Relationship of Paraoxonase 1 (PON1) Gene Polymorphisms and Functional Activity With Systemic Oxidative Stress and Cardiovascular Risk

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Context Paraoxonase 1 (PON1) is reported to have antioxidant and cardioprotective properties. The relationship between PON1 genotypes and functional activity with systemic measures of oxidative stress and cardiovascular disease (CVD) risk in humans has not been systematically investigated.

Objective To investigate the relationship of genetic and biochemical determinants of PON1 activity with systemic measures of oxidative stress and CVD risk in humans.

Design, Setting, and Participants The association between systemic PON1 activity measures and a functional polymorphism (Q192R) resulting in high PON1 activity with prevalent CVD and future major adverse cardiac events (myocardial infarction, stroke, or death) was evaluated in 1399 sequential consenting patients undergoing diagnostic coronary angiography between September 2002 and November 2003 at the Cleveland Clinic. Patients were followed up until December 2006. Systemic levels of multiple structurally defined fatty acid oxidation products were also measured by mass spectrometry in 150 age-, sex-, and race-matched patients and compared with regard to PON1 genotype and activity.

Main Outcome Measures Relationship between a functional PON1 polymorphism and PON1 activity with global indices of systemic oxidative stress and risk of CVD.

Results The PON1 genotype demonstrated significant dose-dependent associations (QQ192 > QR192 > RR192) with decreased levels of serum PON1 activity and with increased levels of systemic indices of oxidative stress. Compared with participants with either the PON1 RR192 or QR192 genotype, participants with the QQ192 genotype demonstrated an increased risk of all-cause mortality (43/681 deaths [6.75%] in RR192 and QR192 and 62/584 deaths [11.1%] in QQ192; adjusted hazard ratio, 2.05; 95% confidence interval [CI], 1.09-2.03; P = .01). The incidence of major adverse cardiac events was significantly lower in participants in the highest PON1 activity quartile (23/315 [7.3%]) compared with those in the lowest activity quartile (78/311 [25.1%] and 75/319 [23.5%]; adjusted hazard ratio, 1.48; 95% CI, 1.09-2.03; P = .01). The incidence of major adverse cardiac events was significantly lower in participants in the highest PON1 activity quartile (23/315 [7.3%]) compared with those in the lowest activity quartile (78/311 [25.1%] and 75/319 [23.5%]; P < .001 for paraoxonase and arylesterase, respectively). The adjusted hazard ratios for major adverse cardiac events between the highest and lowest PON1 activity quartiles were, for paraoxonase, 3.4 (95% CI, 2.1-5.5; P < .001) and for arylesterase, 2.9 (95% CI, 1.8-4.7; P < .001) and remained independent in multivariate analysis.

Conclusion This study provides direct evidence for a mechanistic link between genetic determinants and activity of PON1 with systemic oxidative stress and prospective cardiovascular risk, indicating a potential mechanism for the atheroprotective function of PON1.

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conversely, overexpression of the human PON1 transgene in mice results in reduced aortic lesion size and corresponding decreases in epitopes recognized by antibodies specific for oxidized lipid-protein adducts.\textsuperscript{3,8}

Despite evidence that PON1 prevents atherosclerosis in animal models, it remains to be established whether PON1 possesses atheroprotective and antioxidant properties in humans. Several studies have suggested that PON1 may play an atheroprotective role but the simultaneous associations between PON1 polymorphisms and enzyme activity with cardiovascular disease (CVD) risk have been reported in only some population studies.\textsuperscript{1-3,9-13} Further confounding the relationship in humans has been the discovery of multiple PON1 polymorphisms in both the coding region of the protein and the promoter, some of which reportedly influence overall systemic activity levels.\textsuperscript{14}

In a recent meta-analysis of 43 studies examining multiple single-nucleotide polymorphisms (SNPs) for PON1, the most promising of the genotypes was the Q192R variant, although this SNP demonstrated only a weak overall association with coronary heart disease of uncertain relevance.\textsuperscript{15} However, each of the contributing studies in the meta-analyses involved small cohorts of patients, with variable evaluation of functional activity and no quantitative examination of systemic indices of oxidative stress. The major conclusions of the meta-analyses therefore emphasized the need for both much larger and mechanistic investigations of the role of PON1 in human CVD, particularly since no significant association for the Q192R polymorphism was noted among the larger studies in the meta-analyses, and accompanying simultaneous functional and genetic studies were typically lacking.\textsuperscript{15} Thus, it remains to be established whether genetic and biochemical determinants of PON1 are linked to oxidative stress and CVD risk in humans.

The Q192R polymorphism involves a mutation from glutamine (Q, wild type) to arginine (R, variant) at amino acid position 192 of the protein sequence. Functional PON1 activity can be measured by its ability to hydrolyze exogenous substrates such as paraoxon and phenylacetate, reflecting so-called paraoxonase and arylesterase activity, respectively. Functional differences have been observed in hydrolysis rates of the Q192 vs R192 alloenzymes using paraoxon as substrate, although no difference has been reported regarding their ability to hydrolyze phenylacetate.\textsuperscript{16}

In this present large, prospective clinical study, we report a comprehensive in vivo investigation of a mechanistic link between the functional PON1 Q192R polymorphism, serum PON1 activity using dual enzyme activity measurements to account for the differential rate of hydrolysis of the alloenzymes, multiple indices of systemic oxidative stress, and risk of both prevalent atherosclerotic CVD and near-term incident major adverse cardiovascular events (myocardial infarction [MI], stroke, and death).

**METHODS**

**Study Design and Sample Collection**

PON1 activity and functional polymorphisms were determined in serum and DNA samples of 1399 sequential consenting patients who participated in the GeneBank study between September 2002 and November 2003. GeneBank is a single-site (Cleveland Clinic, Cleveland, Ohio) sample repository generated from patients undergoing elective diagnostic coronary angiography with extensive clinical and laboratory characterization and longitudinal observation.

For systemic measures of oxidative stress, whole blood collected in EDTA tubes was immediately spun, plasma and buffy coat were isolated, and plasma was stored under argon atmosphere with antioxidant cocktail supplement, as previously described.\textsuperscript{17} Serum for PON1 activity measures was obtained from serum separator tubes after 30 to 60 minutes of clotting time at room temperature. All specimens were stored at \(-80^\circ\text{C}\) until time of analysis. Patients were followed up on an annual basis for adjudicated incident major adverse cardiac events and mortality until December 14, 2006. The GeneBank study was approved by the institutional review board of the Cleveland Clinic. All patients provided written informed consent prior to being enrolled in the study.

**Clinical Diagnosis and Definition of Outcomes**

Information regarding demographics, medical history, and medication use was obtained by patient interview and confirmed by chart review. Race information used in analyses was prespecified prior to the study and was based on self-report. All clinical outcomes data were verified by source documentation. Mortality was assessed using the Social Security Death Index.\textsuperscript{18}

Cardiovascular disease was defined by the presence of coronary artery disease or peripheral arterial disease. Coronary artery disease included adjudicated diagnoses of stable or unstable angina, MI (adjudicated definition based on defined electrocardiographic changes or elevated cardiac enzymes), or angiographic evidence of at least 50% stenosis of 1 or more epicardial vessels. Peripheral artery disease was defined as the presence of any extracoronary atherosclerosis and included obstructive disease (including a history of intermittent claudication), amaurosis fugax, history of cerebrovascular accident, or evidence of either arterial stenosis or aneurysmal disease in the thoracic limbs or abdominal aorta on Doppler ultrasound.

Prospective cardiovascular risk was assessed by the incidence of major adverse cardiovascular events (MACE), which included nonfatal and fatal MI, nonfatal and fatal stroke, and all-cause mortality. Nonfatal events were defined as MI or stroke in patients who survived at least for 48 hours following the onset of symptoms. We also assessed the risk of true incidence or the first cardiovascular event in participants without a history of CVD at enrollment (baseline) and the risk for re-
current cardiovascular events in participants with an established diagnosis of CVD at baseline according to levels of PON1 activity.

**Determination of PON1 Activity**

Serum arylesterase and paraoxonase activities were independently measured by UV spectrophotometry in a 96-well plate format (Spectramax 384 Plus, Molecular Devices, Sunnyvale, California) using phenyl acetate or paraoxon (Sigma-Aldrich, St Louis, Missouri) as substrates, respectively. Briefly, for arylesterase assays, initial hydrolysis rates were determined at 270 nm in 50-fold diluted serum (final) in reactions mixtures composed of 3.4mM phenylacetate, 9mM Tris hydrochloride, pH 8, and 0.9mM calcium chloride at 24°C. An extinction coefficient (at 270 nm) of 1310M−1 ·c m−1 was used for calculating units of paraoxonase activity in this healthy, middle-aged population ranged from 169.3 to 814.0 µmol/min/mL, with median levels of 605.8 µmol/min/mL (interquartile range [IQR], 517.6-666.8 µmol/min/mL) of serum. Systemic arylesterase activity in this healthy, middle-aged population ranged from 343.6 to 4025 nmol/min/mL, with median levels of 1264 nmol/min/mL (IQR, 647.0-1865 nmol/min/mL) of serum.

For paraoxonase activity assays, rate of generation of para-nitrophenol was determined at 405 nm in 40-fold diluted serum (final) in reaction mixtures composed of 1.5mM paraoxon, 10mM Tris hydrochloride, pH 8, 1M sodium chloride, and 2mM calcium chloride at 24°C. An extinction coefficient (at 405 nm) of 17 000 M−1 ·c m−1 was used for calculating units of paraoxonase activity, which are expressed as the amount of phenyl acetate hydrolyzed in micromoles per minute per milliliter of serum.

Paraoxonase and arylesterase assays for each sample were performed in duplicates, with average measurements of enzyme activity for each sample calculated. Each 96-well plate included blank samples to monitor spontaneous hydrolysis of substrates and aliquots of serum samples of 3 pooled calibrators (low, mid, and high levels) with known activity levels to ensure assay quality of each plate based on established acceptability criterion. The intra-assay and interassay coefficients of variance for performance of arylesterase were 1.2% and 3.9%, respectively, and the intra-assay and interassay coefficients of variance for performance of paraoxonase activity assays were 2.0% and 5.6%, respectively, on 20 replicates performed on 10 different days.

To establish normal ranges, serum arylesterase and paraoxonase activities were also determined on 100 apparently healthy volunteers (50 men and 50 women) aged 55 years or older (mean, 64 [SD, 4 years]) responding to local advertisements. Systemic arylesterase activity in this healthy, middle-aged population ranged from 169.3 to 814.0 µmol/min/mL, with median levels of 605.8 µmol/min/mL (interquartile range [IQR], 517.6-666.8 µmol/min/mL) of serum. Systemic paraoxonase activity in this healthy, middle-aged population ranged from 343.6 to 4025 nmol/min/mL, with median levels of 1264 nmol/min/mL (IQR, 647.0-1865 nmol/min/mL) of serum.

**Genotyping**

Of the 1399 participants, 1386 DNA samples from the GeneBank cohort were available for genotyping for the PON1 Q192R polymorphism (SNP rs662). Primers for amplifying the sequences containing the SNP were designed using Primer 3 (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Sense and antisense probes used for fluorescence polarization–single base extension detection of the SNP were designed using Primer PREMIER (PREMIER Biosoft International, Palo Alto, California). Each genotyping reaction consisted of a single-plex polymerase chain reaction followed by a single base-pair extension reaction using either the sense or antisense probe and deoxyribonucleotides labeled with TAMRA or R110 for the alternative alleles. The fluorescence signal was detected by fluorescence polarization using the analyst HT (Molecular Devices) and genotypes were determined based on the plot of TAMRA vs R110 signal values.

**Mass Spectrometry Assays**

Plasma levels of structurally specific species of hydroxyicosatetraenoic acids (HETEs), hydroxyoctadecadienoic acids (HODEs), the 8-isoprostane prostaglandin F2α (8-isoPGF2α), and their precursor fatty acids (arachidonic or linoleic acids) were quantified in samples of participants using stable isotope dilution high-performance liquid chromatography with online electrospray ionization tandem mass spectrometry. Prostaglandin F2α-d4 (Cayman Chemicals, Ann Arbor, Michigan) was used as internal standard for calibration of 8-isoPGF2α and 15-HETE-d6 (Cayman Chemicals) for other oxidized fatty acids. Quantification of total plasma levels of each analyte (free plus esterified) were performed following addition of the appropriate isotopically labeled internal standards and base-catalyzed hydrolysis, as previously described.

Analyses were performed on plasma specimens collected from 50 age-, sex-, and race-matched participants of each genotype (QQ192, QR192, and RR192) who were randomly selected from among the 1386 patients. To avoid bias, 50 participants with the genotype of the lowest frequency in our cohort (RR192) were initially randomly selected among the 136 participants who carried that genotype (50% male; 100% white; mean age, 63 [SD, 2 years]). An additional 50 participants from each of the QQ192 and QR192 cohorts, matched with the QQ192 participants for age, sex, and race, were also selected for mass spectrometry analyses.

**Statistical Analyses**

Clinical diagnosis, outcome definition, determination of PON1 activity, genotyping, and mass spectrometry analyses were each performed by investigators who were blinded to CVD status and other measurements. Continuous variables are presented as mean (SD) or median (IQR) for non–normally distributed data and categorical variables as numbers and percentages. Regression analysis was used for calculating the variance (R2) explained by the PON1 polymorphism and activity measures. Quartile cut points were determined from the enzyme activity levels of all 1399 study participants at baseline. Analysis of variance or the Kruskal-Wallis test (for non-
normally distributed data) was used to test the difference in mean oxidized fatty acid levels according to PON1 genotype and tertiles of PON1 activity. Kaplan-Meier methods were used to plot time-to-event curves for PON1 activity quartiles and genotypes and the log-rank test was performed to assess differences between curves.

Logistic regression analysis was used to calculate adjusted odds ratios (ORs) for the association between activity levels and prevalent CVD status after adjusting for the traditional risk factors and selected classes of medications (statins and aspirin) as described herein.

Cox proportional hazard models were performed to determine if PON1 is an independent predictor of future cardiac events. Unadjusted hazard ratios (HRs) for clinical events were calculated with reference to the highest quartile (corresponding to lowest risk). The models were adjusted for all traditional cardiac risk factors, including the Framingham ATP-III risk score (including diabetes status, log C-reactive protein, body mass index, and medication use (statins and aspirin)). Models were created separately for paraoxonase and arylesterase. All variables used in the models met the proportional hazards assumption by testing them as time-dependent covariates in the multivariate model.

Receiver operating characteristic curves were plotted to estimate the C index for MACE with and without PON1 activity as predictors. The C index is analogous to the area under the receiver operating characteristic curve but takes into account right censoring. To evaluate the contribution of PON1 activity as a predictive marker, we calculated the concordance indices with and without each PON1 activity measurement in separate multivariate models that included the variables described herein. The improvement in predictability with the addition of each of these well-established risk factors was assessed by the difference in the concordance indices. The difference of the indices was bias-corrected and bootstrapping was used to generate 95% confidence intervals (CIs). A 1-sample t test was performed to determine if the difference was equal to zero.

Hazard ratios for clinical events were calculated for participants with the PON1 Q192R polymorphism and were adjusted after controlling for differences in the aforementioned traditional cardiac risk factors and medications. An additive model of inheritance was also created to assess the change in risk for having 1 and 2 copies of the Q allele. The genotypes were given values of 0, 1, and 2 and entered in a Cox proportional hazards model. All statistical analyses were performed using SPSS, version 11 (SPSS Inc, Chicago, Illinois) and verified on SAS, version 8.2 (SAS Institute Inc, Cary, North Carolina). All P values are 2-sided, with P < .05 considered significant.

**RESULTS**

**Clinical, Laboratory, and Demographic Characteristics**

The clinical characteristics of participants stratified according to a diagnosis of CVD at time of enrollment are summarized in Table 1. Participants with CVD were older, with a greater prevalence of history of hyperlipidemia, diabetes, and hypertension and greater use of aspirin and statins. While 66% of patients were taking statins, less than 10% of the cohort were taking fenofibrates. Participants with CVD had lower levels of HDL cholesterol (HDL-C) and LDL cholesterol (LDL-C) (the latter presumably because of increased statin use) and higher levels of triglycerides and C-reactive protein.

In the entire cohort, 46.3% (642/1386) had the QR192 genotype, 34.9% (360/1061) had the RR192 genotype, and 9.8% (136/1386) had the QQ192 genotype. Participants with CVD had lower levels of HDL cholesterol (HDL-C) and LDL cholesterol (LDL-C) (the latter presumably because of increased statin use) and higher levels of triglycerides and C-reactive protein.

In the entire cohort, 46.3% (642/1386) had the QQ192 genotype, 43.9% (608/1386) had the QR192 genotype, and 9.8% (136/1386) had the RR192 genotype. Participants with CVD had lower levels of HDL cholesterol (HDL-C) and LDL cholesterol (LDL-C) (the latter presumably because of increased statin use) and higher levels of triglycerides and C-reactive protein.

While 49.4% of participants with the wild-type QQ192 genotype (317/642) were in the lowest quartile of PON1 activity, only 4.4% of participants with the variant RR192 genotype (6/136) were in the lowest activity quartile. In contrast, 3.9% of participants with the QQ192 genotype (52/642) and 83.8% with the RR192 genotype (114/136) were in the highest activity quartile (Table 2).
In the lowest activity quartile, 92.2% of study participants (317/344) had the QQ192 genotype and only 1.7% (6/344) had the RR192 genotype. The reverse trend was observed in the highest activity quartile, with 7.2% of participants (25/348) having the QQ192 genotype and 32.8% (114/344) having the RR192 genotype (Table 3). Regression analysis confirmed that the PON1 Q192R polymorphism accounted for 58.5% ($R^2=0.585$; $P<.001$) of the variation in serum paraoxonase activity levels throughout the population.

**PON1 Genotype, Paraoxonase Activity, and Systemic Oxidative Stress**

Plasma levels of multiple structurally specific oxidized fatty acids were quantified in participants and analyzed for their relationships with the PON1 genotype (Table 4) and paraoxonase activity (Table 5). Participants with the RR192 genotype had lower levels of all measured systemic indices of oxidative stress compared with age-, sex-, and race-matched participants possessing the QQ192 genotype ($P<.001$ for all comparisons). Consistent with these findings, serum paraoxonase activity levels were inversely correlated with multiple direct systemic indices of oxidative stress in a dose-dependent fashion (Table 4). Collectively, these results provide both genetic and biochemical support for the notion that the PON1 Q192R variant strongly influences quantitative measures of systemic oxidative stress in humans.

**Association of the PON1 Q192R Polymorphism With Prevalent CVD and CVD Outcomes**

An increased prevalence of coronary artery disease was observed in participants with the PON1 QQ192 genotype (461/962 [47.9%]) with vs 169/405 [41.7%] without coronary artery disease; $P=.04$). In contrast, participants with the RR192 genotype showed the opposite tendency with lower prevalence of coronary artery disease (Table 5). Those with the PON1 QQ192 genotype were similarly observed

### Table 1. Baseline Characteristics and Prospective Events Among Participants Undergoing Elective Diagnostic Cardiac Catheterization (N = 1399)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>With CVD at Baseline (n = 1116)</th>
<th>Without CVD at Baseline (n = 283)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (SD), y</td>
<td>65.1 (10.9)</td>
<td>57.2 (11.8)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Male</td>
<td>799/1116 (71.6)</td>
<td>135/283 (47.7)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>1023/1111 (92.1)</td>
<td>243/282 (86.2)</td>
<td>.002</td>
</tr>
<tr>
<td>African American</td>
<td>71/1111 (6.4)</td>
<td>38/282 (13.5)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Hypertension</td>
<td>940/1093 (86.0)</td>
<td>168/277 (60.6)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>843/1100 (76.6)</td>
<td>150/282 (53.2)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>433/1093 (39.5)</td>
<td>49/271 (18.1)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Current smoking</td>
<td>163/1116 (14.6)</td>
<td>33/282 (11.7)</td>
<td>.20</td>
</tr>
<tr>
<td>Statin use</td>
<td>717/1088 (65.9)</td>
<td>80/270 (29.6)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Aspirin use</td>
<td>873/1098 (79.5)</td>
<td>176/272 (64.7)</td>
<td>.058</td>
</tr>
<tr>
<td>Body mass index</td>
<td>29.7 (5.9)</td>
<td>29.7 (6.3)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Blood pressure, mean (SD), mm Hg Systolic</td>
<td>134.3 (21.2)</td>
<td>134.8 (21.3)</td>
<td>.96</td>
</tr>
<tr>
<td></td>
<td>74.0 (13.3)</td>
<td>75.5 (12.2)</td>
<td>.20</td>
</tr>
<tr>
<td>Blood pressure, mean (SD), mm Hg Diastolic</td>
<td>46.1 (13.0)</td>
<td>53.9 (15.9)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td></td>
<td>99.0 (37.5)</td>
<td>112.4 (36.6)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Triglycerides, median (IQR), mg/dL</td>
<td>138.0 (99.0-202.0)</td>
<td>115.5 (77.0-173.0)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Total cholesterol, mean (SD), mg/dL</td>
<td>178.4 (46.4)</td>
<td>193.9 (43.5)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>C-reactive protein, median (IQR), mg/L</td>
<td>3.0 (1.5-7.0)</td>
<td>2.4 (1.2-5.9)</td>
<td>.005</td>
</tr>
<tr>
<td>Framingham ATP-III score, mean (SD)</td>
<td>13.3 (3.5)</td>
<td>11.4 (5.0)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Paraoxonase, median (IQR), µmol/min/mL</td>
<td>860.6 (442.6-1599.0)</td>
<td>1164.0 (473.5-1825.0)</td>
<td>.001</td>
</tr>
<tr>
<td>Arylesterase, median (IQR), µmol/min/mL</td>
<td>334.6 (279.5-395.8)</td>
<td>356.9 (296.0-424.4)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Prospective event rates, No. (Kaplan-Meier %) [95% CI]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MI</td>
<td>69 (7.2) [5.6-8.9]</td>
<td>11 (4.4) [1.8-6.9]</td>
<td>.14</td>
</tr>
<tr>
<td>MI/CVA</td>
<td>84 (8.9) [7.1-10.7]</td>
<td>12 (4.7) [2.1-7.4]</td>
<td>.05</td>
</tr>
<tr>
<td>Death</td>
<td>99 (10.3) [8.3-12.2]</td>
<td>7 (2.9) [0.8-5.0]</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>MACE</td>
<td>175 (17.9) [15.5-20.3]</td>
<td>18 (7.2) [4.0-10.4]</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; CVA, cerebrovascular accident; CVD, cardiovascular disease; HDL-C, high-density lipoprotein cholesterol; IQR, interquartile range; MACE, major adverse cardiac events (ie, myocardial infarction, stroke, or death); MI, myocardial infarction; LDL-C, low-density lipoprotein cholesterol; SI conversions: To convert HDL-C, LDL-C, and total cholesterol to mmol/L, multiply by 0.0259; to convert triglycerides to mmol/L, multiply by 0.0113; to convert C-reactive protein to nmol/L, multiply by 9.524.

### Table 2. Distribution of Paraoxonase Activity Quartiles in Each PON1 Q192R Genotype

<table>
<thead>
<tr>
<th>Paraoxonase Quartile, µmol/min/mL</th>
<th>QQ192 (n = 642)</th>
<th>QR192 (n = 608)</th>
<th>RR192 (n = 136)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median (IQR)</td>
<td>453.5 (360.4-560.3)</td>
<td>1436 (1129-1800)</td>
<td>2374 (1978-2851)</td>
</tr>
<tr>
<td>Quartile 4 (&gt;1640)</td>
<td>25 (3.9)</td>
<td>209 (34.4)</td>
<td>114 (83.8)</td>
</tr>
<tr>
<td>Quartile 3 (1640-899.1)</td>
<td>14 (2.2)</td>
<td>321 (52.8)</td>
<td>14 (10.3)</td>
</tr>
<tr>
<td>Quartile 2 (899-450)</td>
<td>286 (44.5)</td>
<td>57 (9.4)</td>
<td>2 (1.5)</td>
</tr>
<tr>
<td>Quartile 1 (&lt;450)</td>
<td>317 (49.4)</td>
<td>21 (3.5)</td>
<td>6 (4.4)</td>
</tr>
</tbody>
</table>

Abbreviations: CVD, cardiovascular disease; IQR, interquartile range; PON1, paraoxonase 1 gene.

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to have an increased likelihood of having a history of coronary artery bypass graft surgery \((P = .03)\) and a tendency toward increased history of percutaneous coronary intervention \((P = .09)\) (data not shown). In contrast, those with the PON1 RR192 genotype demonstrated opposite tendencies (diminished history of revascularization; \(P = .09\)) (data not shown). Individuals carrying the PON1 Q192R genotype showed no observed differences in prevalent peripheral artery disease within the cohort (Table 5).

Of the 1386 participants with genotype information, follow-up data were available in 1265 participants (Q192 genotype, \(n = 584\); Q192 genotype, \(n = 563\); and RR192 genotype, \(n = 118\)). The PON1 functional polymorphism Q192R was significantly associated with CVD outcomes within the cohort. For example, all adverse cardiovascular outcomes monitored were observed to a significantly lesser degree in patients who carried 1 R allele.

Kaplan-Meier estimates of all-cause mortality and MACE revealed a significant trend in our study participants. Homozygous and heterozygous participants (RR192 and QR192 genotypes) had significantly lower event rates for all-cause mortality (43/681 [Kaplan-Meier estimate, 6.7%]) compared with participants with the Q192 genotype (62/584 [Kaplan-Meier estimate, 11.10%]; \(P = .006\)). Similarly, the event rate for MACE over the ensuing 3-year period was lower with the RR192 and QR192 genotypes (88/681 [Kaplan-Meier estimate, 13.6%]) vs the Q192 genotype (102/584 [Kaplan-Meier estimate, 18.0%]; \(P = .03\)) (Table 6 and FIGURE).

In a multivariate model including age, Framingham ATP-III risk score, race, log C-reactive protein, body mass index, and medication (statin and aspirin) use, the addition of the risk allele Q192 showed no association with incident MI and stroke over the ensuing 3 years following enrollment but was significantly associated with increased likelihood of death and MACE (Table 6). Compared with participants with either the RR192 or QR192 genotype, participants with the QQ192 genotype demonstrated an adjusted HR of 2.05 (95% CI, 1.32-3.18) for all-cause mortality \((P = .001)\). Furthermore, MACE were also more likely to occur over the ensuing 3-year period in participants with the QQ192 genotype compared with participants with either the RR192 or QR192 genotype (adjusted HR, 1.48; 95% CI, 1.09-2.03; \(P = .01\)) (Table 6).

In separate analyses, an additive model of inheritance was created to assess the change in risk for having a copy of the Q allele. An adjusted HR for 3-year all-cause mortality of 1.58 (95% CI, 1.12-2.24; \(P = .01\)) was observed for having a Q allele. Furthermore, in this model the presence of a Q allele was also associated with an increased risk of having a MACE over the ensuing 3-year period (HR, 1.32; 95% CI, 1.04-1.69; \(P = .03\)).

### Table 3. Distribution of PON1 Q192R Genotypes in Each Paraoxonase Activity Quartile

<table>
<thead>
<tr>
<th>Paraoxonase Quartile, nmol/min/mL</th>
<th>PON1 Q192R Genotype</th>
<th>Quartile 4 (&lt;1640) (n = 348)</th>
<th>Quartile 3 (1640-899.1) (n = 349)</th>
<th>Quartile 2 (899-450) (n = 345)</th>
<th>Quartile 1 (&lt;450) (n = 344)</th>
</tr>
</thead>
<tbody>
<tr>
<td>QQ192</td>
<td>25 (7.2)</td>
<td>14 (4.0)</td>
<td>286 (82.9)</td>
<td>317 (92.2)</td>
<td></td>
</tr>
<tr>
<td>Q192</td>
<td>209 (60.1)</td>
<td>312 (92.0)</td>
<td>57 (16.5)</td>
<td>21 (6.1)</td>
<td></td>
</tr>
<tr>
<td>RR192</td>
<td>114 (32.8)</td>
<td>14 (4.1)</td>
<td>2 (0.6)</td>
<td>6 (1.7)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: CVD, cardiovascular disease; IQR, interquartile range; PON1, paraoxonase 1 gene.

<table>
<thead>
<tr>
<th>Oxidized Fatty Acid</th>
<th>PON1 Q192R Genotype</th>
<th>Paraoxonase Tertile</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>QQ192 (n = 50)</td>
<td>RR192 (n = 50)</td>
</tr>
<tr>
<td>5-HETE</td>
<td>20.1 (14.3-24.9)</td>
<td>15.5 (12.6-19.2)</td>
</tr>
<tr>
<td>8-HETE</td>
<td>3.0 (2.5-4.4)</td>
<td>2.5 (1.8-3.7)</td>
</tr>
<tr>
<td>9-HETE</td>
<td>42.7 (30.3-63.1)</td>
<td>30.1 (22.0-35.2)</td>
</tr>
<tr>
<td>11-HETE</td>
<td>5.8 (4.8-7.1)</td>
<td>4.5 (3.7-6.1)</td>
</tr>
<tr>
<td>12-HETE</td>
<td>7.6 (5.9-10.4)</td>
<td>6.1 (4.9-8.1)</td>
</tr>
<tr>
<td>15-HETE</td>
<td>24.3 (20.6-30.8)</td>
<td>20.2 (16.2-26.3)</td>
</tr>
<tr>
<td>9-HODE</td>
<td>35.5 (30.0-43.7)</td>
<td>28.8 (22.6-37.1)</td>
</tr>
<tr>
<td>13-HODE</td>
<td>38.7 (30.4-49.0)</td>
<td>29.6 (23.0-38.9)</td>
</tr>
<tr>
<td>8-isoPGB_{\text{Fnu}}</td>
<td>11.1 (4.4-15.6)</td>
<td>11.5 (3.0-28.5)</td>
</tr>
</tbody>
</table>

Abbreviations: HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; PON1, paraoxonase 1 gene; Q192, wild type subjects; QR192, heterozygous subjects; RR192, mutant homozygous subjects; 8-isoPGB, 8-iso-prostaglandin F_{\text{nu}}.

Data are presented as median (interquartile range) of oxidized fatty acid in picomoles per milliliter.

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Association of PON1 Activity With Prevalent CVD and CVD Outcomes

A higher prevalence of CVD was also observed in patients with low serum activity levels of either paraoxonase or arylesterase. Following adjustment for differences in known CVD risk factors, this association was not significant for paraoxonase (OR, 1.5; 95% CI, 0.97-2.2; P = .07 for comparison between upper and lower quartiles) but was significant for arylesterase (OR, 2.1; 95% CI, 1.4-3.1; P = .001). Low systemic levels of enzyme activity were significantly associated with the presence of coronary artery disease (paraoxonase: OR, 1.5; 95% CI, 1.03-2.3; P = .03; arylesterase: OR, 2.0; 95% CI, 1.4-3.1; P = .001) or in combination with peripheral artery disease (paraoxonase: OR, 1.4; 95% CI, 0.9-2.0; P = .11; arylesterase: OR, 1.2; 95% CI, 1.0-2.11; P = .05).

Event rates for all prospective cardiovascular events were significantly lower in participants in the highest PON1 activity quartile compared with participants in the lowest quartile (P < .001 for all prospective events comparisons) (TABLE 7 and Figure). The frequency of incident cases of nonfatal MI or stroke among participants within the highest quartiles of PON1 activity was 2.5% (8/315) based on paraoxonase activity and 2.8% (9/324) using arylesterase activity.

Higher rates of incident nonfatal MI and stroke were observed in participants within the lowest quartile of paraoxonase activity (37/311 [11.9%]) and arylesterase activity (40/319 [12.5%]). Similarly, lower frequency of 3-year incident all-cause mortality was observed in participants within the highest quartiles of paraoxonase activity (17/315 [5.4%]) and arylesterase activity (16/324 [4.9%]) compared with participants within the lowest quartiles of paraoxonase activity (37/311 [11.9%]) and arylesterase activity (41/319 [12.9%]). Lower event rates were also noted for MACE among study participants in the highest quartiles of paraoxonase activity (23/315 [7.3%]) and arylesterase activity (25/324 [7.7%]) compared with event rates for MACE among study participants in the lowest quartiles of paraoxonase activity (78/311 [25.1%]) and arylesterase activity (75/319 [23.5%]) (TABLE 7 and Figure).

Following multivariate analysis, serum PON1 activity measures remained independently associated with prospective risk of cardiac events (TABLE 7). The lowest quartiles of both paraoxonase and arylesterase activity were associated with a greater incident risk of nonfatal MI or stroke (para- oxonase: HR, 4.4; 95% CI, 2.0-9.6; P < .001; arylesterase: HR, 4.5; 95% CI, 2.2-9.4; P < .001) during the 3-year follow-up interval. The risk of all-cause mortality was also greatest in participants in the lowest quartiles of either paraoxonase activity or arylesterase activity (paraoxonase: HR, 2.4; 95% CI, 1.3-4.4; P = .004; arylesterase: HR, 2.2; 95% CI, 1.2-4.2; P = .01). This translated to a greater incidence of MACE in participants in the lowest quartiles of paraoxonase and arylesterase activity (paraoxonase: HR, 3.4; 95% CI, 2.1-5.5; P < .001; arylesterase: HR, 2.9; 95% CI, 1.8-4.7; P < .001).

In further analyses, low systemic levels of arylesterase activity were associated with an increased risk of having a first cardiovascular event (true incidence) among participants without either a history of CVD or angiographic evidence of significant coronary artery disease (defined as ≧50% stenosis) at baseline (adjusted HR, 5.8; 95% CI, 1.2-28.6; P = .03) (TABLE 8). Increased risk of having a recurrent nonfatal MI or stroke, all-cause mortality, and MACE was observed with low levels of paraoxonase and arylesterase activity measurements in participants with an established diagnosis of CVD at enrollment (TABLE 9). The adjusted HR for recurrent nonfatal MI or stroke was 4.5 (95% CI, 1.9-11.0; P = .001) for paraoxonase and 4.2 (95% CI, 1.9-9.6; P = .001) for arylesterase between the highest and lowest activity quartiles. Participants in the lowest activity quartile for either paraoxonase or arylesterase were more likely to have a recurrent MACE compared with participants in the highest activity quartile (paraoxonase: HR, 3.4; 95% CI, 2.0-5.9; P < .001; arylesterase: HR, 2.4; 95% CI, 1.4-3.9; P < .001).

Prognostic Value of PON1

The C index for the outcomes of nonfatal MI and stroke during a minimum 3-year follow-up period was 0.59 (with traditional risk factors as predictors), and significantly increased to either 0.66 (P = .007) or 0.69 (P = .001) in comparisons with traditional risk factors when including systemic measures of either paraoxonase or arylesterase activity, respectively. For the composite MACE outcome, addition of either paraoxonase or arylesterase activity to traditional risk factors also significantly increased the predictive value of the model during a mean of 44 (SD, 7) months. For example, the C index of 0.67 with traditional risk factors alone increased to 0.71 (P = .007) and 0.70

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Nonfatal MI/CVA  
MACE  
All-cause mortality

there has been no definitive in vivo evidence that PON1 promotes systemic antioxidant effects in humans. A recent meta-analysis of 43 studies examining the relationship between PON1 and clinical outcomes concluded that considerable uncertainty remained, since published studies to date primarily involve small cohorts of participants. Moreover, they often failed to simultaneously examine PON1 genotype, systemic PON1 activity measures, and cardiovascular outcomes, and none examined systemic quantitative indices of oxidative stress. In this study, we investigated the potential relationship between PON1 activity, PON1 genotype, systemic oxidative stress, and both coronary and peripheral artery disease in a large, prospective cohort of patients to more fully interrogate the aforementioned relationships. We demonstrate that the PON1 Q192R polymorphism is functional, resulting in increased enzymatic activity. In parallel, we demonstrate that elevated systemic levels of multiple structurally distinct fatty acid oxidation products that are increased in both atherosclerotic plaque and plasma of participants with CVD are associated with low systemic levels of PON1 activity and the PON1 QQ192 genotype. Importantly, the plasma samples analyzed in the present study were collected and processed under conditions designed to prevent artificial oxidation of lipids during both storage and analysis. The finding that levels of 9-HETE, an isomer of arachidonic acid oxidation produced exclusively by free radical–mediated processes, are higher in participants with low levels of paraoxonase activity suggests that PON1 can influence oxidative events beyond the cyclooxygenase and lipoxygenase pathways.

While the mechanism(s) for PON1-mediated systemic antioxidant effects remains to be determined, the present findings strongly support a role for this HDL-associated protein in modulating systemic oxidative stress in humans. Moreover, the present study suggests an important mechanistic link among PON1, systemic oxidative stress, and risk of development of atherosclerotic heart disease and its acute complications.

In a recent publication, amino acid position 192 of PON1 was suggested to participate in HDL binding. The Q192R alloenzyme was shown to bind to the HDL particle with 3-fold lower affinity than the R192 alloenzyme and, consequently, exhibited lower stability, lipoprotein activity, and modulatory effect on macrophage cholesterol efflux. The findings of the present clinical study complement these results, demonstrating that individuals with the arginine (R) mutation at position 192 have higher serum levels of PON1 activity, lower systemic indices of systemic oxidative stress, and corresponding reductions in both prevalent coronary artery disease and prospective cardiac events.

This is, to our knowledge, the first large, prospective study that comprehensively examines the genetics and biochemical activity of PON1 using dual enzyme measurements to predict prevalent disease risks, as well as prospective risk of MACE, while simultaneously also examining whether a potential mechanism for CVD associations is linked to systemic oxidative stress measures in humans. Compared with prior studies that often focused on lower-risk populations, the present cohort is also substantially enriched with patients with both coronary and peripheral artery disease, enabling us to better study the association of the PON1 Q192R variant on

Table 6. Relationship Between PON1 Q192R Genotype and CVD Outcomes

<table>
<thead>
<tr>
<th>CVD Outcomes</th>
<th>RR192 + QR192 (n = 681)</th>
<th>QQ192 (n = 584)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonfatal MI/CVA</td>
<td>51/681 (7.97)</td>
<td>43/584 (7.88)</td>
<td>.95</td>
</tr>
<tr>
<td>Adjusted HR (95% CI)a</td>
<td>1 [Reference]</td>
<td>1.01 (0.65-1.57)</td>
<td>.96</td>
</tr>
<tr>
<td>All-cause mortality</td>
<td>43/681 (6.75)</td>
<td>62/584 (11.10)</td>
<td>.006</td>
</tr>
<tr>
<td>Adjusted HR (95% CI)a</td>
<td>1 [Reference]</td>
<td>2.05 (1.32-3.18)</td>
<td>.001</td>
</tr>
<tr>
<td>MACE</td>
<td>88/681 (13.59)</td>
<td>102/584 (18.04)</td>
<td>.03</td>
</tr>
<tr>
<td>Adjusted HR (95% CI)a</td>
<td>1 [Reference]</td>
<td>1.48 (1.09-2.03)</td>
<td>.01</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; CVA, cerebrovascular accident; CVD, cardiovascular disease; HR, hazard ratio; MACE, major adverse cardiac events (ie, myocardial infarction, stroke, or death); MI, myocardial infarction; PON1, paraoxonase 1 gene.

*Adjusted HRs were calculated for each clinical endpoint by adding the risk allele Q192 to the multivariate model that included the Framingham ATP-III risk score of individual participants (including diabetes and smoking status), race, log C-reactive protein, body mass index, and use of statins and aspirin.

(P = .01) for risk factors plus paraoxonase or arylesterase, respectively.

**Comment**

Oxidative stress is thought to have a pivotal role in the pathogenesis of a number of chronic inflammatory disease processes, including atherosclerosis. The failure of α-tocopherol supplementation studies with alleged antioxidant properties to prevent cardiovascular events has brought the oxidation hypothesis of atherosclerosis into question. Therefore, the search continues to identify effective strategies that promote systemic antioxidant effects in humans and to determine whether they have a beneficial influence on the rate of CVD.

The present genetic and biochemical studies demonstrate that the HDL-associated protein PON1 promotes pronounced systemic antioxidant effects in humans, with coincident links to reduction in coronary artery disease prevalence and prospective risks of MACE. Our results demonstrate that both the PON1 Q192R polymorphism and serum PON1 activity are associated with both prevalent coronary artery disease and incident adverse cardiovascular events.

It has been speculated that PON1 contributes to the atheroprotective property of HDL via promotion of a systemic antioxidant effect. However, there has been no definitive in vivo evidence that PON1 promotes systemic antioxidant effects in humans. A recent meta-analysis of 43 studies examining the relationship between PON1 and clinical outcomes concluded that considerable uncertainty remained, since published studies to date primarily involve small cohorts of participants. Moreover, they often failed to simultaneously examine PON1 genotype, systemic PON1 activity measures, and cardiovascular outcomes, and none examined systemic quantitative indices of oxidative stress.
The paraoxonase 1 (PON1) Q192R genotypes are as follows: RR192 (mutant homozygous), QR192 (heterozygous), and QQ192 (wild type). In the top panels, log-rank \( P \) values are shown for the at-risk genotype QQ192 vs RR192/QR192. PON1 activity (paraoxonase and arylesterase) were categorized into quartiles; Q1: lowest activity quartile; Q4: highest activity quartile. For paraoxonase, Q4 = 1640 nmol/min/mL, Q3 = 1640-899.1, Q2 = 899-450, and Q1 = 450 nmol/min/mL. For arylesterase, Q4 = 403.9, Q3 = 403.9-338.5, Q2 = 338.4-283, and Q1 = 283 µmol/min/mL. In the middle and bottom panels, log-rank \( P \) values across activity quartiles are shown. Days indicates number of days from enrollment to first cardiac event. Event rates were calculated at 6-month intervals. Y-axis scales in blue indicate range from 0% to 15%. MI indicates myocardial infarction; CVA, cerebrovascular accident.
Table 7. Risk of Prospective CVD Events in Relation to PON1 Activity Among Entire Cohort

<table>
<thead>
<tr>
<th>CVD Events</th>
<th>Quartile 4 (&gt; 1640)</th>
<th>Quartile 3 (1640-899.1)</th>
<th>Quartile 2 (899-450)</th>
<th>Quartile 1 (&lt; 450)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 315)</td>
<td>(n = 325)</td>
<td>(n = 326)</td>
<td>(n = 311)</td>
</tr>
<tr>
<td>Nonfatal MI/CVA</td>
<td>8/315 (2.5)</td>
<td>23/325 (7.4)</td>
<td>27/326 (8.3)</td>
<td>37/311 (11.9)</td>
</tr>
<tr>
<td>Unadjusted HR (95% CI)</td>
<td>1 [Reference]</td>
<td>3.0 (1.3-6.7)</td>
<td>3.3 (1.5-7.3)</td>
<td>5.0 (2.3-10.8)</td>
</tr>
<tr>
<td>Adjusted HR (95% CI)a</td>
<td>2.9 (1.3-6.4)</td>
<td>3.1 (1.4-7.0)</td>
<td>4.4 (2.0-9.6)</td>
<td></td>
</tr>
<tr>
<td>All-cause mortality</td>
<td>17/315 (5.4)</td>
<td>21/325 (6.5)</td>
<td>27/326 (8.3)</td>
<td>37/311 (11.9)</td>
</tr>
<tr>
<td>Unadjusted HR (95% CI)</td>
<td>1 [Reference]</td>
<td>1.2 (0.6-2.3)</td>
<td>1.3 (0.7-2.5)</td>
<td>2.7 (1.5-4.7)</td>
</tr>
<tr>
<td>Adjusted HR (95% CI)a</td>
<td>1.1 (0.6-2.2)</td>
<td>1.3 (0.7-2.6)</td>
<td>2.4 (1.3-4.4)</td>
<td></td>
</tr>
<tr>
<td>MACE</td>
<td>23/315 (7.3)</td>
<td>44/325 (13.5)</td>
<td>48/326 (14.7)</td>
<td>78/311 (25.1)</td>
</tr>
<tr>
<td>Unadjusted HR (95% CI)</td>
<td>1 [Reference]</td>
<td>1.9 (1.2-3.2)</td>
<td>2.1 (1.2-3.4)</td>
<td>3.7 (2.3-5.9)</td>
</tr>
<tr>
<td>Adjusted HR (95% CI)a</td>
<td>1.9 (1.1-3.2)</td>
<td>2.0 (1.2-3.4)</td>
<td>3.4 (2.1-5.5)</td>
<td></td>
</tr>
</tbody>
</table>

Table 8. Incidence of First CVD Event in Relation to PON1 Activity Among Patients With No CVD at Baseline

<table>
<thead>
<tr>
<th>First CVD Events</th>
<th>Quartile 4 (&gt; 403.9)</th>
<th>Quartile 3 (403.9-338.5)</th>
<th>Quartile 2 (338.4-283)</th>
<th>Quartile 1 (&lt; 283)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MACE</td>
<td>(n = 80)</td>
<td>(n = 81)</td>
<td>(n = 57)</td>
<td>(n = 56)</td>
</tr>
<tr>
<td>No./total (%)</td>
<td>4/80 (5.0)</td>
<td>3/61 (4.9)</td>
<td>2/57 (3.5)</td>
<td>8/56 (14.3)</td>
</tr>
<tr>
<td>Unadjusted HR (95% CI)</td>
<td>1 [Reference]</td>
<td>1.0 (0.2-4.4)</td>
<td>1.1 (0.2-4.7)</td>
<td>3.0 (0.9-9.8)</td>
</tr>
<tr>
<td>Adjusted HR (95% CI)a</td>
<td>0.6 (0.1-3.1)</td>
<td>1.2 (0.3-5.3)</td>
<td>1.7 (0.5-6.4)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; CVA, cerebrovascular accident; CVD, cardiovascular disease; HR, hazard ratio; MACE, major adverse cardiac events (ie, myocardial infarction, stroke, or death); MI, myocardial infarction; PON1, paraoxonase 1.
aAdjusted HRs were calculated using Framingham ATP-III risk score (including diabetes status), log C-reactive protein, body mass index, and use of statins and aspirin.

coronary and extracoronary atherosclerosis after controlling for established risk factors. However, in the present study, diminished systemic arylesterase activity levels remained significantly associated with 3-year incident MACE (composite of MI, stroke, or death) in the subgroup of participants without history or clinical evidence of either coronary or peripheral artery disease and without significant angiographic evidence of coronary artery disease. Furthermore, the present study also suggests a potential prognostic value of PON1 activity measurement in patients. The addition of systemic PON1 activity measures to traditional risk factors and C-reactive protein provided a significant incremental improvement in the ability to predict clinical outcome during a 3-year period.

In recent genomewide association studies, evidence for association of the PON1 gene with coronary artery disease or MI was not observed. One likely explanation is that genomewide association studies are not ideal for assessing specific candidate genes since the panel of SNPs placed on the chip may not capture all of the genetic variation for any particular gene. Furthermore, genomewide association studies tend to identify genes with the strongest genetic effect because of the stringency in considering what is statistically significant. In this regard, a weak association with PON1 may have been observed but not reported since it did not exceed the threshold for significance based on the thousands of statistical tests performed. Moreover, one of the phenotypes we have studied, namely, mortality within the cohort, was not the exact same as those studied in the genomewide association studies, which could also account for why an association with PON1 was not reported.

The PON1 gene is located in close proximity to 2 other members of the paraoxonase gene family, PON2 and PON3, which raises the possibility that the association we have observed could be due to linkage disequilibrium with a variant in either PON2 or PON3. However, an examination of the HAPMAP database for the white population (which matches the GeneBank
cohort) shows that the PON1 Q192R variant is located within a haplotype block in which the SNPs are in moderate linkage disequilibrium with each other. Moreover, this haplotype block only covers PON1 and does not extend to the PON2 and PON3 genes. Thus, while it is possible that variants of PON2 and PON3 could also contribute to altered PON1 activity and CVD risk, the available HAPMAP data suggest that, in whites, the association observed with the PON1 Q192R variant is not due to linkage disequilibrium with other variants in the adjacent PON2 and PON3 genes, but likely results from altered PON1 function. Although it is possible that other PON1 variants that are in linkage disequilibrium with the Q192R SNP are the causal alleles, recent studies using recombinant PON1 mutants at position 192 have shown that this residue is important for HDL binding\(^2^8\) and suggest that the Q192R SNP is the causal variant underlying the clinical associations that we observe. Further studies to address this issue are required.

A number of caveats to the present study should be noted. All participants presented for elective diagnostic coronary angiography, which limits the generalization of the present findings and raises the possibility of selection bias. However, measurements of both paraoxonase and arylesterase activity in healthy volunteers are higher than in both the CVD and non-CVD participants evaluated in the present study. Given that the majority of participants were white, it also remains to be determined whether the same relationships are observed in large cohorts of other racial/ethnic groups. The lower percentage of nonwhite participants in our cohort (7.9%) limited our ability to analyze the mechanistic link between PON1 and global indices of systemic oxidative stress and thereby its association with CVD in participants of other racial/ethnic backgrounds. It is also uncertain whether concomitant medical problems influenced PON1 activity; however, inclusion of traditional cardiac risk factors including age, sex, race, diabetes, hypertension, smoking, lipids, C-reactive protein, and medication use (statins and aspirin) in the multivariate analyses failed to alter the results.

It also is possible that the reduction in PON1 activity may result from the presence of vascular disease rather than be the direct cause of future CVD events. However, arylesterase activity levels in participants with minimal angiographic evidence of coronary artery disease still remained independently associated with incident cardiovascular events over the ensuing 3-year interval following participant enrollment, consistent with an association of PON1 in early macrovascular atherosclerotic disease processes. Regardless, the current observation of a relationship among the PON1 Q192R polymorphism and PON1 activity, oxidative stress, and CVD outcomes is consistent with findings in murine models and provides evidence that the PON1 protein protects against the development and propagation of CVD.

**CONCLUSION**

The current findings provide direct prospective evidence of an important mechanistic link between the PON1 gene and PON1 systemic activity measures with both multiple quantitative indices of oxidative stress and atherosclerotic heart disease development in humans. Paraoxonase 1 is almost exclusively found to be associated with HDL particles within the circulation and has been argued to promote some of the

<table>
<thead>
<tr>
<th>Recurrent Events</th>
<th>Quartile 1</th>
<th>Quartile 2</th>
<th>Quartile 3</th>
<th>Quartile 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>No./total (%)</td>
<td>40/255</td>
<td>45/265</td>
<td>41/265</td>
<td>22/265</td>
</tr>
<tr>
<td>Unadjusted HR (95% CI)</td>
<td>3.7 (2.2-6.2)</td>
<td>3.4 (1.3-8.1)</td>
<td>3.2 (1.3-7.9)</td>
<td>3.4 (1.4-8.3)</td>
</tr>
<tr>
<td>Adjusted HR (95% CI)</td>
<td>2.4 (1.4-4.6)</td>
<td>2.6 (1.4-4.6)</td>
<td>3.3 (1.3-8.1)</td>
<td>3.4 (1.4-8.3)</td>
</tr>
<tr>
<td>All-cause mortality</td>
<td>4.6 (2.0-10.4)</td>
<td>2.6 (1.4-4.6)</td>
<td>3.3 (1.3-8.1)</td>
<td>3.4 (1.4-8.3)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Arylesterase Activity Quartile, μmol/min/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quartile 1</td>
</tr>
<tr>
<td>No./total (%)</td>
</tr>
<tr>
<td>Unadjusted HR (95% CI)</td>
</tr>
<tr>
<td>Adjusted HR (95% CI)</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; CVD, cardiovascular disease; HDL, high-density lipoprotein; MI, myocardial infarction; PON1, paraoxonase 1.

\(^a\) Adjusted HRs were calculated including the Framingham ATP-III risk score (including diabetes status), log C-reactive protein, body mass index, and use of statins and aspirin.
anti-inflammatory and antioxidant ef-
fects attributed to HDL. Thus, the pres-
ent studies also provide further sup-
port for the concept that functional prop-
erties beyond the ability of HDL and
its associated proteins to promote reverse cholesterol transport contrib-
ute to the overall ability of this lipo-
protein to reduce or prevent develop-
ment of atherosclerosis.

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