TagSNP analyses of the PON gene cluster: effects on PON1 activity, LDL oxidative susceptibility, and vascular disease

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Abstract **Paraoxonase 1 (PON1) activity is consistently predictive of vascular disease, although the genotype at four functional *PON1* polymorphisms is not. To address this inconsistency, we investigated the role of all common *PON1* genetic variability, as measured by tagging single-nucleotide polymorphisms (tagSNPs), in predicting PON1 activity for phenylacetate hydrolysis, LDL susceptibility to oxidation ex vivo, plasma homocysteine (Hcy) levels, and carotid artery disease (CAAD) status. The biological goal was to establish whether additional common genetic variation beyond consideration of the four known functional SNPs improves prediction of these phenotypes. *PON2* and *PON3* tagSNPs were secondarily evaluated. Expanded analysis of an additional 26 tagSNPs found evidence of previously undescribed common *PON1* polymorphisms that affect PON1 activity independently of the four known functional SNPs. PON1 activity was not significantly correlated with LDL oxidative susceptibility, but genotypes at the *PON1*$_{-108}$ promoter polymorphism and several other *PON1* SNPs were. Neither PON1 activity nor *PON1* genotype was significantly correlated with plasma Hcy levels. This study revealed previously undetected common functional *PON1* polymorphisms that explain 4% of PON1 activity and a high rate of recombination in *PON1*, but the sum of the common *PON1* locus variation does not explain the relationship between PON1 activity and CAAD.**—Carlson, C. S., P. J. Heagerty, T. S. Hatsukami, R. J. Richter, J. Ranchalis, J. Lewis, T. J. Bacus, L. A. McKinstry, G. D. Schellenberg, M. Rieder, D. Nickerson, C. E. Furlong, A. Chait, and G. P. Jarvik. **TagSNP analyses of the PON gene cluster: effects on PON1 activity, LDL oxidative susceptibility, and vascular disease.** ***J. Lipid Res.*** **2006.** 47: **1014–1024.**

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Paraoxonase 1 (PON1) is an HDL-associated enzyme whose activity has consistently been associated with vascular disease (1–5). A role of PON1 in vascular disease is strongly supported by knockout and transgenic mouse studies (6, 7). The inhibition of LDL oxidation by HDL, due to metabolism of bioactive lipid hydroperoxidases, appears to be partially attributable to PON1 (8–11). Paraoxonase has been reported to reduce mildly oxidized phospholipids by eliminating these hydroperoxy derivatives of unsaturated fatty acids (9). Watson et al. (10) reported that inactivation of PON1 reduces the ability of HDL to inhibit LDL modification and also reduces the ability of HDL to inhibit monocyte-endothelial interactions, both of which may be important in the inflammatory response in artery wall cells that promotes atherogenesis. Inactivation of PON1 by oxidized LDL can be inhibited by antioxidants (12). PON1 inhibition of HDL oxidation preserves the reverse cholesterol function of HDL (13).

PON1 has four known common functional polymorphisms, two that change amino acids (*PON1*$_{Q192R}$, *PON1*$_{M55L}$), and two that alter promoter activity (*PON1*$_{-108C/T}$ and *PON1*$_{-162A/G}$). Studies of the role of the coding polymorphisms in vascular disease have been contradictory (14–25). We have consistently found that the *PON1*$_{Q192R}$, *PON1*$_{M55L}$, *PON1*$_{-108C/T}$, and *PON1*$_{-162A/G}$ genotypes fail to predict carotid artery disease (CAAD) in modest sample sizes, when PON1 activities are predictive of CAAD (2, 3). Meta-analyses suggest that the *PON1*$_{Q192R}$ and *PON1*$_{M55L}$ (and in one study, *PON1*$_{-108C/T}$) genotypes do not predict cardiovascular disease (CVD) (26–28). Interestingly, a study of severity

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Abbreviations: AIC, Akaike's Information Criterion; CAAD, carotid artery disease; CVD, cardiovascular disease; Hcy, homocysteine; PON, paraoxonase; tagSNP, tagging single-nucleotide polymorphism.

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of CVD did show a $PON1_{Q192R}$ effect (29), and the studies for prediction of stroke or CAAD (22, 30–34) have been more consistently positive for a *PON1* locus effect, although negatives occur (35) and the studies tend to be small. Of note, the polymorphisms previously studied represent only a small fraction of *PON1* genetic variation: reported studies have examined only a handful of the more than 150 known *PON1* region polymorphisms.

The three paraoxonase gene family members are clustered in a segment of 140 kilobase pairs on chromosome 7. The order of the three genes in the cluster is *PON1*, *PON3*, and *PON2*, with *PON1* the most centromeric (5′). PON3 has a tissue distribution similar to that of PON1 (36), but lower expression levels, whereas PON2 is more ubiquitously expressed (37). Both PON2 and PON3 have antioxidant activity (38). The $PON2_{S311C}$ coding SNP has been implicated in CVD (29, 37, 39), particularly in smokers (40). Only PON2 is expressed in human macrophages (38), where it is induced by oxidized LDL (41). However, PON1 appears to mediate macrophage cholesterol efflux (42). Given these data for PON2 and the cooccurrence of PON3 with PON1 on HDL, it is possible that PON2 and PON3 may be important in vascular disease.

PON1 is identical to the enzyme homocysteine (Hcy)-thiolactone hydrolase (43). PON1 has been suggested to protect against the atherosclerotic effects of Hcy-thiolactone (44). The reported rates of Hcy-thiolactone conversion by PON1 are very slow (reported as per hour vs. per minute for phenylacetate); thus, the PON1 metabolism of Hcy-thiolactone may not be physiologically significant. The Framingham study found that Hcy level predicted cerebrovascular, cardiovascular, and all-cause death (45, 46). It has been noted that although most cross-sectional studies support a relationship between moderate Hcy elevation and cardiovascular disease, the prospective studies are less convincing (47). However, like PON1, Hcy has more consistently been associated with cerebrovascular than with CVD (48–51). If PON1 is associated with Hcy level, this may be another possible mechanism of PON1 effects in CAAD.

Known *PON1* polymorphisms do not account for all of the variability in PON1 protein level or activity. It is possible that there are other functional PON1 SNPs that could play a role in prediction of CAAD status. Use of sequencing for detection of all common polymorphisms has found previously undetected functional variability in the coding and noncoding regions of the *APOE* gene that predicts both APOE protein level (52) and lipid effects (53). This approach can detect most of the genetic variance in a trait that is determined at the structural locus (54). Therefore, we used sequencing and tagSNP selection to look for additional functional variability at the *PON1/2/3* cluster.

The goals of this study were 3-fold. First, it has been proposed that the disconnect between PON1 activity, but not genotype, and prediction of vascular disease might be explained by unknown common functional variation in the *PON1/2/3* cluster that impacts disease risk. We addressed this by extending the study of the *PON1* genotype to all common variation in the *PON1/2/3* cluster using a tagging single-nucleotide polymorphism (tagSNP) approach and evaluating the prediction of PON1 activity and CAAD status. Second, we compared phenotype prediction considering tagSNPs versus haplotypes to determine which method best explained the variance. Third, we explored possible mechanisms of PON1 effects in vascular disease by evaluating PON1 activity and all common *PON1/2/3* cluster variance on LDL susceptibility to oxidation ex vivo and Hcy. We have previously shown that LDL susceptibility to oxidation is predictive of CAAD (55).

METHODS

Sample

The sample population included 500 Caucasian males from the previously described, ongoing CLEAR study (3, 55). Briefly, subjects were drawn from the tails of the carotid artery disease distribution. Cases (n = 205) had >80% stenosis of one or both internal carotid arteries, and controls (n = 232) had <15% stenosis bilaterally on duplex ultrasound. Additionally, controls had no known atherosclerotic vascular disease and were age-distribution-matched with the cases based on the age of onset of disease (censored age). The remaining subjects (N = 63) had intermediate internal carotid stenosis levels, between 50% and 79% unilaterally or bilaterally by ultrasound. Because of the substantial differences in allele frequency observed at *PON1* between ethnicities and differences in genotype effects by gender, the study was limited to Caucasian males to avoid population stratification artifacts. Other exclusion criteria included autosomal dominant familial hypercholesterolemia or coagulopathy. Current smoking status was obtained by survey. Use of statin medications was ascertained by report and reconciled with review of pharmacy and medical records. Self-reported race was confirmed by STRUCTURE analyses with three ancestral groups (56), with excellent concordance. Height and weight were measured, with self-report used to complete missing data. The study was approved by both the University of Washington and the Veterans Affairs Puget Sound Health Care System human subject review processes. Subjects gave written informed consent.

Cases had a mean current age of 70.0 years (range 46–89 years), controls had a mean current age of 66.3 years (range 37–83 years), and the 50–79% stenosis subjects had a mean current age of 70.5 years (range 50–85 years). The rate of current smoking differed by group: 37% in cases, 14% in controls, and 26% in 50–79% stenosis subjects.

Illumina system tagSNP genotyping

TagSNPs were genotyped by Illumina on the Illumina BeadStation Laboratory System platform. Illumina technology provides a robust and accurate genotyping platform using highly multiplexed ligation assays with multiple levels of specificity to obtain optimal results (57, 58). Genotypes were called using the Illumina GenCall software package that normalizes and clusters the raw scan data. Other collaborative studies performed in the Nickerson laboratory using this system have resulted in 1,152 SNPs assayed in approximately 1,200 DNA samples with a genotyping call frequency of 99% and a reproducibility of >99%. Quality control on two *PON1* SNPs typed by our lab and also by Illumina detected no Illumina errors in 1,204 genotypes. This low error rate is particularly important when looking for low-risk alleles in case-control data.

TagSNPs were selected for *PON1* and *PON2* from complete resequencing data from the SeattleSNPs Program for Genomic Applications (pga.gs.washington.edu), using the LDselect program. LDselect was run independently in the African American Descent (AD) and European American Descent (ED) SeattleSNPs populations. LDselect parameter thresholds of $r^2 > 0.64$ and minor allele frequencies (MAFs) greater than 5% were used for tagSNP selection in the ED population; thresholds of $r^2 > 0.64$ and MAF > 10% were used in the AD population. Because resequencing data were not yet available from the *PON3* locus, a set of eight SNPs evenly distributed across the locus were selected from dbSNP for genotyping. Resequencing data are now available for *PON3*, and at an r^2 threshold of 0.64, the eight genotyped SNPs from *PON3* tag 52 out of 57 SNPs with MAF > 5% in the ED population. Genotype was scored on all available subjects for 53 SNPs from the PON gene cluster (**Table 1**), with 4 functional and 26 tagSNPs in PON1, 15 tagSNPs in PON2, and 8 tagSNPs in PON3. Forty-six "common" tagSNPs were observed with MAFs greater than 5% in the studied population.

TABLE 1. TagSNP identification, translation, allele frequencies, and Hardy-Weinberg Equilibrium (HWE) test

Hg17 Chr7 Coordinate	DbSNP Rs Number	SeattleSNPs[a]	Functional Alias	Allele 1	SeattleSNPs Europe Frequency	Our Frequency	Allele 2	SeattleSNPs Europe Frequency	Our Frequency	HWE χ^2 *P*
94571235	rs854549	*PON1*$_{29021}$		G	0.61	0.655	T	0.39	0.345	0.773
94572145	rs3735590	*PON1*$_{28107}$		C	0.98	0.933	T	0.02	0.067	0.500
94572327	rs854551	*PON1*$_{27925}$		C	0.89	0.799	T	0.11	0.201	0.573
94572574	rs854552	*PON1*$_{27678}$		A	0.87	0.728	G	0.13	0.272	0.218
94575041	rs854555	*PON1*$_{25209}$		G	0.84	0.651	T	0.16	0.349	0.010
94576362	rs3917564	*PON1*$_{23887}$		T	0.98	0.992	C	0.02	0.008	1.000
94577554	rs3917558	*PON1*$_{22695}$		A	0.96	0.935	G	0.04	0.065	0.504
94579105	rs3917551	*PON1*$_{21144}$		C	0.98	0.943	T	0.02	0.057	0.245
94580779	rs2269829	*PON1*$_{19470}$		T	0.85	0.718	C	0.15	0.282	0.012
94581342	rs3917542	*PON1*$_{18906}$		G	0.87	0.774	A	0.13	0.226	0.007
94582096	rs662	*PON1*$_{18152}$	*Q192R*	Q	0.83	0.707	R	0.17	0.293	0.004
94583436	rs2299255	*PON1*$_{16810}$		A	0.91	0.854	G	0.09	0.146	0.049
94587770	rs3917510	*PON1*$_{12471}$		A	0.91	0.958	C	0.09	0.042	0.620
94590309	rs2301711	*PON1*$_{9932}$		A	0.98	0.94	G	0.02	0.06	0.242
94590734	rs854560	*PON1*$_{9507}$	*M55L*	L	0.5	0.635	M	0.5	0.365	0.493
94592449	rs2074351	*PON1*$_{7792}$		C	0.72	0.706	T	0.28	0.294	0.031
94593399	rs854566	*PON1*$_{6842}$		C	0.87	0.813	T	0.13	0.187	0.887
94593491	rs3917490	*PON1*$_{6750}$		G	0.48	0.511	A	0.52	0.489	0.180
94593979	rs2049649	*PON1*$_{6262}$		T	0.72	0.688	C	0.28	0.312	0.675
94594187	rs2299260	*PON1*$_{6054}$		A	0.91	0.832	G	0.09	0.168	0.428
94594313	rs2299261	*PON1*$_{5928}$		T	0.63	0.647	C	0.37	0.353	0.116
94594578	rs2299262	*PON1*$_{5663}$		G	0.57	0.602	A	0.43	0.398	0.455
94594705	rs854569	*PON1*$_{5536}$		C	0.83	0.787	A	0.17	0.213	0.695
94595415	rs3917481	*PON1*$_{4826}$		G	0.95	0.986	A	0.05	0.014	1.000
94595487	rs2237584	*PON1*$_{4754}$		G	0.89	0.94	A	0.11	0.06	0.095
94596616	rs3917477	*PON1*$_{3625}$		T	1	0.969	C	0	0.031	0.011
94598545	rs705379	*PON1*$_{1696}$	*−108*	C	0.41	0.502	T	0.59	0.498	0.930
94598599	rs705381	*PON1*$_{1642}$	*−162*	G	0.82	0.754	A	0.18	0.246	0.401
94599269	rs854571	*PON1*$_{972}$		G	0.78	0.715	A	0.22	0.285	0.450
94599346	rs854572	*PON1*$_{895}$		C	0.68	0.534	G	0.32	0.466	0.587
94634323	rs17881344	*PON3*$_{42471}$		G	0.7	0.807	A	0.3	0.193	0.030
94637558	rs17878844	*PON3*$_{39236}$		G	0.5	0.542	A	0.5	0.458	0.206
94641563	rs17883057	*PON3*$_{35231}$		A	0.48	0.53	C	0.52	0.47	0.242
94646008	rs17883039	*PON3*$_{30785}$		A	0.96	0.943	T	0.04	0.057	0.251
94663443	rs17881737	*PON3*$_{13350}$		G	0.98	0.996	A	0.02	0.004	0.008
94666453	rs17879428	*PON3*$_{10340}$		A	0.83	0.735	C	0.17	0.265	0.109
94666548	rs17883873	*PON3*$_{10245}$		A	0.52	0.542	G	0.48	0.458	0.204
94670810	rs2072200	*PON3*$_{5982}$		G		0.804	C		0.196	0.046
94679425	rs17876171	*PON2*$_{30199}$	*C311S*	C	0.83	0.741	G	0.17	0.259	0.204
94681642	rs17876159	*PON2*$_{27982}$		G	0.46	0.524	A	0.54	0.476	0.587
94685666	rs17876142	*PON2*$_{23956}$	*A148G*	C	0.8	0.741	G	0.2	0.259	0.205
94692127	rs17876116	*PON2*$_{17484}$		G	0.88	0.96	T	0.12	0.04	0.619
94692480	rs17876115	*PON2*$_{17131}$		G	0.61	0.593	A	0.39	0.407	0.198
94698907	rs17876087	*PON2*$_{10704}$		T	0.93	0.882	A	0.07	0.118	0.832
94699627	rs17876082	*PON2*$_{9984}$		C	0.67	0.791	T	0.33	0.209	0.011
94700098	rs17876189	*PON2*$_{9513}$		A	0.93	0.986	G	0.07	0.014	1.000
94701589	rs17876075	*PON2*$_{8018}$		A	0.7	0.831	G	0.3	0.169	0.113
94704892	rs17876067	*PON2*$_{4715}$		G	0.68	0.791	A	0.32	0.209	0.501
94704963	rs17876066	*PON2*$_{4644}$		T	0.8	0.676	C	0.2	0.324	0.188
94705978	rs10261470	*PON2*$_{3628}$		C	0.83	0.873	T	0.17	0.127	0.115
94707443	rs17876185	*PON2*$_{2155}$		G	0.8	0.91	A	0.2	0.09	0.608
94707920	rs17876056	*PON2*$_{1678}$		T	0.76	0.702	C	0.24	0.298	0.456
94708352	rs17876053	*PON2*$_{1246}$		A	0.83	0.872	C	0.17	0.128	0.159

PON, paraoxonase; TagSNP, tagging single-nucleotide polymorphism.

[a] SeattleSNPs numbering for PON1, PON2, and PON3 polymorphism is relative to GenBank records AF539592, AY210982, and AY805220, respectively.

PON1 functional genotypes

DNA was prepared from buffy coat preparations by a modification of the procedure of Miller, Dykes, and Polesky (59) using Puregene reagents (Gentra; Minneapolis, MN). The genotypes of the $PON1_{Q192R}$, $PON1_{L55M}$, $PON1_{-108C/T}$, and $PON1_{-162A/G}$ polymorphisms were determined as published (60–62). Genotyping personnel were blinded to case status. All genotype distributions were tested for departure from Hardy-Weinberg equilibrium proportions, and no significant departures were detected after correction for multiple tests (Table 1).

PON1 hydrolysis phenotypes

The activity of PON1 in the hydrolysis of paraoxon, diazoxon, and phenylacetate (arylesterase activity) was measured by a continuous spectrophotometric assay with lithium heparin plasma, as described elsewhere (62–65). These are termed POase, DZOase, and Arylase activities, respectively. PON1 Arylase activity showed the strongest predictive value for CAAD case control status in this cohort ($P < 0.001$; POase and DZOase, $P > 0.05$, considering square root (sqrt)-transformed activities), so we focused our efforts on predicting Arylase activity, which allows comparison across $PON1_{Q192R}$ genotypes. Arylesterase activity is a good reflection of the levels of PON1 present, because the catalytic efficiency of Arylase activity is not affected by the $PON1_{Q192R}$ polymorphism, allowing for comparison of PON1 levels across $PON1_{Q192R}$ genotypes (66), and Arylase activity predicts vascular disease.

Oxidative measures

LDL oxidation susceptibility was measured essentially as described by Esterbauer et al. (67), as modified by Crawford et al. (68), and as validated in the prediction of CAAD (55). LDL (ρ = 1.019–1.063 g/ml) was isolated from plasma by density gradient ultracentrifugation. The concentration of LDL was kept constant at 100 μg/ml based on protein, and oxidation was initiated by the addition of 1.66 μM freshly prepared copper sulfate solution to the LDL, to a final concentration of 5 μM, incubated at 37°C. The kinetics of LDL oxidation were determined by the change in the absorbance at 234 nm on a Beckman DU-70 spectrometer allowing measurement of six samples simultaneously. The susceptibility to oxidation is described by *1*) the lag time in minutes, defined as that interval between initiation and intercept of the tangent of the slope of the absorbance curve (lagtime); *2*) the maximal rate of oxidation (LDLrate), defined as the slope of the absorbance curve during the propagation phase; and *3*) the maximal change in oxidation (LDLmaxox), also determined with this assay by measuring the change of absorbance over time. These measures were available on a subset of 387 subjects. LDLrate is correlated with both lagtime and LDLmaxox, but lagtime and LDLmaxox are uncorrelated (55).

Homocysteine

Fasting plasma Hcy was measured after all forms were reduced with sodium borohydride and tris-(2-carboxyethyl) phosphine. After addition of the internal standard (cysteamine), the thiol group of homocysteine and the other thiol compounds present in the sample were derivatized with monobromobimane. The excess monobromobimane was removed by glacial acetic acid extraction. Derivatized Hcy was quantified by high-pressure liquid chromatography developed by an acetonitrile gradient. Derivatized Hcy was detected by fluorescence.

Analysis

Missing genotype data were inferred for each PON locus independently using PHASE v2.0 software (69). Regression analysis was performed in the R statistical environment (Raqua version 2.1.1, Mac OSX 10.4.2) using either the standard regression tools available within R or the haplo.glm tools from the haplo.glm package (70). Models incorporating haplotype were built using the haplo.glm package, which infers a matrix of haplotype probabilities for each individual using the expectation maximization algorithm and which estimates regression coefficients corresponding to each haplotype. To evaluate the candidate SNPs, model comparison (criticism) was performed using Akaike's Information Criterion (AIC) to evaluate the fit of each model to the observed data, starting from a base model, with age, smoking status, and genotype at the four previously described SNPs as covariates. AIC aims to identify models with good predictive characteristics, and is particularly useful in comparing models with different numbers of explanatory variables, because the criterion is based on the maximized log likelihood plus a penalty for the number of explanatory variables. Genotypes for SNPs were encoded as allele counts (0, 1, or 2) for genotype-based regressions. Statin drug use was considered as a covariate for prediction of Arylase and LDLmaxox. It was not considered as a predictor of CAAD status, due to confounding of subjects with CAAD being placed on statins at higher rates than subjects without CAAD with similar lipid profiles.

RESULTS

PON1 functional SNP analysis of Arylase prediction

Arylase activity showed an approximately normal distribution in the population, with an observed mean of 104.6 U/l and a standard deviation (SD) of 42.3. Previous work has identified four functional polymorphisms in the PON1 gene, $PON1_{Q192R}$, $PON1_{M55L}$, $PON1_{-108C/T}$, and $PON1_{-162A/G}$. A regression model incorporating these genotypes, smoking status, and age as predictors explained 25.9% more of the variance in Arylase activity than did a model with age and smoking but without genotype data (**Table 2**). Inclusion of statin drug use as a predictor did not significantly improve Arylase prediction. No interactions between case status and *PON1* genotypes were significant at a 0.1 level in the prediction of Arylase; examination of the coefficients for cases and controls separately (Table 2) showed a high degree of similarity. Alternatively, a smoking by case status interaction was significant in the prediction of Arylase ($P = 0.003$); cases had a higher rate of smoking than did controls. Of those who smoked, cases smoked 1.24 packs per day (SD 0.60) on average and controls 1.19 packs per day (SD 0.54).

Significant linkage disequilibrium (LD) exists between any pair of these four SNPs, but $|D'|$ is less than 1 for all pairwise comparisons, indicating the presence of all four possible haplotypes of the two SNPs. This suggests one of three possibilities: recombination in the region between each pair of SNPs, recurrent mutation at several of the SNPs, or gene conversion at several of the SNPs. Thirteen out of 16 possible haplotypes were inferred by PHASE v2.0 for these four SNPs in the data set, with eight haplotypes at greater than 2% frequency. In a nonrecombinant region, at most five haplotypes would be expected with four SNPs; consequently, we believe that the most likely explanation for the high haplotype diversity is high rates of recombi-

TABLE 2. *PON1* functional and tagSNP type models for PON1 Arylase activity prediction

Variable	Coefficient (All)	SE	*t*-Statistic[a]	*P*	Coefficient (Case-only)	Coefficient (Control-only)
Four functional *PON1* SNPs						
(Intercept)	190.8	13.9				
$PON1_{1642}A$ (−162)	8.6	3.3	2.587	0.010	11.4	10.9
$PON1_{1696}T$ (−108)	−19.7	2.9	−6.888	$<10^{-5}$	−17.6	−21.7
$PON1_{9507}A$ (55Met)	−9.9	3.0	−3.28	0.001	−14.8	−7.1
$PON1_{18152}G$ (192Arg)	−10.1	3.1	−3.243	0.001	−11.5	−12.3
Age	−0.8	0.2	−4.394	1.36×10^{-5}	−1.2	−0.3
Current smoker	−8.2	3.8	−2.127	0.034	−18.0	3.5
Best-fit model for PON1 functional and tagSNPs						
(Intercept)	175.3	16.0				
$PON1_{1642}A$ (−162)	10.1	3.7	2.733	0.007	12.3	11.3
$PON1_{1696}T$ (−108)	−9.2	4.7	−1.980	0.048	−10.0	−13.5
$PON1_{9507}A$ (55Met)	−21.6	5.0	−4.313	1.95×10^{-5}	−20.6	−23.8
$PON1_{18152}G$ (192Arg)	2.2	10.9	0.200	0.842	9.3	−39.4
Age	−0.8	0.2	−4.444	1.10×10^{-5}	−1.1	−0.3
Current smoker	−9.8	3.8	−2.594	0.010	−18.9	0.8
$PON1_{6842}T$	−10.6	4.3	−2.480	0.014	−9.0	−10.3
$PON1_{29021}T$	9.2	4.5	2.051	0.041	6.1	11.0
$PON1_{895}G$	13.0	4.9	2.677	0.008	12.4	9.4
$PON1_{12471}C$	16.6	6.9	2.424	0.016	−1.1	23.6
$PON1_{23887}C$	−39.0	18.1	−2.153	0.032	−34.2	−45.7
$PON1_{19470}C$	−16.5	10.8	−1.520	0.129	−24.4	23.1

[a] *t*-Statistics and *P* values were calculated from the coefficients (for all subjects) and standard errors within the best-fit multivariate model by the glm function in R. Case- and control-only coefficients are provided for comparison.

nation throughout the region. Because of the high haplotype diversity, using the four known functional SNPs in *PON1*, we built a series of regression models for Arylase activity prediction in order to explore the possibility that haplotype-based analysis might increase the percent variance explained for Arylase activity. We built a model incorporating haplotype (**Table 3**) using haplo.glm. The haplotype-based model explained a slightly higher proportion of the residual variance (30.2% vs. 25.9%), but this was expected, because the haplotype model, with twelve haplotype coefficients, had more parameters than did the genotype-based model, with four genotype coefficients. Comparison of AIC between the haplo.glm model and the genotype model showed a slightly better fit to the data for the haplo.glm model. This might reflect interactions between regulatory polymorphisms and coding polymorphisms within the same haplotype in this region of high haplotype diversity.

PON1 tagSNP analysis of Arylase prediction

To explore the possibility of unknown common functional PON1 polymorphisms, we genotyped an additional 26 tagSNPs within PON1. These were selected to comprehensively describe patterns of common variation within the gene (71). Thus, if additional common polymorphisms of functional import exist within *PON1*, then such variation would either be selected as a tagSNP or be in strong linkage disequilibrium with a tagSNP.

AIC was used to assess whether the additional polymorphisms within *PON1* provided a better fit to the Arylase activity data. Starting from a base model with age, smoking status, and genotype at the four known functional SNPs as predictor variables, we ran a forward model analysis allowing the additional 26 *PON1* tagSNPs to enter the model one at a time. Table 2 shows that the best-fit model incorporated an additional six SNPs into the model (SNPs in order of model entry: $PON1_{6842}$, $PON1_{29021}$, $PON1_{895}$, $PON1_{12471}$, $PON1_{23887}$, and $PON1_{19470}$). Results from a stepwise regression model comparison, allowing explanatory variables to enter or leave the model at each step, were the same, with the exception that $PON1_{18152}$ was dropped from the model. The AIC is a relatively lenient criterion for model

TABLE 3. Haplotype analyses considering four functional *PON1* SNPs for Arylase activity prediction

Variable	Haplotype Frequency[a]	Coefficient	SE	*t*-Statistic[b]	*P*
(Intercept)		126.8	13.6		
ACLQ[c]	0.17834	37.5	3.6	10.491	$<10^{-5}$
ACLR	0.03989	19.6	6.9	2.824	0.005
ACMQ	0.01797	45.7	10.0	4.564	$<10^{-5}$
ATLQ	0.00371	12.6	21.7	0.584	0.559
ATLR	0.00342	51.7	15.3	3.382	0.001
ATMQ	0.00315	52.2	10.7	4.869	$<10^{-5}$
GCLQ	0.08352	33.4	5.1	6.547	$<10^{-5}$
GCLR	0.12416	23.1	4.2	5.433	$<10^{-5}$
GCMQ	0.04855	19.4	6.8	2.868	0.004
GCMR	0.01058	−2.6	13.0	−0.199	0.843
GTLQ	0.08606	10.6	4.5	2.339	0.020
GTLR	0.11571	0.8	4.5	0.185	0.854
GTMQ	0.28493	Referent			
Age		−0.8	0.2	−4.170	<0.001
Current smoker		−8.9	3.8	−2.331	0.020

[a] Haplotype frequencies were inferred using the expectation maximization algorithm within the haplo.stats package.

[b] *t*-Statistics and *P* values were calculated from the coefficients and standard errors within the best-fit multivariate model by the haplo.glm function from the haplo.stats R package.

[c] The four sites represented are, in order, $PON1_{-162A/G}$, $PON1_{-108C/T}$, $PON1_{M55L}$, and $PON1_{Q192R}$. These are identical to $PON1_{1642}$, $PON1_{1696}$, $PON1_{9507}$, and $PON1_{18152}$, respectively (see Table 1). The alternative alleles included in each haplotype are noted by the first letter of the base for the promoter region SNP and the first letter of the amino acid for the coding SNPs.

comparison, and is expected to allow approximately 15% of null explanatory variables to enter the best-fit model. Thus, given 26 additional SNPs considered, on average four ($26 \times 15\% = 3.9$) null genotype variables would be expected to enter the model by chance, and perhaps fewer, given that correlations between tagSNPs (LD) effectively reduce the number of independent explanatory variables. Again, no *PON1* genotype by case status interactions were found to be significant in the prediction of Arylase, and smoking by case status genotype was significant ($P = 0.009$).

We then explored the best-fit *PON1* tagSNP model using haplo.glm (70). Using stepwise forward model regression, and starting from the base 4 SNP model, we allowed the six tagSNPs identified by the genotype-based stepwise analysis to enter the model. The best-fit haplotype model incorporated four of these SNPs: $PON1_{29021}$, $PON1_{895}$, $PON1_{23887}$, and $PON1_{19470}$. Thus, it appears that some, but not all, of the additional tagSNPs in the best-fit genotype model were tagging haplotypes distinct from the four known, functional SNPs.

We further explored, by running forward stepwise regression, whether common variation at the *PON2* and *PON3* loci might impact Arylase activity. Starting from a model with the four functional *PON1* genotypes, age, and smoking status as predictor variables, we ran a forward model analysis allowing the additional 49 tagSNPs from *PON1*, *PON2*, or *PON3* to enter the model one at a time. The best-fit model, as judged by the minimum AIC, incorporated an additional seven SNPs (in order of model entry: $PON1_{6842}$, $PON1_{29021}$, $PON1_{895}$, $PON1_{12471}$, $PON2_{4715}$, $PON1_{23887}$, and $PON1_{5663}$). It is interesting to note that all but one of the tagSNPs that entered the model were within PON1, and all but one of the tagSNPs that entered the PON1-only model were also in this model ($PON1_{6842}$, $PON1_{29021}$, $PON1_{895}$, $PON1_{12471}$, and $PON1_{23887}$), with the $PON1_{19470}$ site replaced by $PON1_{5665}$ when $PON2_{4715}$ was in the model. Given the number of tagSNPs considered, the entry of one PON2 and no PON3 genotypes into the model suggests little if any role for these genes in Arylase prediction.

Prediction of homocysteine and LDL ex vivo oxidation

Homocysteine levels ranged from 5.5 μmol/l to 119.9 μmol/l, with a mean of 15.44 and a roughly exponential distribution, so we explored the relationship between PON1 genotypes and log-transformed Hcy levels. Again, regression-based model comparison was used to explore this relationship. As judged by AIC, the null model with only age and smoking status as explanatory variables was the best fit model for log Hcy levels; Arylase activity did not significantly predict Hcy, and neither PON1 functional SNPs nor tagSNPs improved Hcy prediction.

Three measures of LDL oxidative susceptibility were available from the same assay of LDL (see METHODS) for 387 subjects. LDLmaxox showed the strongest predictive value for case control status within this study (55), so we assessed how well *PON1* genotypes predicted LDLmaxox (**Table 4**). The initial round of analysis identified an overly influential outlier, so this data point was discarded from analysis. There was no correlation between LDLmaxox and Arylase, apolipoprotein [A-I], or lipoprotein [a] in either cases or controls (the absolute value of correlations were <0.1, all $P > 0.1$). Age, smoking, and statin drug use were considered as possible covariates, but only statin use was predictive. Genotypes at six *PON1* loci entered the best-fit model: $PON1_{3625}$, $PON1_{6054}$, $PON1_{1696}$, $PON1_{12471}$, $PON1_{27678}$, and $PON1_{23887}$. Three of these SNPs ($PON1_{1696}$, *PON112471*, and $PON1_{23887}$) overlapped with the best-fit model for Arylase. Statin use was associated with a higher LDLmaxox, which is correlated with CAAD. Thus, it is likely that the statin effect was acting as a surrogate for disease status and was not indicative of the effect of the drug. Inclusion of statin use as a predictor only modestly influenced the *PON1* tagSNP regression coefficients, and no significant *PON1* genotype by statin use interaction effects were detected. Allowing additional tagSNPs from the *PON2* and *PON3* genes to enter the model added two SNPs: $PON2_{23956}$ and $PON3_{13350}$.

Predicting disease status

Within the study, 205 individuals had >80% carotid stenosis (cases) and 232 samples had <15% stenosis (controls). Age ($P < 0.001$), smoking status ($P < 0.001$), and sqrt-Arylase activity ($P < 0.001$) all independently predict case control status; age is an artifact of the matching on censored (onset) age versus current age. Genotypes at the four functional SNPs have not previously shown significant associations with disease in a subset of this cohort (3). Using logistic regression, we used stepwise model comparison to evaluate whether models incorporating the 26 tagSNPs and 4 functional *PON1* genotypes provided a better

TABLE 4. Best fit *PON1* tagSNP model for prediction of LDL susceptibility to oxidation ex vivo (LDLmaxox)

Variable	Coefficient (All)	SE	*t*-Statistic[a]	*P*	Coefficient (Case-only)	Coefficient (Control-only)
(Intercept)	1.74	2.00×10^{-2}				
$PON1_{3625}C$	5.96×10^{-2}	2.86×10^{-2}	2.081	0.038	6.42×10^{-2}	6.49×10^{-2}
$PON1_{6054}G$	-4.20×10^{-2}	1.39×10^{-2}	−3.016	0.003	-3.72×10^{-2}	-4.45×10^{-2}
$PON1_{1696}T$ (−108)	-3.02×10^{-2}	1.15×10^{-2}	−2.635	0.009	-3.59×10^{-2}	-2.33×10^{-2}
$PON1_{12471}C$	-8.20×10^{-2}	2.83×10^{-2}	−2.900	0.004	-1.31×10^{-1}	-5.04×10^{-2}
$PON1_{27678}G$	-3.01×10^{-2}	1.24×10^{-2}	−2.412	0.016	-4.04×10^{-2}	-2.57×10^{-2}
$PON1_{23887}C$	1.13×10^{-1}	5.79×10^{-1}	1.959	0.051	1.37×10^{-1}	1.36×10^{-1}
Statin use	5.62×10^{-2}	1.41×10^{-2}	3.981	<0.001	4.83×10^{-2}	3.79×10^{-3}

[a] *t*-Statistics and *P* values were calculated for all subjects from the coefficients and standard errors within the best fit multivariate model by the glm function in R.

TABLE 5. *PON1* functional and tagSNP genotype final models for case control prediction

Variable	Coefficient	SE	*t*-Statistic	*P*
Best-fit model considering all PON1 SNPs				
(Intercept)	5.49	0.95		
Age	−0.07	0.01	−5.532	$<10^{-5}$
Current smoker	−1.76	0.27	−6.510	$<10^{-5}$
$PON1_{23887}C$	−1.84	1.10	−1.672	0.095
$PON1_{29021}T$	0.25	0.16	1.586	0.113
Model including Arylase				
(Intercept)	4.35	1.15		
Age	−0.07	0.01	−5.239	$<10^{-5}$
Current smoker	−1.73	0.27	−6.364	$<10^{-5}$
Sqrt-Arylase	0.08	0.05	1.732	0.083
$PON1_{23887}C$	−1.72	1.10	−1.556	0.119
$PON1_{29021}T$	0.32	0.16	1.947	0.052
Model including LDLmaxox				
(Intercept)	9.56	1.41		
Age	−0.11	0.016	−6.44	$<10^{-5}$
Current smoker	−1.83	0.32	−5.72	$<10^{-5}$
Ln-LDLmaxox	−4.82	1.45	−3.31	0.0009
$PON1_{23887}C$	−1.15	1.14	−1.015	0.31
$PON1_{29021}T$	0.28	0.19	1.474	0.14

Ln,

fit than a null model with only age and smoking status as independent variables. Two tagSNPs entered the model and only one, $PON1_{23887}$ ($P = 0.095$) with $P < 0.1$ (**Table 5**), suggesting a false positive. This SNP is rare in Caucasians, MAF = 0.008, and does not fall into the category of common variation. Of the eight heterozygous subjects, six were cases, one was a control, and one had 50%–79% stenosis. However, this rare SNP also predicted Arylase and LDLmaxox. To further explore this relationship, we examined a base model with age, smoking status, $PON1_{23887}$ ($P = 0.119$), $PON1_{29021}$ ($P = 0.052$), and sqrt-Arylase ($P = 0.083$) activity as independent variables. Adding sqrt-Arylase as a predictor only modestly influenced the $PON1_{23887}$ coefficient. Allowing ln-LDLmaxox as a predictor reduced the impact of $PON1_{23887}$. Consideration of neither Arylase nor LDLmaxox reduced the coefficient for the marginal effect of $PON1_{29021}$, which did not predict either of these phenotypes. This is consistent with either a spurious effect of this SNP or a mechanism not mediated through Arylase or LDLmaxox.

DISCUSSION

We have examined the relationship between all common genetic polymorphisms in the paraoxonase gene cluster and related phenotypes using a tagSNP-based approach. The findings reported here shed light upon both the specific biology of this system and the methodologies we have used. Four common functional SNPs in *PON1* were known prior to the beginning of this analysis, two of which change amino acids and two of which alter promoter activity. Although significant linkage disequilibrium exists between these SNPs, thirteen out of sixteen possible recombinant haplotypes were observed, suggesting a high frequency of recombination (3). Whether haplotype-based analysis is substantially more powerful than genotype-based analysis is an important question to consider as whole genome association studies become feasible. The results presented here demonstrate that in the *PON1* gene, which has substantial haplotype diversity and recombinant haplotypes, haplotype-based approaches afford a modest advantage over SNP-based approaches for capturing *cis*-variation effects on PON1 levels. Similar results have been seen for *APOE* (52, 53).

We also investigated whether other common polymorphisms in the region might explain substantially more of the Arylase activity phenotype than did the previously described functional polymorphisms. By genotyping a total of 30 tagSNPs across *PON1*, we now have a comprehensive collection of common variation within the gene, and regression analysis suggests that additional common polymorphisms beyond the four previously described polymorphisms probably do contribute to the Arylase phenotype. However, genotype at the four known functional polymorphisms alone explains 25.9% of residual phenotypic variance, after adjustment for age and smoking, whereas the best-fit model (with an additional six SNPs) explains 29.8% of this phenotypic variance. This difference is much more modest than the previously reported 20.4% to 33.4% variance explained by five PON1 genotypes ($PON1_{-909}$, $PON1_{-162}$, $PON1_{-108}$, $PON1_{55}$, and $PON1_{192}$) or haplotypes derived from those five sites (72). So although additional functional variation at *PON1* exists, most *cis* variation has already been accounted for by the four known functional SNPs. Rare SNPs with large effects on PON1 activity are known (73), as are environmental factors that modestly influence PON1 activity, such as age and smoking (3). However, there may also be genetic variability at other loci that modulates PON1 levels. Although *PON1* knockout mice have no measurable paraoxonase activity (6), this does not rule out modifier genes.

Interestingly, among the additional tagSNPs associated with Arylase activity, several SNPs that are strongly, but not perfectly, correlated with the known functional SNPs entered the model. For example, $PON1_{895}$ is in strong LD ($r^2 = 0.72$) with the promoter $PON1_{-108C/T}$ SNP ($PON1_{1696}$), suggesting that additional regulatory polymorphisms in the promoter region are likely to be relevant in predicting Arylase activity. Similarly, the $PON1_{19470}$ SNP is strongly correlated with the $PON1_{Q192R}$ polymorphism ($r^2 = 0.92$), and the $PON1_{29021}$ SNP is strongly correlated with $PON1_{M55L}$ ($r^2 = 0.67$), but in each case, the correlation is imperfect, suggesting additional functional variation within the context of the major functional polymorphisms. Finally, the $PON1_{23887}$ SNP is a rare polymorphism in Europeans, but is more frequent in African Americans and is strongly associated with $PON1_{27737}$ in African Americans ($r^2 = 0.68$). Polymorphisms that are frequent in African Americans may be rare in Europeans due to founder effects, drift, or negative selection. $PON1_{27737}$ lies in the 3′ untranslated region, making it an attractive candidate for regulatory function via RNA stability, secondary structure, or other 3′ effects. The exact nature of the functional alterations associated with each tagSNP will require further exploration with in vitro studies.

Extending this analysis beyond *PON1* genotypes confirmed the expectation that *PON2* and *PON3* polymorphisms contribute relatively little to the PON1 Arylase activity phenotype, inasmuch as all but one of the tagSNPs in the best-fit model using all variation in the region were within *PON1*. This is consistent with biochemical results indicating that PON2 and PON3 have negligible Arylase activity (74, 75).

Neither *PON1* genotypes nor Arylase activity predicted homocysteine level. Although PON1 has been shown to have some Hcy-thiolactonase activity in previous studies, this has been argued to be at physiologically irrelevant rates due to the very low catalytic efficiency of PON1. The results of this study were consistent with this assertion.

The relationship between *PON1* variation and LDLmaxox is substantially more interesting than the negative Hcy results. LDLmaxox was the LDL oxidation measure most strongly correlated with CAAD in this study. Although Arylase activity was not significantly correlated with LDLmaxox, a substantial number of *PON1* SNPs appear to be related to this oxidative phenotype, including the functional $PON1_{1696}$ promoter SNP ($PON1_{-108C/T}$). This result has been reported previously by others (76). This SNP has a strong impact on expression levels of PON1, and thereby upon PON1 mass and Arylase activity. However, as mentioned previously, Arylase activity shows no significant correlation with LDLmaxox. No active PON1 was present in the LDL oxidation assay, both because HDL is removed and because the EDTA destroys the activity of PON1, a calcium-dependent enzyme. Thus, PON1 effects on the oxidation assay would depend on the underlying LDL oxidative status at the time of sampling.

The effects of PON1 on LDL oxidation are controversial (74, 75, 77–79). Newer studies have questioned whether the antioxidant effects of PON1 ex vivo shown in prior work might be, in part, due to cross contamination (74, 77). However, work on recombinant PON1, which is not susceptible to similar purification issues, confirmed that PON1 did influence oxidized LDL levels as measured by the oxidized LDL-stimulated MCP-1 secretion, whereas HDL without PON1 did not (78). Another study of recombinant PON1, PON2, and PON3 without HDL present did not find that any had an effect on ex vivo oxidative susceptibility of LDL, considering the lag time phenotype (75), which is uncorrelated with LDLmaxox and a weaker predictor of CAAD in our cohort (55). HDL from *PON1* knockout mice does not protect LDL from oxidation (80), and *PON1* transgenic mice have improved protection of LDL from oxidation (81).

If *PON1* genotype predicts LDL oxidative susceptibility, this strongly suggests a PON1 role. It is not yet clear how *PON1* genotypes are related to LDLmaxox when Arylase is not. One possibility is that the PON1 activity for Arylase does not reflect the PON1 activity for the LDLmaxox relevant substrate. This is consistent with the finding that site-specific mutagenesis of $PON1_{284CYS}$ decreases PON1 antioxidant activity, but not its Arylase activity (82). We did not detect $PON1_{192}$ effects on LDLmaxox; prior studies of this polymorphism for the phenotype lag time have been mixed (76, 83). A $PON1_{-108}$ effect on both lag time and LDL oxidation rate has been reported (76).

Extending our analysis from intermediate phenotypes to the clinical phenotype of CAAD, our previous analyses (3) showed that although *PON1* functional SNP genotypes predict a significant portion of Arylase activity, and Arylase activity predicts disease status, *PON1* functional SNPs are not significantly correlated with disease status. This finding is robust to analysis of additional tagSNPs in the gene, suggesting that *PON1* tagSNPs are an inadequate proxy for Arylase activity in CAAD prediction. Why common *PON1* genotypes that are correlated with the CVD risk factors Arylase and LDLmaxox, particularly $PON1_{-108}$, do not predict CVD remains unclear. This may be related to the observation in this study that *cis* variation in the *PON* gene cluster accounts for less than one-third of the overall variance in PON1 activity levels. Genetic variation at other loci may influence PON1 activity, or rare variation may be important, as suggested for the rare SNP $PON1_{23887}$, which was found to predict Arylase, LDLmaxox, and, marginally, case status. Larger studies will be required to determine whether that is a spurious result.

In conclusion, our analysis of genetic variation at the *PON1* gene revealed several important results. First, haplotype-based analysis afforded a modest advantage over genotype-based models of the Arylase activity quantitative trait, but the same panel of tagSNPs were identified as important in either analysis. Second, comprehensive tagSNP analysis of the *PON1* gene suggested that additional functional polymorphisms exist, in addition to the known four functional polymorphisms, but that the majority of the *cis* effects are attributable to the known functional variants. Because the additional SNPs do not appear to be coding SNPs, they may include polymorphisms that affect regulation or splicing efficiency. Third, neither PON1 Arylase activity nor *PON1* genotype predicted plasma Hcy levels. Additionally, although Arylase activity failed to predict the LDL ex vivo oxidation measure LDLmaxox, a number of *PON1* genotypes were correlated with this variable. Taken together with the observed correlation between LDLmaxox and CAAD, this suggests that it may be regulatory variation, and not coding region variation of the PON1 on HDL particles, that is important in preventing oxidative damage, consistent with our earlier studies (3). Thus, although *PON1* genotype accounts for some variability in the CAAD risk factors Arylase and LDLmaxox, capturing common genetic variation comprehensively at the *PON1* structural locus is not an adequate substitute for measuring Arylase activity in the prediction of CAAD.

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