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Clinical and Genetic Association of Serum Paraoxonase and Arylesterase Activities With Cardiovascular Risk

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Objective—Diminished serum paraoxonase and arylesterase activities (measures of paraoxonase-1 [PON-1] function) in humans have been linked to heightened systemic oxidative stress and atherosclerosis risk. The clinical prognostic use of measuring distinct PON-1 activities has not been established, and the genetic determinants of PON-1 activities are not known.

Methods and Results—We established analytically robust high-throughput assays for serum paraoxonase and arylesterase activities and measured these in 3668 stable subjects undergoing elective coronary angiography without acute coronary syndrome and were prospectively followed for major adverse cardiovascular events (MACE= death, myocardial infarction, stroke) over 3 years. Low serum arylesterase and paraoxonase activities were both associated with increased risk for MACE, with arylesterase activity showing greatest prognostic value (quartile 4 versus quartile 1; hazard ratio 2.63; 95% CI, 1.97–3.50; $P<0.01$). Arylesterase remained significant after adjusting for traditional risk factors, C-reactive protein, and creatinine clearance (hazard ratio, 2.20; 95% CI, 1.60–3.02; $P<0.01$), predicted future development of MACE in both primary and secondary prevention populations, and reclassified risk categories incrementally to traditional clinical variables. A genome-wide association study identified distinct single nucleotide polymorphisms within the PON-1 gene that were highly significantly associated with serum paraoxonase (1.18×10^{-303}) or arylesterase (4.99×10^{-116}) activity but these variants were not associated with either 3-year MACE risk in an angiographic cohort ($n=2136$) or history of either coronary artery disease or myocardial infarction in the Coronary Artery Disease Genome-Wide Replication and Meta-Analysis consortium ($n\approx 80\,000$ subjects).

Conclusion—Diminished serum arylesterase activity, but not the genetic determinants of PON-1 functional measures, provides incremental prognostic value and clinical reclassification of stable subjects at risk of developing MACE.

Heightened oxidative stress in the form of oxidation of lipids and proteins by reactive oxidant species adversely contributes to disease progression in cardiovascular disease.¹ Paraoxonase-1 (PON-1) belongs to a family of high-density lipoprotein-associated enzymes that show hydrolytic activity toward a variety of substrates, including toxins in the environment² and oxidized lipids in the body.³ Consequently, diminished activities of PON-1 and other paraoxonases have been associated with the development of cardiovascular disease.^{4,5} PON-1 (EC 3.1.1.2) activity in serum is classically named after the substrate used to monitor enzymatic function, namely, paraoxonase activity (using paraoxon as substrate) and arylesterase activity (using phenyl acetate as substrate).⁶

Our group has recently observed the relationship between a specific PON-1 genotype and functional activity with multiple systemic measures of oxidative stress and cardiovascular disease risk in humans.^{7,8} Based on these encouraging findings, we developed analytically validated semiautomated high throughput methods for arylesterase and paraoxonase activity assays amenable to large scale clinical and genetic studies. We sought to expand and validate our findings in an independent cohort of stable patients undergoing cardiac evaluation to examine and contrast the potential role of distinct circulating PON-1 activity measures to predict adverse disease progression. In addition, we sought to identify genetic loci controlling paraoxonase and arylesterase activity by carrying

out an unbiased genome-wide association study (GWAS) and determine whether these genetic factors were associated with incident risks of adverse cardiac events or prevalent coronary artery disease (CAD).

Patients and Methods

Study Population

The Cleveland Clinic GeneBank study is a large, prospective cohort study from 2001 to 2006 that established a well-characterized clinical repository with clinical and longitudinal outcomes data obtained from consenting subjects undergoing an elective diagnostic cardiac catheterization procedure. All the GeneBank participants gave written informed consent approved by the Cleveland Clinic Institutional Review Board.

This study involved a total of 3668 subjects in the GeneBank study who underwent coronary angiography in the absence of acute coronary syndrome and confirmed by including only those with cardiac troponin I <0.03 ng/mL, with no history of revascularization within 30 days before enrollment, and with at least 3 years of long-term follow-up. Adjudicated outcomes were ascertained over the ensuing 3 years for all subjects after enrollment. Framingham risk factors age, sex, cigarette smoking, low-density lipoprotein and high-density lipoprotein cholesterol, systolic blood pressure, and diabetes mellitus were identified. We defined secondary prevention cohort by a known history of CAD (including stenosis of any coronary artery $\geq 50\%$ at the time of catheterization), prior myocardial infarction (MI), known history of peripheral artery disease, history of transient ischemic attack, stroke, or known cerebrovascular disease, or previous percutaneous or surgical revascularization. Those who did not fulfill secondary prevention cohort criteria were assigned to primary prevention cohort.

An estimate of creatinine clearance was calculated using the Cockcroft–Gault equation, because a majority of the patients have preserved renal function. High-sensitivity C-reactive protein (hsCRP), cardiac troponin I, serum creatinine, fasting blood glucose, and lipid profiles were also measured on the Architect ci8200 platform (Abbott Laboratories, Abbott Park, IL). Absolute neutrophil counts were analyzed by the Advia 120 Automated Hematology Analyzer (Siemens Healthcare Diagnostics, Deerfield, IL). Major adverse cardiovascular events (MACE) were defined as death, nonfatal MI, or nonfatal cerebrovascular accident after enrollment.

Serum Paraoxonase Activity and Arylesterase Activity Assays

Serum paraoxonase and arylesterase activities were measured by spectrophotometry in an open channel on the aforementioned Architect ci8200 platform, and in a 96-well plate format (Spectramax 384 Plus, Molecular Devices, Sunnyvale, CA), respectively. For serum paraoxonase activity, the rate of generation of paranitrophenol was determined at 405 nm in 40-fold diluted serum (final) in reaction mixtures composed of 1.5 mmol/L paraoxon (Sigma-Aldrich, St. Louis, MO), 10 mmol/L Tris hydrochloride, pH 8, 1 mol/L sodium chloride, and 2 mmol/L calcium chloride at 24°C. An extinction coefficient (at 405 nm) of 17000 mol/L⁻¹·cm⁻¹ was used for calculating units of paraoxonase activity, which is expressed as nanomoles of paranitrophenol produced per minute per milliliter of serum. The intra-assay and inter-assay coefficients of variance for the high-throughput paraoxonase activity assay were 1.9% and 3.3%, respectively, on 30 replicates performed on 15 different days. For serum arylesterase activity measurement, initial hydrolysis rates were determined at 270 nm in 50-fold diluted serum (final) in reactions mixtures composed of 3.4 mmol/L phenylacetate (Sigma-Aldrich), 9 mmol/L Tris hydrochloride, pH 8, and 0.9 mmol/L calcium chloride at 24°C. An extinction coefficient (at 270 nm) of 1310 mol/L⁻¹·cm⁻¹ was used for calculating units of arylesterase activity, which are expressed as micromoles of phenyl acetate hydrolyzed per minute per milliliter of serum. The intra-assay and interassay coefficients of variance for arylesterase activity assay were 3.4% and 3.9%, respectively, on 20 replicates performed on 10 different days.

Genotyping

Genotyping was performed on the Affymetrix Genome-Wide Human single nucleotide polymorphism (SNP) Array 6.0 platform. Using these data and those from 120 phased chromosomes from the HapMap CEU samples (HapMap r22 release, National Center for Biotechnology Information build 36), genotypes were imputed for untyped SNPs across the genome using MACH 1.0 software.⁹ All imputations were done on the forward (+) strand using 562554 genotyped SNPs that had passed quality control filters. QC filters for the imputed data set excluded SNPs with Hardy–Weinberg equilibrium *P* values <0.0001 or minor allele frequencies <1%, and individuals with <95% call rates. This resulted in 2421770 autosomal SNPs that were available for analysis.

Statistical Analyses

The Student *t* test or Wilcoxon–Rank sum test for continuous variables and χ^2 test for categorical variables were used to examine the difference between the groups. Kaplan–Meier analysis with Cox proportional hazards regression was used for time-to-event analysis to determine hazard ratio (HR) and 95% CI for MACE. Adjustments were made for individual traditional cardiac risk factor (including age, sex, diabetes mellitus, hypertension, former or current cigarette smoking, prior CAD), log-transformed hsCRP, and creatinine clearance. The R package Mclust was used for discriminant analysis. The clustering process is based on multivariate normal mixture models. The optimal model parameters and the number of clusters were determined via Bayesian information criterion. All analyses were performed using R 2.13.1 (Vienna, Austria) and *P* values <0.05 were considered statistically significant.

Genome-wide linear regression analyses were used to identify loci associated with serum paraoxonase and arylesterase activity after adjustment for age and sex under an additive model. Genetic analyses were carried out with PLINK (v1.07) using both untransformed (arylesterase activity) and inverse-normal transformed (paraoxonase activity) values. Relative risk for experiencing a MACE as a function of genotype was assessed using Cox proportional hazard models with adjustment for age, sex, Framingham ATP-III risk score (which includes diabetes mellitus status), and medication use (aspirin and statins). Adjusted HR and 95% CI are reported with 2-sided *P* values. A haplotype score test was also used to test all haplotypes with >1% frequency, as implemented in the Haplo.Stats package. All genetic analyses were performed using SAS version 9.2 (SAS Institute Inc, Cary, NC) or R 2.10.1 (<http://www.R-project.org>).

Associations With CAD

The Coronary Artery Disease Genome-Wide Replication and Meta-analysis (CARDIoGRAM) Consortium represents a GWAS meta-analysis of CAD comprising a discovery set of ≈ 22000 cases and ≈ 65000 controls.¹⁰ For each cohort in CARDIoGRAM, logistic regression was first used to test for association with CAD using a log-additive model with adjustment for age and sex and taking into account the uncertainty of possibly imputed genotypes. Subsequently, a meta-analysis was performed separately for every SNP from each study that passed the quality control criteria using a fixed effect model with inverse variance weighting.¹⁰ The results of this default meta-analysis were used to determine whether SNPs influencing PON-1 functional activity measures were associated with CAD.

Results

Study Population

Table 1 describes the baseline characteristics of our primary study population of 3668 subjects. Serum arylesterase activity levels were normally distributed, with a mean of 104 ± 25 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mL}^{-1}$. However, serum paraoxonase activity levels were not normally distributed, with a median

Table 1. Baseline Subject Characteristics From GeneBank Cohort (n=3668)

Variable	Value
Age, y	63±11
Sex, male, %	65
Body mass index, kg/m ²	29.6±6
Race,(Caucasian), %	97
Diabetes mellitus, %	29
Hypertension, %	70
Smokers (former/current), %	65/11
Prior coronary artery disease, %	67
LDL cholesterol, mg/dL	95 (78, 117)
HDL cholesterol, mg/dL	34 (28, 41)
Triglycerides, mg/dL	114 (83, 162)
hsCRP, mg/L	2.00 (0.91, 4.51)
Creatinine clearance, mL/min	99.9 (76.8, 126.3)
Total leukocyte count, ×10 ⁹ cells/L	6.1 (5, 7.4)
Absolute neutrophil count, cells/μL	3.9 (3.1, 5)
Baseline medications, %	
ACE inhibitors/ARBs	49
β-blockers	61
Statin	59
Aspirin	73
Serum paraoxonase activity, nmol·min ⁻¹ ·mL ⁻¹	562 (315–1045)
Serum arylesterase activity, μmol·min ⁻¹ ·mL ⁻¹	104±25

LDL indicates low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol; hsCRP, high-sensitivity C-reactive protein; ACE, angiotensin converting enzyme; ARB, angiotensin receptor blocker.

Values expressed in mean±SD or median (interquartile range).

of 562 μmol·min⁻¹·mL⁻¹ (interquartile range, 315–1045 μmol·min⁻¹·mL⁻¹). Both serum paraoxonase and arylesterase levels were lower in men than in women. Compared with those in the highest quartile, subjects in the lowest quartile of serum paraoxonase and arylesterase activity were more likely to have significantly obstructive (≥50% stenosis) CAD (odds ratio, 1.86 [95% CI, 1.53–2.27] *P*<0.01 for arylesterase, and 1.48 [95% CI, 1.22–1.81] *P*<0.01 for paraoxonase). However on adjustment of cardiovascular risk factors, such differences were no longer apparent. In addition, there were very weak (but statistically significant) correlations between serum arylesterase activity and hsCRP (*r*=-0.09; *P*<0.001), estimated creatinine clearance (*r*=0.15; *P*<0.001), and absolute lymphocyte count (*r*=0.09; *P*<0.001). In contrast, there was no statistically significant relationship between serum arylesterase activity and leukocyte count (*r*=0.02; *P*=0.28) or absolute neutrophil count (*r*=0.002; *P*=0.91). These findings were similar when correlations were performed within the Q192R genotype subgroups (data not shown).

PON-1 Activities and Major Adverse Cardiac Outcomes

In the 3668 subjects, a total of 417 cardiac events were recorded within the 3-year period of follow-up. Lower serum paraoxonase and arylesterase activity levels were associated with poorer long-term outcomes when stratified by quartiles

(Table 2, Figure 1). After adjusting for Framingham risk factors, estimated creatinine clearance, diabetes mellitus and log-transformed hsCRP, lower serum arylesterase activity (HR 2.20 [95% CI, 1.60–3.02]; *P*<0.01) and to a lesser extent lower serum paraoxonase activity (HR 1.39 [95% CI, 1.04–1.85]; *P*<0.05) demonstrated increased risk in developing future MACE. Even when cardiac troponin I levels were added to the model (within the normal range of 0.001–0.029 mg/dL), lower serum arylesterase levels still maintain a 2-fold increased risk in MACE at 3 years (HR, 2.04 [95% CI, 1.49–2.79]; *P*<0.01). The separation is particularly apparent between the lowest quartile and the upper 3 quartiles at the cutoff of 87 μmol·min⁻¹·mL⁻¹ (Figure 1). The addition of serum arylesterase activity or serum paraoxonase activity to the model results in significant improvement in risk classification with net reclassification index of 7.9% for arylesterase activity (*P*=0.003) and 7.2% for paraoxonase activity (*P*=0.002). The prognostic value of serum arylesterase activity was observed within the secondary prevention cohort (Table 2), as well as within subjects who demonstrated a recent normal catheterization (ie, no significant [>50%] angiographic evidence of CAD in any major vessel or preceding history of CAD, the primary prevention cohort; Table 2). Serum arylesterase activity also remained a prognostic indicator within each Framingham risk factor subcohort and among those with low hsCRP (Figure 2), or in those without statin therapy (n=1512). In addition to serum arylesterase remaining a significant predictor of MACE after addition of higher sensitivity troponin testing to traditional risk factors and laboratory risk markers in the models, we further observed an increased risk of developing subclinical myocardial necrosis (troponin levels that are detectable but remain below the 99th percentile diagnostic cutoff among healthy subjects used to define cutoff for MI) with decreasing quartiles of serum arylesterase levels (odds ratio 2.01 [95% CI, 1.53–2.64]; *P*<0.001; Figure 3); this trend was not observed with serum paraoxonase activity levels (Figure 3).

GWAS for PON-1 Activities

We next performed a GWAS for serum paraoxonase and arylesterase activity in 2136 GeneBank subjects (all of Caucasian ancestry) for whom both genotype and PON-1 functional data were available. The genomic inflation factors for paraoxonase and arylesterase activity were 1.015 and 1.013, respectively, indicating that the GWAS results were not confounded by underlying population stratification, and the Q-Q plots are shown in Figure I in the online-only Data Supplement. Serum paraoxonase activity was controlled by a major locus on chromosome 7 containing the PON-1, PON-2, and PON-3 genes (Figure 4A). The lead SNP for serum paraoxonase activity at this locus (rs2057681) is located within the PON-1 gene and yielded a highly significant *P* value of 1.18×10⁻³⁰³ (Figure 4A, Table 3). Based on our genotype data, rs2057681 is in near complete linkage disequilibrium (LD) (*r*²=0.99) with a functional amino acid substitution in PON-1 (rs662; Q192R), which is also associated with increased paraoxonase activity (*P*=3.31×10⁻²⁹⁵; Table 3). Of interest, while rs2057681 and rs662 are associated with significant

Table 2. Unadjusted and Adjusted HR for Major Adverse Cardiovascular Events at 3-Year Follow-Up According to Serum Arylesterase Activity and Paraoxonase Activity Quartiles, Stratified According to Primary Versus Secondary Prevention

	Serum Arylesterase Activity, $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mL}^{-1}$				Serum Paraoxonase Activity, $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mL}^{-1}$			
	Quartile 4	Quartile 3	Quartile 2	Quartile 1	Quartile 4	Quartile 3	Quartile 2	Quartile 1
All subjects (n=3668)								
Range	≥ 121	103–121	87–103	< 87	≥ 1045	562–1045	315–562	< 315
Unadjusted HR	1	1.50 (1.1–2.05)*	1.45 (1.06–1.99)*	2.63 (1.97–3.50)**	1	1.39 (1.04–1.84)*	1.1 (0.82–1.48)	1.63 (1.24–2.14)**
Adjusted HR [†]	1	1.44 (1.05–1.97)*	1.34 (0.96–1.86)	2.20 (1.60–3.02)**	1	1.19 (0.89–1.6)	1.04 (0.77–1.4)	1.39 (1.04–1.85)*
Adjusted HR [‡]	1	1.40 (1.02–1.92)*	1.27 (0.91–1.77)	1.85 (1.35–2.55)**	1	1.1 (0.82–1.49)	1.00 (0.74–1.36)	1.27 (0.95–1.70)
Secondary prevention subjects (n=2636)								
Range	≥ 119	101–119	86–101	< 86	≥ 1022	549–1022	308–549	< 308
Unadjusted HR	1	1.24 (0.89–1.73)	1.26 (0.9–1.75)	2.01 (1.49–2.72)**	1	1.35 (0.98–1.84)	1.2 (0.87–1.65)	1.45 (1.07–1.97)*
Adjusted HR [†]	1	1.23 (0.88–1.72)	1.21 (0.86–1.72)	1.78 (1.28–2.48)**	1	1.2 (0.87–1.66)	1.14 (0.83–1.57)	1.30 (0.94–1.79)
Adjusted HR [‡]	1	1.17 (0.83–1.63)	1.13 (0.80–1.61)	1.51 (1.08–2.11)*	1	1.12 (0.81–1.56)	1.12 (0.81–1.55)	1.18 (0.86–1.64)
Primary prevention subjects (n=1032)								
Range	≥ 125	107–125	91–107	< 91	≥ 1107	644–1107	335–644	< 335
Unadjusted HR	1	0.91 (0.35–2.35)	2.63 (1.21–5.72)*	4.38 (2.11–9.09)**	1	1.20 (0.61–2.38)	0.71 (0.33–1.55)	2.15 (1.16–3.97)*
Adjusted HR [†]	1	0.83 (0.32–2.18)	2.50 (1.08–5.79)*	4.00 (1.82–8.82)**	1	1.09 (0.55–2.18)	0.71 (0.33–1.51)	1.85 (0.99–3.45)
Adjusted HR [‡]	1	0.84 (0.32–2.21)	2.44 (1.02–5.84)*	3.02 (1.35–6.80)**	1	0.92 (0.46–1.84)	0.65 (0.30–1.42)	1.68 (0.88–3.19)

HR indicates hazard ratio.

[†]Model 1: Adjusted for traditional risk factors (include age, sex, systolic blood pressure, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, cigarette smoking, and diabetes mellitus).

[‡]Model 2: Adjusted for traditional risk factors (Model 1), plus log-transformed high-sensitivity C-reactive protein levels, creatinine clearance, race, body mass index, and statin.

* $P < 0.05$ and ** $P < 0.01$.

increase in paraoxonase activity, they are also associated with significant decrease in arylesterase activity (Table 3). Another known coding SNP in PON-1 (rs854560; L55M) exhibited significant association with paraoxonase activity as well ($P = 1.27 \times 10^{-140}$; Table 3) but is in relatively weak LD ($r^2 = 0.20$) with the aforementioned lead SNPs (rs2057681 and rs662). The GWAS analysis for serum arylesterase activity also revealed that this chromosome 7 region was the major locus controlling this measure of PON-1 functional activity (Figure 4B). For example, the lead SNP for serum arylesterase activity, rs854572, is located in the promoter region of PON-1 and yielded a highly significant P value of 4.99×10^{-116} (Table 3). This SNP (rs854572) also was associated with the increase in paraoxonase activity. Of note, the 4 lead SNPs for arylesterase activity differ from those identified for paraoxonase activity, although they do exhibit some association with paraoxonase activity and vice versa (Table 3). However, the lead SNPs for arylesterase and paraoxonase activity are in weak LD ($r^2 < 0.23$), suggesting that the reciprocal associations are for the most part independent. Further, the lead SNPs for both paraoxonase (rs2057681) and arylesterase (rs854572) activities are localized in the PON-1 gene and not in LD with variants in either PON-2 or PON-3 genes. We next determined whether

the lead SNPs on chromosome 7 influenced other CAD risk factors but did not observe any significant evidence for such associations (Table I in the online-only Data Supplement). Because body mass index and statin use have been shown to potentially affect serum paraoxonase activity,^{11–13} we also adjusted the genetic analyses for these potential confounders. However, the strength of the association of the lead SNPs with paraoxonase and arylesterase activities was not diminished when either body mass index or statin use alone was included with age and sex in the genetic models or in a fully adjusted model that included all 4 covariates (Table II in the online-only Data Supplement). Thus, the effect of the lead SNPs on paraoxonase and arylesterase activities are robust and independent of traditional cardiovascular risk factors.

Given the strong effect of the chromosome 7 locus on PON-1 function, we also carried out GWAS analyses for paraoxonase and arylesterase activity conditioned on the lead SNP for each respective trait. These analyses did not reveal other loci in the genome that were significantly associated with either measure of PON-1 activity. To gain further insight into the effect of the chromosome 7 variants on PON-1 function, we also examined the relationship between serum paraoxonase and arylesterase activities in our cohort. As illustrated in Figure 5,

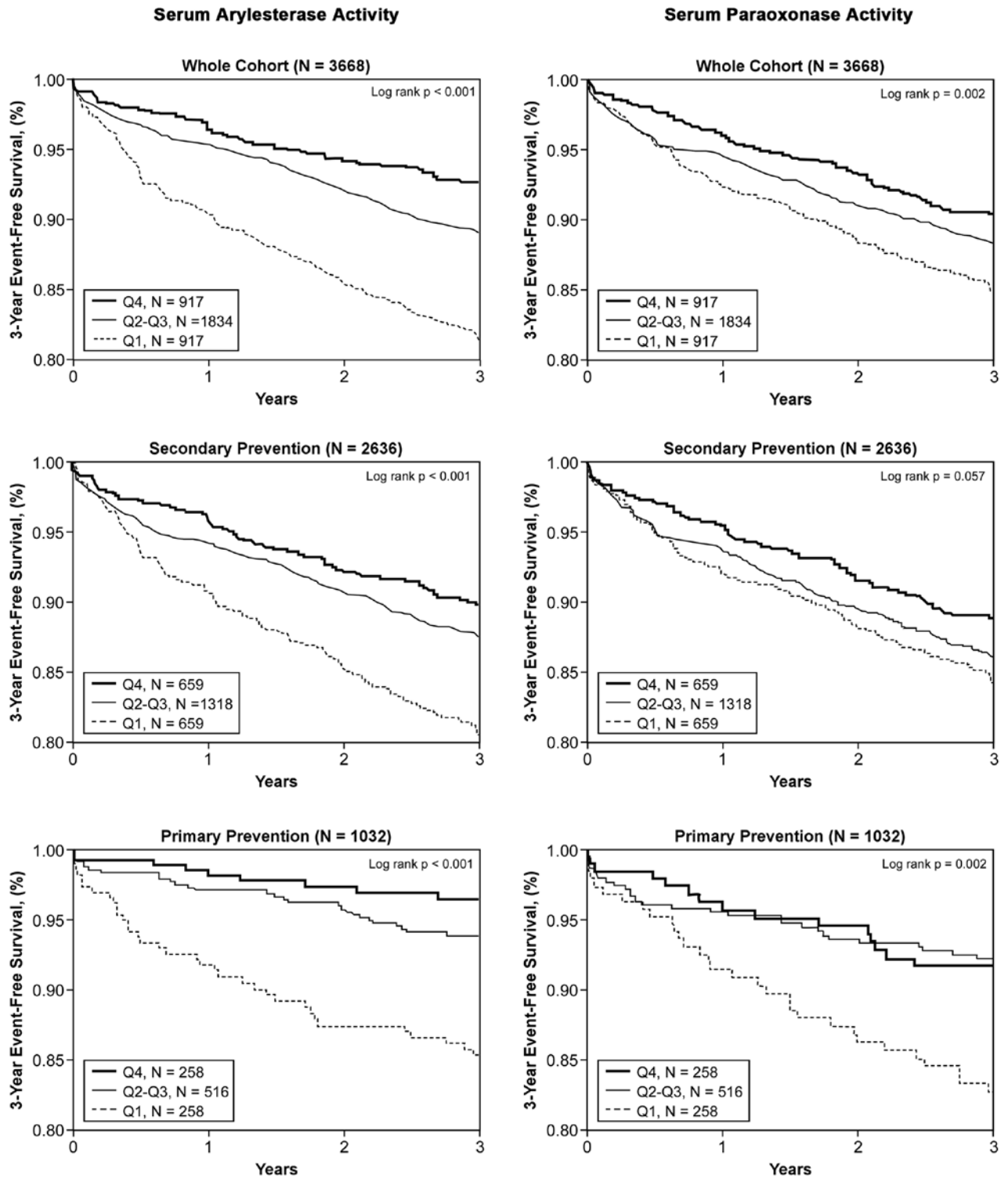


Figure 1. Kaplan–Meier analysis for long-term major adverse cardiac events stratified by serum arylesterase (left column) and paraoxonase (right column) activities quartiles in overall (top row), secondary prevention (middle row), and primary prevention (bottom row) populations.

both activities were significantly correlated overall ($r=0.30$; $P<0.001$) and exhibited a striking grouping of 3 distinct patterns when plotted against each other. These 3 clusters were found to correspond nearly exactly with the 3 genotype groups of the Q192R (rs662) polymorphism (Figure 5A), with 97.2% with the genotype QQ in the top cluster, 97.2% with genotype QR in the middle cluster, and 91.3% with genotype RR in the

bottom cluster. However, such a pattern was not observed with the 3 genotype groups of the lead SNP for serum arylesterase activity (rs854572; G>C, Figure 5B). Quantitative analysis of the separation of the 3 genotypes, GG, GC, and CC, across the 3 clusters showed 50.7% with genotype CG in the top cluster, 48.4% with genotype CG in the middle cluster, and 51.4% with genotype CG in the bottom cluster.

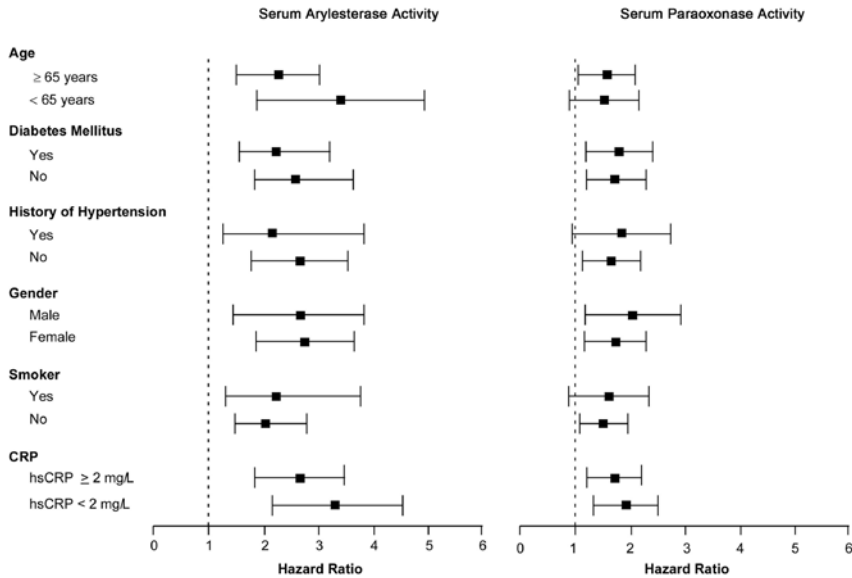


Figure 2. Forest plot regarding hazard ratios of serum arylesterase and paraoxonase activities according to traditional cardiac risk factors in subgroups of patients. hsCRP indicates high-sensitivity C-reactive protein.

Association of PON-1 Variants With Incident MACE and Prevalent Cardiovascular Disease

We next sought to determine whether the lead variants influencing paraoxonase and arylesterase activity were associated with the development of MACE. These analyses included the 2136 subjects used in the quantitative association described above plus an additional 567 subjects for whom genotype and MACE data were also available (total $n=2703$). As shown in Table 4, there were no individual effects of the Q192R (rs662) and L55M (rs854560) substitutions on incident risk of MACE in GeneBank subjects. Given the differential effects of the associated variants on PON-1 function (eg, rs662 is associated with increase in paraoxonase activity but decrease in arylesterase activity), we also constructed haplotypes with rs662, rs854560, rs854570, and rs854572 and specifically tested the ATAG haplotype, which leads to modest decreases in both paraoxonase and arylesterase activity (data not shown). However, risk of future MACE was not significantly increased in subjects carrying 1 (HR=0.96; 95% CI, 0.68–1.35) or 2 (HR=0.91; 95% CI, 0.50–1.63) copies of this haplotype ($P=0.72$; Table 4). Similarly, an analysis using a haplotype score test that included all haplotypes with >1%

frequency also did not reveal any associations with MACE (data not shown).

To further evaluate the genetic contribution of these SNPs to cardiovascular risk, we used the large CARDIOGRAM consortium, which comprises $\approx 80,000$ case–controls subjects. As shown in Table 5, no significant evidence for association of these SNPs was observed with risk of prevalent CAD. A sub-analysis with only cases that had a positive history for MI also did not reveal any associations (Table 5).

Discussion

PON-1 is an atherosclerosis protective enzyme associated with high-density lipoprotein and systemic antioxidant function. Its catalytic activity within crude serum mixtures has traditionally been measured by quantifying enzymatic hydrolysis rates of 2 known in vitro substrates, with functional activities named paraoxonase and arylesterase activities.^{4,5} There are several novel findings in this report that describe the clinical and genetic associations of PON-1 activities with cardiovascular risks that differ from prior observations from smaller cohorts. First, we demonstrated the prognostic value of serum arylesterase activity (and to a lesser extent serum paraoxonase activity) in predicting long-term cardiovascular risk in a wide range of subjects already treated with contemporary medical therapy. We observed that diminished serum arylesterase activity, particularly within the lowest quartile range, was predictive of adverse long-term cardiac events independent of standard clinical and biochemical risk factors and provided incremental value in reclassifying subjects who are at higher risk of long-term MACE. Furthermore, the prognostic value of serum arylesterase activity was evident in both primary as well as secondary prevention subjects. The fact that arylesterase activity provided prognostic value consistently within subjects with or without underlying angiographic evidence of significant CAD implies that a lack of systemic antioxidant defense mechanisms (a primary function of PON-1) may both promote greater vulnerability to oxidative stress, as well as increase risk for development and progression of CAD in subjects. Moreover, the ability of low serum arylesterase activity

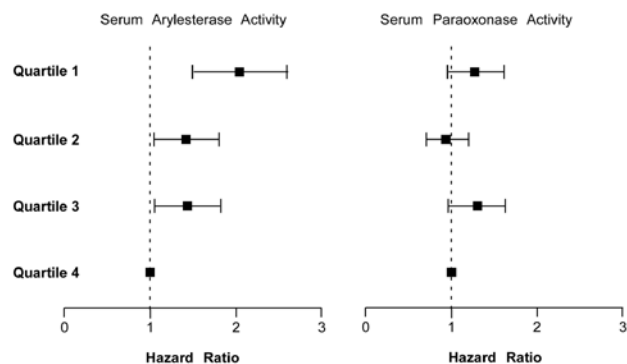


Figure 3. Adjusted odds ratio across serum arylesterase and paraoxonase activity quartiles with prevalent subclinical myocardial necrosis (defined as cardiac troponin I ≥ 0.009 ng/mL).

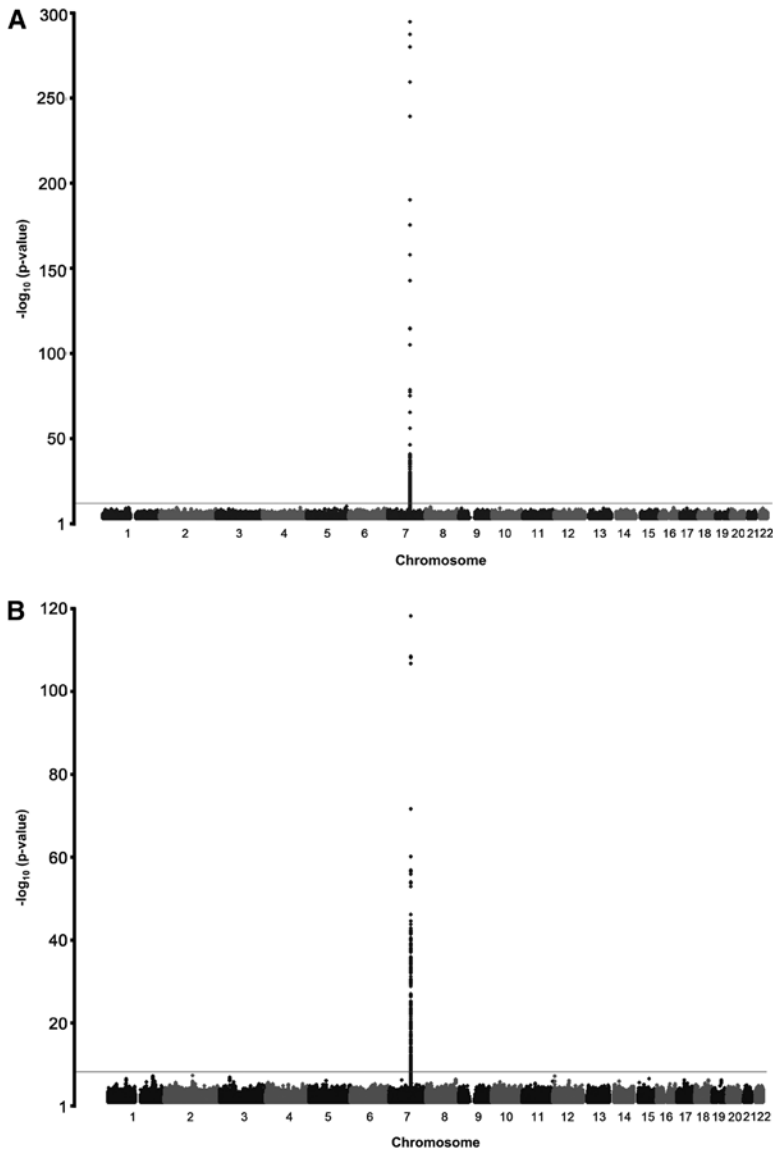


Figure 4. Manhattan plots for Genome-Wide Association Studies identifying highest single nucleotide polymorphisms associated with serum paraoxonase (A), and arylesterase (B) activity levels.

to identify those at significant increased risk of MACE, even among primary prevention subjects with recent coronary angiographic data showing <50% stenosis in all major coronary vessels, suggests this assay has prognostic value and can identify a vulnerable cohort of subjects who otherwise are not identified as being at high risk. Taken together, the present results suggest that enzymatic activity measures with arylesterase, more so than paraoxonase, serve as a powerful prognostic indicator of cardiovascular risk in a broad spectrum of subjects.

Studies on PON-1 activities have relied on quantifying its wide range of enzymatic activities in breaking down *in vitro* substrates like paraoxon (paraoxonase activity) and phenylacetate (arylesterase activity). These activities are often reported together and the findings are commonly concordant. Hence, the distinction between serum paraoxonase and arylesterase activities in predicting future adverse cardiac events is somewhat unexpected, even though historically the correlation between these 2 measures has not been particularly tight. The unique relationship between the 2

PON-1 activity measurements is largely the result of a very strong association between serum paraoxonase activity and its underlying genetic determinants, which may also explain why serum arylesterase activity is normally distributed whereas serum paraoxonase activity was not. Meanwhile, the strong association between the PON gene cluster and serum arylesterase activity confirms the long-standing assumption that this locus harbors important genetic determinants of serum arylesterase activity.

The significant genetic associations revealed by the GWAS analyses for paraoxonase and arylesterase activities are of further interest for several reasons. First, there was a stronger relationship between serum paraoxonase activity levels and its genetic determinants than those with serum arylesterase activity. Second, our results confirm the strong association of PON-1 variants with serum paraoxonase and arylesterase activities and demonstrate that the 2 measured enzymatic functions are associated in a Mendelian-like fashion by a single, major locus on chromosome 7 containing the PON gene cluster. This is supported by the

Table 3. Mean Serum Paraoxonase and Arylesterase Activity as a Function of Genotype for Lead SNPs on Chromosome 7

Position (bp)*	SNP	Alleles†	MAF‡	Serum Paraoxonase Activity, nmoles·min ⁻¹ ·mL ⁻¹				Serum Arylesterase Activity, μmoles·min ⁻¹ ·mL ⁻¹			
				0	1	2	PValue‡	0	1	2	PValue‡
94 774 065	rs2269829	A/G	0.28	389±269 (n=1078)	934±361 (n=905)	1434±492 (n=153)	3.27×10 ⁻²⁸⁸	105±25 (n=1106)	100±24 (n=930)	93±20 (n=157)	4.22×10 ⁻¹¹
94 775 382	rs662 (Q192R)	A/G	0.29	382±261 (n=1062)	930±358 (n=914)	1424±497 (n=160)	3.31×10 ⁻²⁹⁵	105±25 (n=1088)	100±24 (n=940)	94±21 (n=165)	9.43×10 ⁻¹¹
94 776 193	rs2057681	A/G	0.29	377±253 (n=1055)	929±353 (n=919)	1433±501 (n=162)	1.18×10 ⁻³⁰³	105±25 (n=1080)	100±24 (n=946)	94±21 (n=167)	2.11×10 ⁻¹⁰
94 784 020	rs854560 (L55M)	A/T	0.36	906±489 (n=877)	620±398 (n=1020)	311±280 (n=269)	1.27×10 ⁻¹⁴⁰	109±23 (n=868)	99±24 (n=1053)	90±22 (n=272)	2.03×10 ⁻³⁸
94 790 628	rs854570	A/C	0.36	670±470 (n=898)	711±469 (n=987)	721±455 (n=251)	2.90×10 ⁻⁰⁹	91±21 (n=920)	107±23 (n=1014)	122±21 (n=259)	5.10×10 ⁻¹⁰⁶
94 792 632	rs854572	G/C	0.46	572±407 (n=645)	716±474 (n=1075)	831±494 (n=416)	1.23×10 ⁻³⁵	88±21 (n=659)	104±22 (n=1104)	119±22 (n=430)	4.99×10 ⁻¹¹⁶
94 793 157	rs705382	G/C	0.36	670±470 (n=897)	710±469 (n=989)	723±455 (n=250)	2.92×10 ⁻⁰⁹	91±21 (n=919)	107±23 (n=1016)	122±21 (n=258)	1.98×10 ⁻¹⁰⁶
94 793 464	rs757158	C/T	0.42	569±403 (n=725)	731±478 (n=1053)	843±501 (n=358)	3.97×10 ⁻³⁸	89±22 (n=741)	105±22 (n=1084)	120±22 (n=368)	1.04×10 ⁻¹⁰⁴

SNPs indicate single nucleotide polymorphisms; MAF, minor allele frequency.

Data are shown as mean±SD as a function of carrying 0, 1, or 2 copies of the minor alleles for lead genome-wide association study SNPs.

*Base pair positions on chromosome 7 are given according to National Center for Biotechnology Information build 36.1 of the reference human genome sequence.

†Major/minor alleles are given for Caucasians based on the forward (+) DNA strand.

‡P values are obtained from multiple linear regression using inverse normal-transformed values for paraoxonase activity and untransformed values for arylesterase activity, adjusted for age and sex.

GWAS analyses conditioned on the lead SNPs, which did not identify other genomic regions associated with either paraoxonase or arylesterase activity. Furthermore, the lead SNPs for either enzymatic activity localize to the PON-1 gene, are distinct from each other, and are not in LD with SNPs in the neighboring PON-3 and PON-2 genes. However, although the PON-1 variants exhibited strong (if not opposed) effects on PON function, they were not associated with future risk of MACE, either individually or as a haplotype that modestly decreased both paraoxonase and arylesterase activity. These results are consistent with our analyses from the CARDIoGRAM consortium, which did not reveal an

association of these SNPs with either prevalent CAD or history of MI in ≈80 000 subjects. It should be noted, however, that because the lead SNPs only contributed to an estimated 15% of the PON-1 activity variation, other processes, such as posttranslational modifications, could also play a role in determining the ultimate functionality of PON-1. For example, PON-1 is known to be sensitive to posttranslational oxidative modification and inactivation.¹⁴ It is also possible that our study was underpowered to detect genetic effects on prospective risk of MACE because only 311 subjects out of the ≈2700 subjects included in these analyses experienced a MACE over 3 years of follow-up.

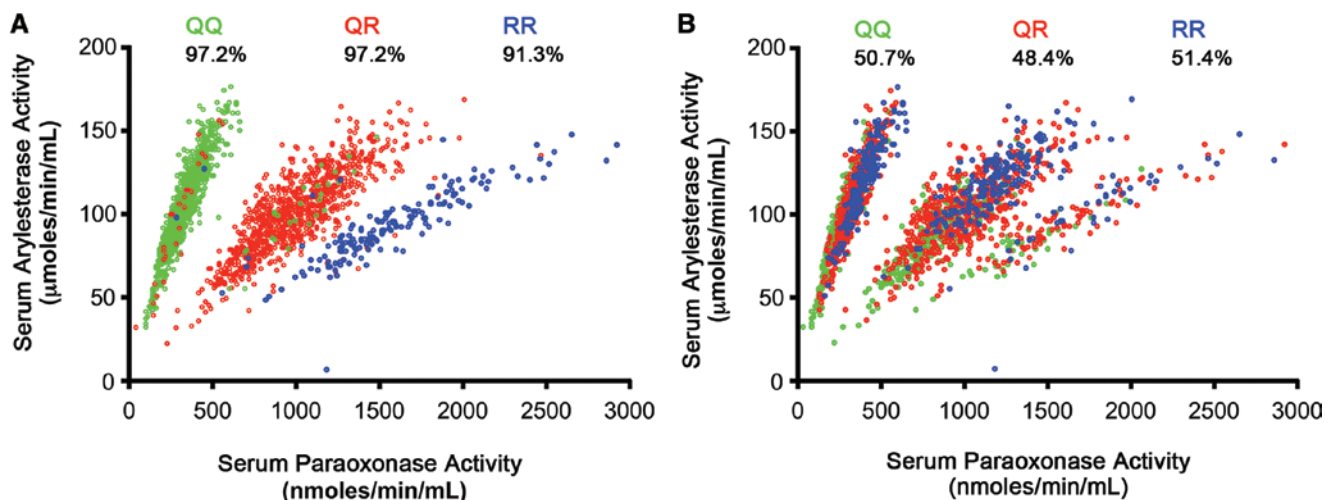


Figure 5. Relationship between serum arylesterase and paraoxonase activity levels stratified according to (A) the lead single nucleotide polymorphism (SNP) for serum paraoxonase activity (rs662; Q192R); (B) the lead SNP for serum arylesterase activity (rs854572; G>C). Percentages reflect separation of the 3 genotypes within each stratified cluster of each genotype.

Table 4. Association of Variants Controlling Plasma Paraoxonase or Arylesterase Activity With MACE

SNP/Haplotype	HR (95% CI)			P Value
	0	1	2	
Rs2057681	1 (n=1341)	1.18 (0.94–1.49) (n=1155)	0.94 (0.60–1.49) (n=207)	0.48
Rs854572	1 (n=794)	1.18 (0.91–1.54) (n=1358)	1.12 (0.81–1.56) (n=551)	0.44
ATAG	1 (n=1212)	0.96 (0.68–1.35) (n=1236)	0.91 (0.50–1.63) (n=255)	0.72

MACE indicates major adverse cardiovascular events; SNPs indicate single nucleotide polymorphisms; HR, hazard ratio.

HRs are shown as a function of carrying 0, 1, or 2 copies of the minor allele for the lead SNP for paraoxonase (rs2057681) and arylesterase (rs854572) activity or the ATAG haplotype of rs662, rs854560, rs854570, and rs854572. HR are adjusted for age, sex, Framingham ATP-III risk score, and medication use (aspirin and statins). 2703 subjects were used in these analyses, of which 311 experienced a MACE (death, MI, or stroke) over 3 y of follow-up.

The lack of associations between the lead SNPs for PON and arylesterase activities with CAD and MI risks in humans in our study are in direct contrast to what has been observed in mice where PON-1 deficiency leads to increased aortic lesion formation³ and transgenic mice over expressing PON-1 are protected.^{15,16} It is of interest that the PON-1 transgenic mouse models demonstrating protective effects have 50% to 400% increased serum arylesterase activity,^{15,16} far in excess of the modest changes in activity associated with the peak SNP identified in the arylesterase GWAS. If arylesterase activity is the more important atheroprotective aspect of PON-1 function, as our clinical associations with MACE suggest, then it may not be surprising that an association was not observed with the SNPs identified. For example, the lead SNP for arylesterase activity (rs854572) only increases activity by $\approx 16\%$ per minor allele copy. It is, thus, possible that the genetic effects on arylesterase activity from this SNP are too weak to observe, especially if a minimum biological threshold of activity change is needed to influence risk of prevalent CAD, history of MI, or incident risk of MACE.

The ability for serum arylesterase activity to identify a high-risk population in the primary prevention cohort that just underwent cardiac catheterization and showed no

significant evidence of stenoses in any major vessel may have important clinical implications. Even though our subset analysis was limited by the relatively smaller sample size and low event rates, a significant 4-fold increase risk in long-term MACE in these otherwise low risk subjects was identified. Use of serum arylesterase activity may thus have clinical use and help identify an important patient cohort that may warrant more aggressive risk factor reduction treatment strategies who might otherwise not be targeted for aggressive preventive intervention. In addition to low serum arylesterase activity predicting increased risk for MACE in primary prevention subjects, there was a direct association also noted between lower serum arylesterase activity level and increased prevalence of significantly obstructive CAD by angiography. Of note, we did not find a strong relationship between systemic inflammatory biomarkers, such as CRP or leukocyte parameters, with serum arylesterase levels in this group of stable cardiac patients. This observation is consistent with previous reports that distinguished systemic inflammatory from oxidative stress processes.

Several limitations of our study should be noted. Serum paraoxonase and arylesterase activities used substrates that are not the endogenous substrates for PON-1, but are used because they are not readily influenced by other esterases/lactonases in serum, and are presumed to reflect the underlying catalytic activity of PON-1. The GWAS results observed demonstrate these assumptions are reasonable, as only genetic variations in the PON-1 gene were observed to be associated with variations in paraoxonase and arylesterase activities. A further potential limitation is that the measurements were only made under fasting conditions at a single time point. Hence, we are unable to determine the variability and prognostic value of level changes over time and the impact of dietary or therapeutic interventions on serum arylesterase activity level. Selection bias may also be present for those undergoing cardiac catheterization for symptomatic evaluation and management of cardiac diseases at a tertiary care setting, but the large sample size and event rates of the patient population, together with careful phenotypic evaluation, including angiographic data, provides unique insights and adequate power to adjust for clinical and biomarker variables.

Table 5. Association of Identified SNPs With Risk of Prevalent CAD and Risk of MI in the CARDIoGRAM Consortium

SNP	Allele	Frequency	Risk of CAD			Risk of MI		
			OR (95% CI)	P Value	n	OR (95% CI)	P Value	N
rs2269829	G	0.28	0.99 (0.97–1.03)	0.74	83 324	0.97 (0.94–1.01)	0.17	52 973
rs662 (Q192R)	G	0.30	0.99 (0.96–1.02)	0.60	79 262	0.98 (0.94–1.02)	0.24	52 306
rs2057681	G	0.28	0.99 (0.96–1.02)	0.44	84 106	0.97 (0.93–1.00)	0.09	53 649
rs854560 (L55M)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
rs854570	C	0.41	1.02 (0.99–1.05)	0.14	81 019	1.01 (0.98–1.05)	0.46	51 143
rs854572	C	0.52	1.01 (0.99–1.04)	0.33	83 486	1.00 (0.97–1.04)	0.74	53 204
rs705382	C	0.41	1.02 (1.0–1.05)	0.09	83 367	1.02(0.98–1.05)	0.40	53 035
rs757158	C	0.54	0.99 (0.96–1.02)	0.50	78 275	0.99 (0.96–1.03)	0.71	51 559

SNP indicates single nucleotide polymorphism; CAD, coronary artery disease; MI, myocardial infarction; OR, odds ratio; N/A, not available; CARDIoGRAM, Coronary Artery Disease Genome-Wide Replication And Meta-Analysis.

Conclusions

Diminished serum arylesterase activity can provide incremental prognostic value and clinical reclassification of stable patients at risk of developing MACE, even among primary prevention subjects who just demonstrated no significant coronary stenoses by angiography and might otherwise be dismissed as low risk. Despite the strong genetic effects of the PON locus on serum paraoxonase and arylesterase activities, the identified variants were not associated with risk of incident MACE or prevalent CAD.

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Disclosures

Dr Tang has received research grant support from Abbott Laboratories, Inc. Dr Hazen reports being listed as coinventor on pending and issued patents held by the Cleveland Clinic relating to cardiovascular diagnostics and therapeutics. Dr Hazen reports having been paid as a consultant or speaker for the following companies: Abbott Diagnostics, Cleveland Heart Lab, Esperion, Lilly, Liposcience Inc, Merck & Co, Inc, and Pfizer Inc. Dr Hazen reports receiving research funds from Abbott, Cleveland Heart Lab, Liposcience Inc, and Pfizer Inc. Dr Hazen reports having the right to receive royalty payments for inventions or discoveries related to cardiovascular diagnostics or therapeutics from the companies shown below: Abbott Laboratories, Inc, Cleveland Heart Lab, Esperion, Frantz Biomarkers, LLC, Liposcience Inc, and Siemens. The other authors have no conflicts to report. There are no medical writers or editors involved in the preparation of the manuscript.

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Supplemental Table I Association of Lead SNPs on Chromosome 7 with Adjustment for BMI and Statin Use.

SNP	Serum Paraoxonase Activity				Serum Arylesterase Activity			
	p-value ^a	p-value ^b	p-value ^c	p-value ^d	p-value ^a	p-value ^b	p-value ^c	p-value ^d
rs2269829	3.27x10 ⁻²⁸⁸	4.02x10 ⁻²⁸⁸	2.83 x10 ⁻²⁸⁸	3.49 x10 ⁻²⁸⁸	4.22x10 ⁻¹¹	3.54x10 ⁻¹¹	3.96x10 ⁻¹¹	3.33x10 ⁻¹¹
rs662 (Q192R)	3.31x10 ⁻²⁹⁵	4.36 x10 ⁻²⁹⁵	2.43 x10 ⁻²⁹⁵	3.20 x10 ⁻²⁹⁵	9.43x10 ⁻¹¹	8.19x10 ⁻¹¹	8.63 x10 ⁻¹¹	7.50 x10 ⁻¹¹
rs2057681	1.18x10 ⁻³⁰³	1.56 x10 ⁻³⁰³	9.92 x10 ⁻³⁰⁴	1.32 x10 ⁻³⁰³	2.11x10 ⁻¹⁰	1.83 x10 ⁻¹⁰	1.98 x10 ⁻¹⁰	1.71 x10 ⁻¹⁰
rs854560 (L55M)	1.27x10 ⁻¹⁴⁰	1.22 x10 ⁻¹⁴⁰	1.31 x10 ⁻¹⁴⁰	1.26 x10 ⁻¹⁴⁰	2.03x10 ⁻³⁸	2.48 x10 ⁻³⁸	2.14 x10 ⁻³⁸	2.61 x10 ⁻³⁸
rs854570	2.90x10 ⁻⁰⁹	2.87 x10 ⁻⁰⁹	3.00 x10 ⁻⁰⁹	2.97 x10 ⁻⁰⁹	5.10x10 ⁻¹⁰⁶	4.98 x10 ⁻¹⁰⁶	3.12 x10 ⁻¹⁰⁶	3.07 x10 ⁻¹⁰⁶
rs854572	1.23x10 ⁻³⁵	1.18 x10 ⁻³⁵	1.32 x10 ⁻³⁵	1.27 x10 ⁻³⁵	4.99x10 ⁻¹¹⁶	7.21 x10 ⁻¹¹⁶	3.73 x10 ⁻¹¹⁶	5.42 x10 ⁻¹¹⁶
rs705382	2.92x10 ⁻⁰⁹	2.90 x10 ⁻⁰⁹	3.03 x10 ⁻⁰⁹	3.00 x10 ⁻⁰⁹	1.98x10 ⁻¹⁰⁶	1.92 x10 ⁻¹⁰⁶	1.15 x10 ⁻¹⁰⁶	1.12 x10 ⁻¹⁰⁶
rs757158	3.97x10 ⁻³⁸	3.87 x10 ⁻³⁸	4.28 x10 ⁻³⁸	4.17 x10 ⁻³⁸	1.04x10 ⁻¹⁰⁴	1.28 x10 ⁻¹⁰⁴	8.10 x10 ⁻¹⁰⁵	1.00 x10 ⁻¹⁰⁴

^ap-values obtained with adjustment for age and gender.

^bp-values obtained with adjustment for age, gender, and BMI.

^cp-values obtained with adjustment for age gender, and statin use.

^dp-values obtained with adjustment for age, gender, BMI, and statin use.

Supplemental Table II Association of Identified SNPs with CAD Biomarkers.

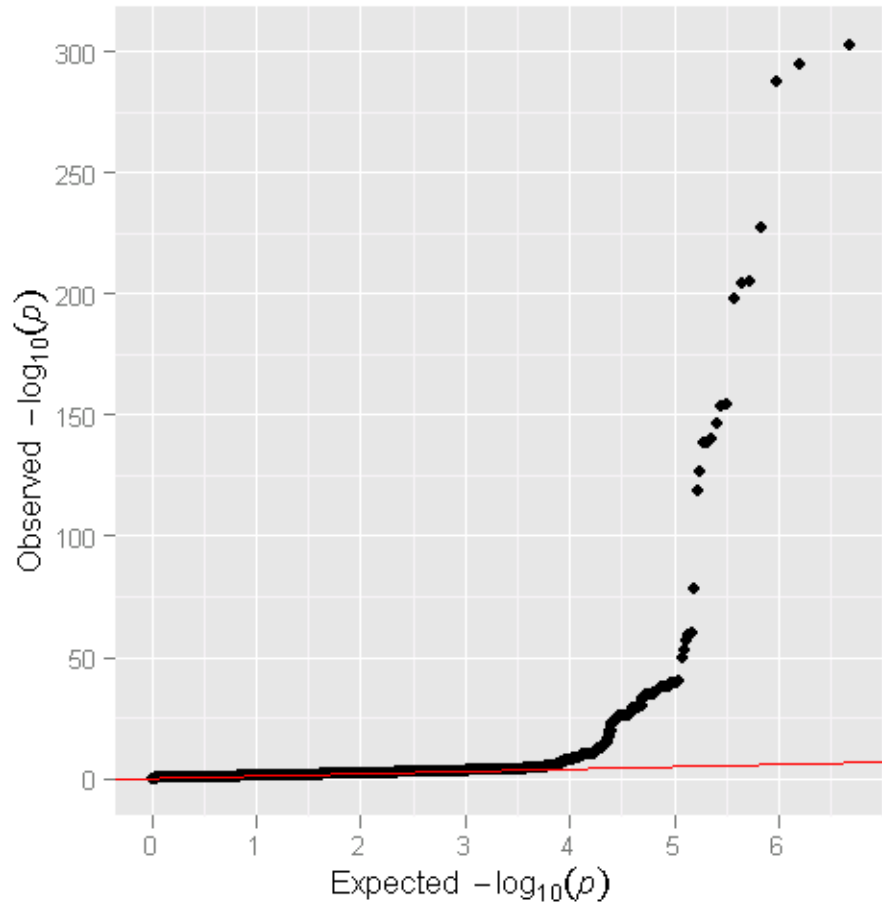
SNP	Total Cholesterol	LDL cholesterol	HDL cholesterol	^bC-reactive Protein	Creatinine clearance
rs2269829	0.12	0.12	0.77	0.29	0.19
rs662 (Q192R)	0.13	0.11	0.76	0.31	0.28
rs2057681	0.13	0.12	0.92	0.35	0.30
rs854560 (L55M)	0.76	0.54	0.40	0.49	0.15
rs854570	0.58	0.17	0.57	0.31	0.87
rs854572	0.83	0.33	0.52	0.70	0.82
rs705382	0.57	0.17	0.55	0.32	0.86
rs757158	0.94	0.43	0.78	0.68	0.88

^ap-values are shown for association of lead SNPs on chromosome 7 with CAD biomarkers, with adjustment for age and gender.

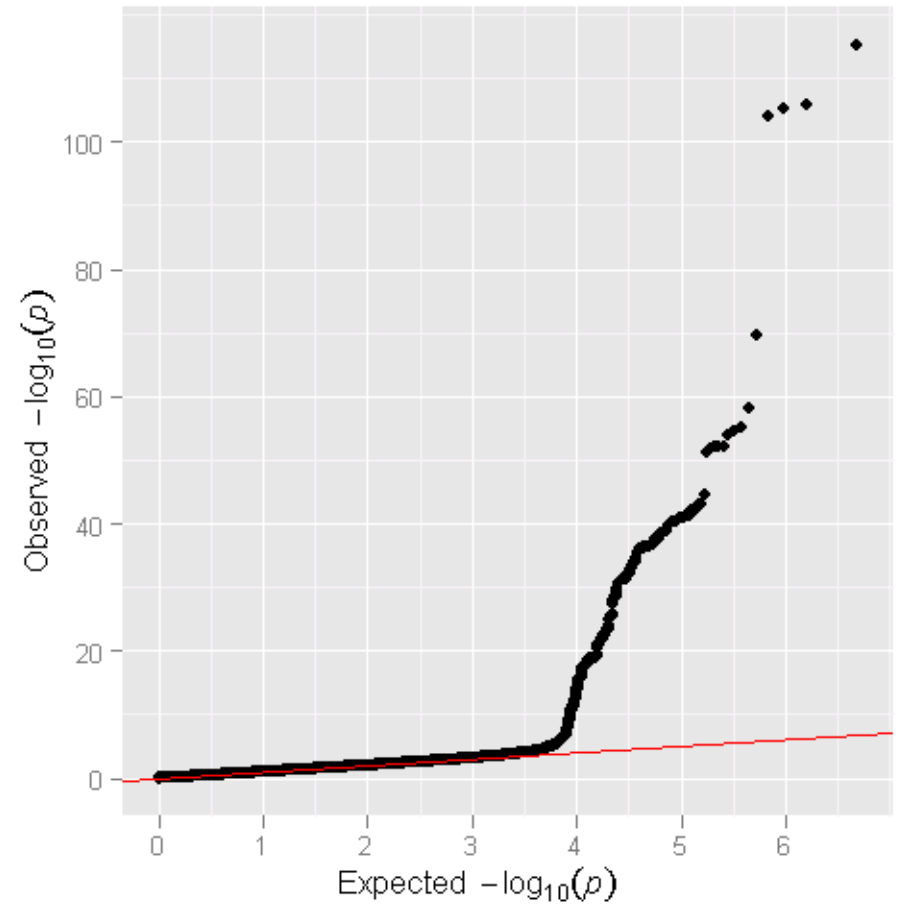
^blog-transformed prior to analysis.

Supplemental Figure I Q-Q plots for GWAS of paraoxonase and arylesterase activity.

Paraoxonase Activity



Arylesterase Activity



Supplement: The CARDIoGRAM Consortium

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