol/L glycine buffer, pH 10.5, with 1.0 mmol/L CaCl\(_2\) with or without 1 mol/L NaCl. The reaction was initiated by 20 μL diluted sample (1:20 in 25 mmol/L triethanolamine-hydrochloride, pH 7.4, 1.0 mmol/L CaCl\(_2\)) to 360 μL working reagent. The rate of p-nitrophenol formation was measured at 405 nm over 200 s with a 25 s lag time. The activity was expressed as U/L based on the molar absorptivity (18 290) of p-nitrophenol at 405 nm, at pH 10.5. Peraxonase is a neurotoxic substance and safety measures included use of dedicated sample and reagent needles and treatment of the on-board wastewater receptacle with concentrated sodium hydroxide.

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\(^5\) Human gene: PON1, paraoxonase 1.
ARYLESTERASE ACTIVITY WITH PHENYL ACETATE AS SUBSTRATE
The working reagent consisted of 25 mmol/L Tris-HCl, 1 mmol/L PA, pH 8.0, with 1.0 mmol/L CaCl2. The reaction was initiated by 20 μL of diluted sample (1:3 in TRIS) to 3.0 mL of the working reagent at 25 °C. The reaction was recorded for 60 s after a 20 s lag time on the 160-U spectrophotometer. The activity, expressed as kU/L, was based on the molar absorptivity (1310) of phenol at 270 nm, at pH 8.0. For automated assay on the Cobas Fara, serum samples were automatically diluted 1:4 in TRIS buffer and the enzymatic reaction was initiated by addition of 5 μL of diluted sample to 0.3 mL of the working reagent at 25 °C. The activity, expressed as kU/L, was based on the molar absorptivity (188) of phenol at 285 nm, at pH 8.0 and determined on the 160U spectrophotometer.

ARYLESTERASE ACTIVITY WITH P-NITROPHENYL ACETATE AS SUBSTRATE AND INHIBITION OF P-NITROPHENYLACETATE HYDROLYSIS BY PHENYL ACETATE
The working reagent consisted of 25 mmol/L triethanolamine-hydrochlorine buffer, pH 7.4, with 1.0 mmol/L CaCl2 with or without 1 mmol/L phenyl acetate. The start reagent consisted of 2.5 mmol/L p-nitrophenyl acetate in water. The reaction was initiated by addition of 20 μL diluted sample (1:20 in triethanolamine-hydrochlorine buffer) to 288 μL working reagent followed by 72 μL of start reagent. The rate of formation of p-nitrophenol was determined at 405 nm at 25 °C over 225 s after a 100 s lag time. The activity, expressed in kU/L, was based on the molar absorptivity (14 000) of p-nitrophenol at 405 nm, at pH 7.4 (24).

ENZYMATIC ACTIVITY RATIOS
To differentiate between PON1 phenotypes we calculated activity ratios (24,26). The salt-stimulation ratio (SALT/PA) was defined as the salt-stimulated paraoxonase activity (SALT) over arylesterase activity, with PA as substrate (24). The inhibition ratio [(IA-IAO)/NIA] was defined as the PA-inhibited arylesterase activity (IA) with p-nitrophenyl acetate as substrate minus the estimate of influence of nonspecific arylesterase activity of other carboxylic ester hydrolases (IAO) divided by the noninhibited arylesterase activity (NIA) with p-nitrophenylacetate alone as substrate (26). Finally, a double ratio, dubbed the PON salt-stimulation/similar-substrate inhibition (PON 4SI) ratio, was defined as (SALT/PA)/(IA-IAO)/NIA. The activity of PON to hydrolyze paraoxon in the absence of 1 mol/L NaCl (PXON) was not used in the calculation of ratios but was measured nonetheless as a further indicator of PON1 activity.

PARTIAL PURIFICATION OF PON 1 FROM HUMAN SERUM
PON1Q and PON1R alloenzymes were partially purified from 200 mL of pooled human serum according to the methods described by Gan et al. (32). The final DEAE anion exchange fractions were screened for total protein and arylesterase activity and the highest specific activity fractions were used as a QC material.

TOTAL CHOLESTEROl MEASUREMENT
Total serum cholesterol was determined by cholesterol oxidase methodology with reagents, calibrators, and controls from Wako Diagnostics, Inc.

METHOD PERFORMANCE CHARACTERISTICS
QC materials consisted of 3 human serum pools (QQ, QR, and RR) and 2 preparations (QQ and RR) of partially purified PON1. Within-run precision was calculated on 20 replicates of each material. Between-run precision was estimated by analysis of 5 replicates per day on 5 consecutive days. Precision was expressed as the CV and the percentage of total sum of squares (SS) attributed to each component, where within-run SS + between-run SS = Total SS. Correlation among PON1 activity assays was calculated by Pearson regression. Agreement between manual and automated arylesterase activity measurements was calculated as the interclass correlation coefficient.

METHOD ACCURACY
PCR/AlwI genotype analysis was used as the standard to compare the efficiency [correctly classified phenotypes/ (correctly classified phenotypes + incorrectly classified phenotypes)] of activity ratios to classify QQ homozygotes vs QR heterozygotes and for QR heterozygotes vs RR homozygotes. Cutoff points for classifying genotype by activity ratio(s) were determined by plotting the distribution of ratio values for each genotype and identifying the point of overlap between adjacent distributions.

BIOLOGICAL VARIABILITY
To describe the observed variability we used an unconditional hierarchical nested random effects model. The model assumes the total variance in the population is represented by \( \sigma^2_{IA} = \sigma^2_{IA} + \sigma^2_{IAO} + \sigma^2_A \), and variance within an individual is represented by \( \sigma^2_{A+} = \sigma^2_A + \sigma^2_A \), where the subscript G = among group, I = within individual, and A = analytical or within replicate. IA and NIA were log transformed to meet statistical assumptions. Estimates of the variance obtained from the fitted model \( \sigma^2_{IA} \) and the percentage each component represents in terms of the total were computed, as were the CVs \( CV_{IA}, CV_{IAO} \) and \( CV_A \). The index of individuality \( I = \frac{S_{A+}}{S_G} \) where \( S_{A+} = \left( \sigma^2_A + \sigma^2_A \right)^{1/2} \). To determine the number of measurements \( k \) needed to be taken on an individual so that \( I \) was at least as small as the cholesterol index of individuality, \( I_{CHOL} \).
used the formula, $k = \frac{1}{(\text{cholesterol}_{\text{mean}})^2}$. In this context cholesterol was used as a point of reference to a well-established biomarker of CHD risk. We also calculated the analytical acceptability index, defined as $\frac{CVA}{CVI} \times \frac{SA}{SI}$, an index of analytical error. The standard criterion states that the maximum allowable error should be less than or equal to half the intraindividual biological variation (33). SAS version 9.0 statistical software was used for all analyses.

### Results

#### PCR GENOTYPING

The results for the uncut and cut PON1 PCR products indicate that the digestion products were of the correct size predicted by the position of the AlwI restriction site. The observed distribution of genotypes were 52% QQ, 39% QR, and 9% RR; the expected distribution (assuming the population to be in Hardy–Weinberg equilibrium) was 46% QQ, 44% QR, and 10% RR, with respect to the 2 alleles.

#### PON1 ENZYME ASSAYS

The descriptive statistics, stratified by PON1 genotype, are listed in Table 1. These values agree closely with previous reports of PON1 activity against the substrates and reaction conditions selected for this study (24, 26). The correlations for all pairs of analyses (with different substrates and reaction conditions) were $>0.64$, with most $>0.90$ (Table 2). The automated and manual PA methods were highly correlated (Pearson $r = 0.96$, interclass correlation coefficient $= 0.929$), but imprecision of the automated method was higher (Table 3). A Bland–Altman plot indicated that results for the automated method were slightly higher than expected at high activity and slightly lower than expected at low activity (data not shown). Phenotypes assigned by the SALT/PA ratio, however, were identical for the 2 PA methods, and the automated PA values were used for all subsequent data analyses.

The distribution of the IA-IAO/NIA ratio was trimodal (Fig. 1A). We used the Kolmogorov–Smirnov test to test for gaussian distribution of the ratio values for each

### Table 1. Descriptive statistics of PON1 activities and PON1 activity ratios stratified based on PCR genotype for 79 study participants.

<table>
<thead>
<tr>
<th>PON Genotype</th>
<th>PXON, U/L</th>
<th>PA, kU/L</th>
<th>SALT, U/L</th>
<th>Stimulation by salt</th>
<th>SALT/PA</th>
<th>NIA, kU/L</th>
<th>IA, kU/L</th>
<th>Inhibition, %</th>
<th>I-AO/NIA</th>
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<tr>
<td>QQ (n = 41)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>146.02</td>
<td>57.68</td>
<td>229.81</td>
<td>183.69</td>
<td>4.01</td>
<td>2.60</td>
<td>1.18</td>
<td>54.30</td>
<td>0.44</td>
</tr>
<tr>
<td>SD</td>
<td>62.22</td>
<td>14.04</td>
<td>55.52</td>
<td>95.39</td>
<td>0.47</td>
<td>0.66</td>
<td>0.29</td>
<td>2.17</td>
<td>0.02</td>
</tr>
<tr>
<td>Minimum</td>
<td>22.40</td>
<td>20.59</td>
<td>104.00</td>
<td>87.80</td>
<td>2.73</td>
<td>1.19</td>
<td>0.64</td>
<td>46.46</td>
<td>0.40</td>
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<tr>
<td>Maximum</td>
<td>272.30</td>
<td>108.35</td>
<td>391.22</td>
<td>520.53</td>
<td>5.04</td>
<td>4.96</td>
<td>2.23</td>
<td>57.95</td>
<td>0.51</td>
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<td>QR (n = 31)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>387.80</td>
<td>65.73</td>
<td>902.76</td>
<td>238.75</td>
<td>13.81</td>
<td>4.13</td>
<td>1.41</td>
<td>66.05</td>
<td>0.33</td>
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<tr>
<td>SD</td>
<td>106.19</td>
<td>13.24</td>
<td>208.92</td>
<td>31.82</td>
<td>1.92</td>
<td>0.82</td>
<td>0.30</td>
<td>1.41</td>
<td>0.01</td>
</tr>
<tr>
<td>Minimum</td>
<td>202.00</td>
<td>32.63</td>
<td>534.00</td>
<td>186.77</td>
<td>10.34</td>
<td>2.55</td>
<td>0.83</td>
<td>63.35</td>
<td>0.28</td>
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<tr>
<td>Maximum</td>
<td>623.54</td>
<td>90.25</td>
<td>1394.80</td>
<td>344.32</td>
<td>18.26</td>
<td>5.71</td>
<td>2.01</td>
<td>70.19</td>
<td>0.35</td>
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<td>RR (n = 7)</td>
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<tr>
<td>Mean</td>
<td>670.03</td>
<td>84.48</td>
<td>1632.82</td>
<td>247.31</td>
<td>21.55</td>
<td>6.26</td>
<td>1.84</td>
<td>70.43</td>
<td>0.29</td>
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<tr>
<td>SD</td>
<td>230.64</td>
<td>23.22</td>
<td>481.03</td>
<td>17.87</td>
<td>1.73</td>
<td>1.59</td>
<td>0.46</td>
<td>1.25</td>
<td>0.01</td>
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<tr>
<td>Minimum</td>
<td>363.24</td>
<td>40.92</td>
<td>831.62</td>
<td>228.94</td>
<td>18.09</td>
<td>3.17</td>
<td>1.01</td>
<td>68.16</td>
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<tr>
<td>Maximum</td>
<td>920.88</td>
<td>111.62</td>
<td>2319.28</td>
<td>270.11</td>
<td>22.78</td>
<td>8.14</td>
<td>2.45</td>
<td>71.76</td>
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<td>All (n = 79)</td>
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<td></td>
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<tr>
<td>Mean</td>
<td>281.96</td>
<td>62.99</td>
<td>628.47</td>
<td>211.32</td>
<td>9.39</td>
<td>3.53</td>
<td>1.33</td>
<td>39.72</td>
<td>0.40</td>
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<tr>
<td>SD</td>
<td>180.85</td>
<td>15.85</td>
<td>510.23</td>
<td>76.14</td>
<td>6.13</td>
<td>1.39</td>
<td>0.36</td>
<td>6.64</td>
<td>0.07</td>
</tr>
<tr>
<td>Minimum</td>
<td>22.40</td>
<td>20.59</td>
<td>104.00</td>
<td>87.80</td>
<td>2.73</td>
<td>1.19</td>
<td>0.64</td>
<td>28.24</td>
<td>0.28</td>
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<tr>
<td>Maximum</td>
<td>920.88</td>
<td>108.35</td>
<td>2319.28</td>
<td>520.54</td>
<td>23.23</td>
<td>8.15</td>
<td>2.46</td>
<td>53.54</td>
<td>0.54</td>
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### Table 2. Pearson correlations (intercept/slope) of PON1 activity among multiple substrates.

<table>
<thead>
<tr>
<th></th>
<th>PA</th>
<th>PA Automated</th>
<th>SALT</th>
<th>NIA</th>
<th>IA</th>
<th>PXON</th>
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<tr>
<td>PA</td>
<td>1.00</td>
<td></td>
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<tr>
<td>PA Automated</td>
<td>0.96 (8.95/0.8)</td>
<td>1.00</td>
<td></td>
<td></td>
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<tr>
<td>SALT</td>
<td>0.68 (−47.9/1.95)</td>
<td>0.66 (−31.2/1.58)</td>
<td>1.00</td>
<td></td>
<td></td>
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<tr>
<td>NIA</td>
<td>0.84 (−96.4/6.63)</td>
<td>0.83 (−43.3/5.5)</td>
<td>0.95 (−28.3/0.35)</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IA</td>
<td>0.88 (−7.5/1.79)</td>
<td>0.91 (4.4/1.54)</td>
<td>0.74 (−37.1/1.05)</td>
<td>0.90 (24.6/0.23)</td>
<td>1.00</td>
<td></td>
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<tr>
<td>PXON</td>
<td>0.70 (−17.5/0.78)</td>
<td>0.69 (−10.7/0.63)</td>
<td>0.95 (−4.7/2.48)</td>
<td>0.93 (−7.9/13)</td>
<td>0.74 (−12.0/0.41)</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Dependant variables are in rows and independent variables are in columns.
genotype. A log transform was applied to nongaussian data, the transformed mean and SD were calculated, and the inverse log was taken to plot a distribution for each genotype (as determined by PCR). The cutoff points for assigning phenotype based on activity ratio are shown in Fig. 1A and 1B. Similar plots for the SALT/PA ratio are shown in Fig. 2.

The SALT/PA method correctly classified 78 of 79 genotypes, and the IA-IAO/NIA method correctly classified 77 of 79. Both ratios are able to completely distinguish between QQ and QR phenotypes, and the efficiency for each method was 100% with respect to these 2 genotypes. There was misclassification, however, between QR and RR genotypes. The SALT/PA method misclassified one RR genotype as a QR genotype. The area of overlap between QR and RR distributions appeared to be less than the one QR genotype as a RR genotype. The correct classification rates of the 2 methods were 97.4% and 94.7%, respectively. Combining these ratios into the PON 4SI ratio completely separated QR and RR genotypes. The SALT/PA method misclassified one QR genotype as a RR genotype. The IA-IAO/NIA method misclassified one RR genotype as a QR genotype. The area of overlap between QR and RR distributions appeared to be less than the one QR genotype as a RR genotype.

The QC materials reflected the activity found in serum in that the QQ material had the lowest activity, the RR material had the highest activity, and the RR activities were intermediate. All assays demonstrated CV <5% except the IA method, which had imprecision that increased from 3% to 8% as the enzyme activity decreased. The NIA method also demonstrated an increase in imprecision at lower enzyme activity. We attribute this observation to both nonenzymatic hydrolysis of the p-nitrophenylacetate substrate during the reaction and non-paraoxonase-specific hydrolysis. Although water blanks were used to account for this nonenzymatic hydrolysis, we found these blank measurements to be highly variable as well. The imprecision of the blank contributed more to the total change in absorbance per minute at lower enzyme activity, which was reflected in increased imprecision in the QQ control material relative to the RR and RR control material. We further noted a decrease in imprecision in the partially purified PON1 preparations relative to frozen serum samples for the para-nitrophenylacetate assays, which we attributed to removal of non-paraoxonase-specific hydrolysis by the purification steps. ANOVA of the QC data showed that the within-run sum of squares comprised >90% of the total sum of squares when within-run and between-run measurements were combined, indicating that nearly all the analytical variation was due to within-run imprecision.

Results of ANOVA separating the sources of variation into analytical variance (S_A^2), intraindividual variance (S_I^2), and interindividual or group variance (S_G^2) are shown in Table 3. For assays using PA and paraoxonase, <10% of the total variance was attributed to analytical and intraindividual variability combined, indicating that the majority of difference within our study population was due to differences among individuals. The assays using p-nitrophenyl acetate as substrate had higher analytical variability than did paraoxon and PA assays. We hypothesize that the nonenzymatic and non-paraoxonase-specific hydrolysis of p-nitrophenylacetate in the reaction is the major contributor to this variability. Before modification of the NIA and IA assays (i.e., removing the substrate from the working reagent into a separate start reagent and thus minimizing the nonspecific hydrolysis of the substrate), the estimates of analytical imprecision (within-run and between-run CVs) and the estimated percentage of variance attributable to analytical variation (%S_A^2) were 50% to 60% higher (data not shown).

For optimal test interpretation, the analytical acceptability index specifies having CV_A ≤ 1.5 CV_R or CV_A/CV_R ≤ 0.5 (33). None of the described assays had an analytical acceptability index < 0.5 except for total cholesterol. The paraoxon- and PA-based assays had indices of 0.58 – 0.68, and the 4 nitrophenol-based assays had indices of 1.79 – 3.69. The intraindividual ranges for assay results were narrow compared with group ranges, and the values of the index of individuality were lower than those of cholesterol for all assays except the IA assay.

Table 3. Summary of sources of variability for enzyme kinetic assays of PON1 activity and PON1 activity ratios by ANOVA.

<table>
<thead>
<tr>
<th>Method</th>
<th>%S_A^2</th>
<th>%S_I^2</th>
<th>%S_G^2</th>
<th>CV_A</th>
<th>CV_I</th>
<th>CV_G</th>
<th>Analytical acceptability index CV_A/CV_I</th>
<th>Index of individuality I = CV_I/CV_G</th>
<th>No. of measurements required to achieve the I of cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paraoxon</td>
<td>1.15</td>
<td>2.45</td>
<td>96.40</td>
<td>9.17</td>
<td>13.40</td>
<td>84.05</td>
<td>0.68</td>
<td>0.19</td>
<td>1</td>
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<tr>
<td>PA</td>
<td>2.45</td>
<td>6.16</td>
<td>91.38</td>
<td>4.13</td>
<td>6.55</td>
<td>25.23</td>
<td>0.63</td>
<td>0.31</td>
<td>1</td>
</tr>
<tr>
<td>SALT</td>
<td>0.28</td>
<td>0.85</td>
<td>98.86</td>
<td>4.64</td>
<td>8.02</td>
<td>86.39</td>
<td>0.58</td>
<td>0.11</td>
<td>1</td>
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<tr>
<td>SALT/PA</td>
<td>0.56</td>
<td>0.31</td>
<td>99.13</td>
<td>5.21</td>
<td>3.89</td>
<td>69.48</td>
<td>1.34</td>
<td>0.09</td>
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<tr>
<td>NIA</td>
<td>10.92</td>
<td>0.93</td>
<td>88.15</td>
<td>13.10</td>
<td>3.82</td>
<td>37.21</td>
<td>3.43</td>
<td>0.37</td>
<td>1</td>
</tr>
<tr>
<td>IA</td>
<td>23.67</td>
<td>1.74</td>
<td>74.59</td>
<td>98.65</td>
<td>26.74</td>
<td>175.10</td>
<td>3.69</td>
<td>0.58</td>
<td>2</td>
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<tr>
<td>IA-IAO/NIA</td>
<td>8.05</td>
<td>1.46</td>
<td>90.49</td>
<td>3.90</td>
<td>1.66</td>
<td>13.08</td>
<td>2.35</td>
<td>0.32</td>
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<tr>
<td>PON 4SI</td>
<td>0.74</td>
<td>0.23</td>
<td>99.03</td>
<td>6.90</td>
<td>3.85</td>
<td>80.01</td>
<td>1.79</td>
<td>0.10</td>
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<tr>
<td>Cholesterol</td>
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<td>19.06</td>
<td>78.58</td>
<td>2.99</td>
<td>8.50</td>
<td>17.26</td>
<td>0.35</td>
<td>0.52</td>
<td>reference</td>
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</table>

Variance is expressed as percent of total variance attributed to analytical variation (%S_A^2), intraindividual variation (%S_I^2), and interindividual variation (%S_G^2) from 17 persons at 3 different time points, with 3 replicate measurements at each time point. The CV is defined as 100 percent X (standard deviation/mean) and was calculated for each component of variance (CV_A, CV_I, CV_G). # Log transformation of the dependant variable used.
Epidemiological studies of the role of PON1 in disease, particularly CHD, have been inconsistent. Genetic studies have been more common than studies that focused on PON1 status (i.e., activity and/or concentration), and there has recently been a call for standardized assays to facilitate comparability among studies (34).

We modified the molecular methods (7) by using different forward and reverse primers to lengthen the PCR product from a 99-bp to a 176-bp amplimer. We had difficulty resolving the 99-bp amplimer cut and uncut fragments from the 22 and 23mer PCR primers on 3% agarose, and the 176-bp product was more easily resolved, as was the 118-bp digestion product. Thermocycler parameters were adjusted to 35 cycles to increase the PCR product without having to do a secondary amplification, decreasing the number of steps and the potential for contamination.

Five kinetic enzyme methods were adapted to the Cobas Fara II autoanalyzer. Manual PON 1 arylesterase activity with PA as substrate at 270 nm was compared with the Cobas Fara II, which has a lower wavelength limit of 285 nm. The suboptimal wavelength on the Cobas Fara II caused an increase in assay CVs but did not change the genotype assignment by activity ratios. Paraoxonase assay parameters were only minimally modified, and our results closely parallel other reports (24). The PON1-inhibition technique (26) was modified more substantially. Para-nitrophenylacetate is subject to significant spontaneous hydrolysis in the reagent buffer system originally described. This hydrolysis was grossly apparent in the yellow color of the reagent. We removed this substrate from the working reagent buffer and prepared it in water as a separate starting reagent, which remained colorless. The starting reagent was added to initiate the kinetic reaction, and this modification reduced the CV of

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Fig. 1. (A) Distribution of the quotient of inhibited arylesterase - IA/ noninhibited arylesterase and the calculated gaussian distribution of quotient values within each PCR/AlwI genotype (QQ, QR, and RR) for 79 study sera. Arrows and values indicate the point of overlap between adjacent distributions used as cut-points to assign phenotype based on activity ratio, depicted graphically in (B).

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Fig. 2. (A) Distribution of the quotient of salt-stimulated paraoxonase activity (SALT)/aryl esterase activity using PA and the calculated gaussian distribution of quotient values within each PCR/AlwI genotype (QQ, QR, and RR) for 79 study sera. Arrows and values indicate the point of overlap between adjacent distributions used as cut-points to assign phenotype based on activity ratio depicted graphically in (B).
both sample and sample blank readings and improved the reproducibility of the assay.

In addition to using QC material to generate estimates of analytical imprecision and describing the proportion of total variance attributed to analytical variance, we calculated an index of analytical acceptability defined as $CVA_{\frac{1}{2}} CVI$. The rationale for selecting the fraction $\frac{1}{2}$ has been previously described (33). This index has been used to judge the ability of analytical methods to detect individual variations that indicate a disease state or significant changes over time. Based on this criterion, the assays described here are not analytically acceptable to detect small changes within an individual over the 1-month time frame studied.

The index of individuality is a means to assess the usefulness of conventional population-based reference intervals. If the index is $>1.4$, the test results from an individual can be compared usefully to the reference interval. If the index is $<0.6$, reference intervals are of limited value because of limited day-to-day variation and/or a large variation between individuals in the population (35). The low index of individuality found in this study reflects a large variation in serum PON1 activity between individuals and indicates that although these assays (except for the measurement of IA) can detect differences between individuals in a population, the use of population-based reference intervals based solely on PON1 activity will have limited utility. Because each genotype is associated with significant differences in activity it may be more useful to consider PON1 status or genotype-specific reference intervals with regard to PON1 activity in epidemiological studies.

Fig. 3. (A) Distribution of the quotient of the PON 4SI double ratio $[(IA - IA_0) / (NIA) / (SALT/PA)]$ and the calculated gaussian distribution of quotient values within each PCR/AlwI genotype (QQ, QR, and RR) for 79 study sera. Arrows and values indicate the point of overlap between adjacent distributions used as cut-points to assign phenotype based on activity ratio depicted graphically in (B).
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References