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DNA Polymorphisms in Two Paraoxonase Genes (PON1 and PON2) Are Associated with the Risk of Coronary Heart Disease

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Summary

A common polymorphism at codon 192 in the human paraoxonase (PON) 1 gene has been shown to be associated with increased risk for coronary heart disease (CHD) in Caucasian populations. However, these findings have not been reported consistently in all Caucasian and non-Caucasian populations, suggesting that this is not a functional mutation but may mark a functional mutation present in either PON1 or a nearby gene. Recently, two other PON-like genes, designated "PON2" and "PON3," have been identified, and they are linked with the known PON1 gene on chromosome 7. Identification of additional polymorphisms in the PON-gene cluster may help to locate the functional polymorphism. In this report, we describe the existence of a common polymorphism at codon 311 (Cys→Ser; *PON2*S*) in the PON2 gene, as well as its association with CHD alone and in combination with the PON1 codon 192 polymorphism in Asian Indians. The frequency of the *PON2*S* allele was significantly higher in cases than in controls (.71 vs. .61; $P = .016$). The age- and sex-adjusted odds ratio (OR) was 2.5 (95% confidence interval [95% CI] = 1.8–3.1; $P = .0090$) for the *PON2*S* allele carriers. Further stratification of the *PON2*S* association, on the basis of the presence or absence of the *PON1*B* allele, showed that the CHD risk associated with the *PON2*S* allele was confined to *PON1*B*-allele carriers. Likewise, the *PON1*B*-allele risk was present only among *PON2*S* carriers. Age- and sex-adjusted ORs for the *PON2*S* and *PON1*B* were 3.6 (95% CI = 2.6–4.6; $P = .011$) and 2.9 (95% CI = 2.4–3.5; $P = .0002$) among the *PON1*B* and *PON2*S* carriers, respectively. Our data indicate that both polymorphisms synergistically contribute to the CHD risk in this sample and that this genetic risk is independent of the conventional plasma lipid profile.

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Introduction

Oxidized LDL is believed to play an important role in initiation of atherosclerosis. Numerous studies suggest the formation of fatty-acid streaks in response to a series of events, followed by the migration of oxidized LDL-loaded monocytes into the subendothelial space of the arteries (Navab et al. 1996). HDL has been shown to prevent oxidative modification of LDL in vivo (Klimov et al. 1993) as well as in vitro (Mackness et al. 1993). Recent studies indicate that two HDL-associated enzymes, PON and platelet-activating–factor acetyl hydrolase, are responsible for its antioxidative and anti-inflammatory properties (Stafforini et al. 1993; Watson et al. 1995). In vitro studies indicate that PON can significantly reduce lipid peroxide generation during LDL oxidation and thus may provide HDL-associated protection against atherosclerosis. Furthermore, serum PON activity has been found to be lower in patients with myocardial infarction (MI) (McElveen et al. 1986), diabetes and familial hypercholesterolemia (Mackness et al. 1991), fish-eye disease (Mackness et al. 1987), and Tangier disease (Mackness et al. 1989). PON tightly binds to HDL subfractions that also contain apolipoprotein (apo) A-I and apo J (Blatter Garin et al. 1993; Kelso et al. 1994). PON has largely been studied for its role in hydrolyzing a large number of organophosphate compounds used in pesticides, insecticides, and nerve gases (La Du 1992; Davies et al. 1996). Serum PON levels and activity vary widely among populations of different ethnic backgrounds (Diepgen et al. 1986; Roy et al. 1991). Human serum PON (now called "PON1") is genetically polymorphic, because of the occurrence of two common isoforms that differ by substitution of an amino acid (Gln→Arg) at codon 192 (Adkins et al. 1993; Humbert et al. 1993). Individuals homozygous for Gln (the A allele) have lower PON activity than is seen in individuals homozygous for Arg (the B allele), the latter having eight times more activity than has Gln in hydrolyzing paraoxon (Davies et al. 1996; La Du 1996). PON activity is substrate dependent and may be quite opposite for different substrates (Furlong et al. 1988; La Du et al. 1988; Davies et al. 1996). Notably, compared with the PON1 A variant, the PON1 B variant is more effi-

Table 1

Demographic, Clinical, and Lipid Profile of Asian Indian Controls and Cases

	Values in[a]		
	Controls	Cases	*P*
Males/Females	144/45	115/14	...
Age (Years)	49.6 ± .8	55.20 ± .8	.001
BMI (kg/m^2)	24.9 ± .3	24.85 ± .3	.79
Smokers (0/1/2)[b]	166/0/23	71/26/29[c]	...
Nondiabetic/ diabetic	169/20	89/40	...
Non-MI/MI	189/0	46/69[d]	...
HDL cholesterol (mg/dl)	41.3 ± 1.0	27.3 ± .8	.0001
LDL cholesterol (mg/dl)	165.5 ± 4.3	155.5 ± 3.9	.11
Total cholesterol (mg/dl)	241.4 ± 5.3	216.3 ± 4.1	.001
Triglycerides (mg/ dl)[e]	149.0 ± 6.0	150.7 ± 6.3	.84
Apo A-I (mg/dl)	141.9 ± 2.0	98.3 ± 5.0	.0001
Apo B (mg/dl)	136.0 ± 2.7	105.4 ± 2.6	.000

[a] Note.—Mean ± SE values are for continuous variables.

[b] The three numerals indicate, respectively, the number of individuals who have never been smokers, who once were smokers, and who currently are smokers.

[c] Smoking history was not known for 3 individuals.

[d] MI information was not available for 15 individuals.

[e] Values are log transformed.

cient in hydrolyzing paraoxon but is less efficient in hydrolyzing three additional organophosphates: diazoxon, sarin, and soman (Davies et al. 1996). Recently, the B allele of the PON1 codon 192 polymorphism has been found to be associated with an increased risk of CHD (Ruiz et al. 1995; Serrato and Marian 1995). Our own study, conducted in Asian Indians and Chinese, has indicated that the B allele is a significant risk factor for CHD in Asian Indians but not in Chinese (Sanghera et al. 1997). Two additional studies, in Caucasians, reported a lack of association of the B allele with CHD (Antikainen et al. 1996; Herrman et al. 1996), suggesting that the B allele may act merely as a marker for an as yet unidentified functional mutation in either PON1 or a nearby gene.

In addition to the known human PON1 gene, two additional PON-like genes, designated "PON2" and "PON3," have been identified, and all three genes have been mapped to chromosome 7q21-q22 (Primo-Parmo et al. 1996*a*, 1996*b*). In the present study, we describe the existence of a common polymorphism at codon 311 in the PON 2 gene, as well as its association with CHD risk both alone and in combination with the PON1 codon 192 polymorphism in Asian Indians.

Subjects and Methods

All subjects included in this study are Asian Indians and are second- or third-generation descendants of immigrants who migrated from the Indian subcontinent to Singapore during the early 19th century. There is a very low degree of interracial marriage in Singapore. On the basis of self-reports, no subjects with mixed ancestry were encountered.

CHD Cases

The study samples comprised 129 unrelated CHD cases (115 males and 14 females) with an age range of 32–83 years (mean ± standard error [mean ± SE] 55.2 ± 0.8 years). The cases were consecutive CHD patients admitted to the Cardiothoracic Surgery Unit at the Singapore General Hospital, for coronary-artery-bypass graft, between August 1989 and December 1992. Blood samples from cases were collected either during preoperative review or, for those with a history of MI, ⩾3 mo after full recovery. Cases with a positive stress test (Bruce method) were evaluated for the presence of CHD by means of coronary angiography. Inclusion criteria were >50% narrowing of the lumina of at least one of the major coronary arteries. A detailed family history of CHD, hypertension, diabetes mellitus, angina, and smoking history was obtained from each case by a certified physician and cardiologist. MI had occurred in ~60% of the cases, as judged both by typical electrocardiogram (ECG) changes (Minnesota code 1.1 or 1.2 in the ECG) and by changes in serum enzymes (aspartate aminotransferase, lactate dehydrogenase, and creatine kinase); and nearly 31% of the cases were diabetic. Cases with less obstruction or with valvular disease were excluded.

Controls

Unrelated controls ($n = 189$) of age >32 years (mean ± SE 49.6 ± 0.8 years) were selected from a larger, population-based control sample of 470 Asian Indians, to match the age range of cases. The control samples were collected as part of the Healthy Lifestyle Promotion Exercise study. All participants completed

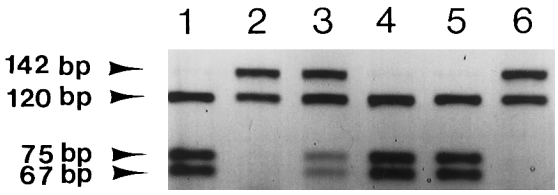

Figure 1 Restriction patterns of the PCR-amplified 262-bp fragment after digestion with *Dde*I. Fragment sizes of 142 bp and 120 bp correspond to Cys311 (C allele), whereas 75 bp and 67 bp are diagnostic bands for Ser311 (S allele). Note that a 120-bp band is present in all genotypes, because of the presence of a common *Dde*I site. Lanes 1, 4, and 5, SS genotype. Lanes 2 and 6, CC genotype. Lane 3, CS genotype.

Table 2

Distribution of PON1 and PON2 Genotype and Allele Frequencies in Cases and Controls

	Controls (n = 190)	Cases (n = 157)
PON1:		
Genotype:		
AA[a]	91 (47.9%)	42 (26.8%)
AB[b]	76 (40.0%)	85 (54.1%)
BB[c]	23 (12.1%)	30 (19.1%)
Allele:		
A (Gln)	.679	.538
B (Arg)[d]	.321	.462
	(n = 189)	(n = 129)
PON2:		
Genotype:		
CC	33 (17.5%)	14 (10.9%)
CS	80 (42.3%)	48 (37.2%)
SS[e]	76 (40.2%)	67 (51.9%)
Allele:		
C (Cys)	.386	.295
S (Ser)[f]	.614	.705

[a] Significant difference between cases and controls: $P < .0001$.
[b] Significant difference between cases and controls: $P < .01$.
[c] Significant difference between cases and controls: $P < .025$.
[d] Significant difference between cases and controls: $P = .0001$.
[e] Significant difference between cases and controls: $P < .05$.
[f] Significant difference between cases and controls: $P = .016$.

questionnaires including a complete medical and family history of hypertension, CHD, any other significant illness, and (in women) hormone therapy. Only those subjects who were healthy and had no family history of CHD were included in the control group. Individuals were selected after careful physical examination, chest x-ray, ECG, and urine and blood tests including those for hemoglobin and glucose.

Clinical and Metabolic Estimations

Recumbent blood pressure and 12-lead ECG were recorded on each subject after a 30-min rest on a couch. Body weight, height, and waist and hip measurements were recorded, and blood samples were collected by venipuncture after an overnight fast. Details of methods of quantitative estimations of apolipoproteins and lipoprotein lipids have been provided elsewhere (Sanghera et al. 1997).

PON-Polymorphism Screening

DNA was extracted from buffy coats as described by Parzer and Mannhalter (1991) and was used to amplify the target region in the PON1 and PON2 genes by PCR. Details of primers and PCR conditions for the PON1 codon 192 polymorphism have been described elsewhere (Sanghera et al. 1997).

On the basis of a comparison between the DNA sequence submitted to the dbEST database and those obtained by Primo-Parmo et al. (1996*b*) in their cDNA and genomic DNA for PON2, the occurrence of a possible polymorphism, having either TCT (Ser) or TGT (Cys), was predicted at codon 311. Using DNA data submitted to GSDB, we designed primers flanking the region containing codon 311, to amplify a 262-bp fragment, which was followed by restriction digestion with *Dde*I, and we found the existence of a common polymorphism in Asian Indians. The forward primer PON2 F9 (5′-ACA TGC ATG TAC GGT GGT CTT ATA-3′) and the reverse primer PON2 R9 (5′-AGC AAT TCA TAG ATT AAT TGT TA-3′) were used to amplify the target region. One microgram of genomic DNA was amplified in 50 μl of reaction mixture containing 0.3 μM of each primer, 200 μM of each dNTP (Pharmacia), 5 μl of 10 × reaction buffer (100 mM Tris-HCL [pH 9.0], 500 mM KCL, and 1% Triton X-100), 5% dimethyl sulfoxide, 1.5 mM $MgCl_2$, and 1.25 units *Taq* DNA polymerase. After the DNA was denatured for 4 min at 94°C, the reaction mixture was subjected to 30 cycles of denaturation for 1 min at 94°C, annealing for 1.5 min at 50°C, and extension for 2 min at 72°C. The PON1 and PON2 polymorphisms were detected by digesting the PCR-amplified product with the *Alw*I and *Dde*I restriction enzymes, respectively, followed by size fractionation in 3% Nusieve (FMC) agarose gels and visualization of bands with ethidium bromide.

Statistical Analyses

Allele frequencies were calculated by allele counting. Concordance of genotype frequencies with Hardy-Weinberg equilibrium was tested by a χ^2 goodness-of-fit test. Observed heterozygosity was estimated on the basis of the observed proportion of heterozygotes in the sample, ht, with the 95% CI given by $\pm 1.96 \times \sqrt{ht(1-ht)/n}$, where n is the total number of individuals in the sample; the expected heterozygosity was calculated as $ht = 2p(1-p)$, where p is the allele frequency. Comparison of the genotype frequencies in cases versus those in controls was calculated by a χ^2 test for a $2 \times k$ contingency table.

Table 3

Assessment of Hardy-Weinberg Equilibrium for PON Polymorphisms

	Heterozygosity (95% CI)			
	Observed	Expected	χ^2 [a]	P [a]
PON1:				
Controls	.40 (.35–.45)	.44	1.28	.26
Cases	.54 (.49–.60)	.50	1.25	.26
PON2:				
Controls	.42 (.37–.47)	.47	2.18	.14
Cases	.37 (.31–.43)	.42	1.44	.23

[a] Calculated by use of all three genotypes, for each PON genotype and subject sample.

Table 4

OR for Risk of Development of CHD, Estimated by Logistic Regression

	OR (95% CI); *P* Value[a]			
	PON1 Only	PON2 Only	PON1 and PON2	Interaction
Age[b]	3.2 (2.7–3.6); <.0001	3.4 (2.9–3.9); <.0001	3.2 (2.7–3.7); <.0001	3.2 (2.7–3.7); <.0001
Sex[c]	.4 (0–1.0); .0095	.4 (0–1.0); .0048	.4 (0–1.1); .0054	.4 (0–1.1); .0054
PON1[d]	2.6 (2.1–3.0); .0001	…	2.4 (1.9–2.9); .0007	.9 (0–2.2); .850
PON2[e]	…	2.5 (1.8–3.1); .0090	2.1 (1.4–2.8); .040	1.1 (.1–2.1); .870
PON1 × PON2	…	…	…	3.3 (1.9–4.7); .090
χ^2	358.3	311.8	302.9	300.9

[a] Data are for Wald's test; note that the 95% CI and Wald's test assume that the OR has an approximate Gaussian distribution. An ellipsis (…) denotes the term was not a part of the model.

[b] Entered as a dichotomy: <50 years versus ⩾50 years, with <50 years as the referent group

[c] Entered as a dichotomy, with males as the referent group.

[d] Considered as a dominant effect and entered as a dichotomy, AA versus (AB, BB), with AA as the referent group.

[e] Considered as a dominant effect and entered as a dichotomy, CC versus (CS, SS), with CC as the referent group.

Approximate normality of the sampling distribution of each dependent variable was tested by Lilliefor's test for normality (a modified Kolmogorov-Smirnov test). The distributions of triglycerides in controls, as well as the distributions of apo A-I, triglyceride, and total cholesterol in cases, were not normal and, therefore, were log transformed. Significant covariates for each dependent trait were identified by Spearman's correlations and stepwise multiple linear regression with an overall 5% level of significance. The covariates considered were the linear effects of age, body-mass index (BMI), waist/hip ratio, smoking history, and triglyceride levels, as covariates known to affect lipoprotein-lipid levels. Age and BMI were found to be the significant covariates in all quantitative traits. Each dependent quantitative variable was then adjusted to remove the effects of significant covariates, and these adjusted variables were used in one-way analysis of variance (ANOVA) to determine the impact of the PON2 polymorphism on plasma lipid levels. In genotype-lipid analysis, individuals with triglycerides >300 mg/dl were excluded.

The contribution of each polymorphism to CHD risk was estimated on the basis of logistic regression for unmatched data, to obtain ORs for both PON polymorphisms and for their interactions (e.g., age × PON genotype), adjusted for the effects of age and sex. Included ORs were tested for significance by Wald's test, whereas overall significance for the difference between the logistic regression models was tested by the deviance as an approximate χ^2 statistic. All calculations used SPSS for Windows statistical analysis software.

Results

Clinical and Demographic Information

Age, BMI, smoking history, and medical history, along with the distributions of the mean quantitative lipid and apolipoprotein levels, for controls and for cases, are summarized in table 1.The cases were older and more frequently were either current smokers or ex-smokers than were the controls. Plasma LDL-cholesterol and triglyceride levels did not differ appreciably between cases and controls, whereas plasma HDL-cholesterol and apo A-I levels were significantly lower in cases than in controls ($P = .0001$). Plasma apo B ($P = .0001$) and total cholesterol ($P = .001$) were significantly lower in cases than in controls, which could be the result of controlled diet and medication in the cases after they had undergone coronary-bypass surgery.

PON2 Codon 311 Polymorphism and Its Distribution in Cases and Controls

The restriction patterns indicating the presence of three genotypes due to the existence of two common alleles, S (Ser311) and C (Cys311), are shown in figure 1. The distributions of the PON2 codon 311 polymorphism in controls and cases are given in table 2. This table also includes the PON1 codon 192 polymorphism for the expanded sample size for Asian Indian cases, versus that which we had previously reported (Sanghera et al. 1997) ($n = 157$ vs. $n = 122$). The distribution of the PON2 polymorphism was in Hardy-Weinberg equilibrium, among both cases and controls (table 3). The cases had a significantly higher frequency of the PON2/SS genotype (52% vs. 40%; $P < .05$) than was seen in the controls. Consequently, the frequency of the *PON2*S* allele was significantly higher (.70 vs. .61; $P = .016$) and the frequency of the *PON2*C* allele was significantly lower (.29 vs. .39; $P = .016$) in cases than in controls. Compared with the PON2/CC genotype, age- and sex-adjusted ORs for the development of CHD in the *PON2*S* carriers (CS and SS genotypes) was 2.5 (95% CI = 1.8–3.1; $P = .0090$) (table 4, "PON2 Only" model). A more general, codominant model of risk for the *PON2*S* allele—that is, with separate ORs for CS and SS genotypes—did not give a significantly better fit

to the data ($P > .10$) than was seen with this dominant model. The B allele of the PON1 polymorphism was also overrepresented in cases compared with controls (.46 vs. .32; $P = .0001$). Cases had a significantly higher frequency of the PON1/BB (19% vs. 12%; $P < .01$) and PON1/AB (54% vs. 40%; $P < .01$) genotypes and significantly lower frequency of the PON1/AA genotype (27% vs. 48%; $P < .0001$) than was seen in controls. The age- and sex-adjusted ORs for the development of CHD in the *PON1*B* carriers (AB and BB genotypes) was 2.6 (95% CI = 2.1–3.0; $P = .0001$) (table 4, "PON1 Only" model). Again, a codominant model for risk of the *PON1*B* allele did not give a significantly better fit to the data ($P > .10$) than was seen with the dominant model. The association of both polymorphisms with the risk of CHD was independent of the diabetic status, because the removal of the 40 diabetic CHD cases and of the 20 diabetic controls (table 1) did not affect these associations (data not shown).

Relationship between PON1 and PON2 Polymorphisms and CHD Risk

To investigate whether the CHD risks attributable to PON1 (B allele) and PON2 (S allele) are additive or interactive between the two loci, we determined the distribution of the PON2 genotypes with regard to the PON1 polymorphism, stratified by the presence (AB and BB genotype) or absence (AA genotype) of the *PON1*B* allele, and the distribution of the PON1 genotypes with regard to the PON2 polymorphism, stratified by the presence (CS and SS genotype) or absence (CC genotype) of the *PON2*S* allele (table 5). Although the distribution of the PON2 genotype among non-*PON1*B* carriers was similar among cases and controls, it was significantly different among *PON1*B* carriers. Among *PON1*B* carriers, the frequency of the PON2/CC genotype was significantly lower (4% vs. 13%; $P < .03$) and the frequency of the PON2/SS genotype was significantly higher (60% vs. 42%; $P < .01$) in cases than in controls. Although the frequency of the *PON2*S* allele was comparable in cases and controls having the PON1/AA genotype (.51 vs. .60; $P = .20$), it was significantly higher in cases than in controls, among *PON1*B* carriers (.78 vs. .64; $P < .003$). Also, the distribution of the *PON1*B* allele was similar between cases and controls having the PON2/CC genotype (.18 vs. .26; $P = .39$) but was significantly higher among *PON2*S* carriers (.47 vs. .33; $P < .001$).

An interesting feature about the distribution of the *PON1*B*-allele frequency among the PON2 genotypes in CHD cases was that it increased gradually, from .18 in the PON2/CC genotype to .39 in the PON2/CS genotype and to .51 in the PON2/SS genotype (fig. 2). This difference was significant between the PON2/CC and CS genotypes ($P = .015$) and between the PON2/CC and SS genotypes ($P < .0001$) (see fig. 2). This increase is also seen in the age- and sex-adjusted ORs for the PON1 dominant model: OR = 0.9 (95% CI = 0–2.2; $P = .84$) within the PON2/CC genotype, but OR = 2.9 (95% CI = 2.4–3.5; $P = .0002$) within the PON2/CS and SS genotypes. A similar gradual increase in the distribution of the *PON2*S* allele among the PON1 genotypes was observed in the cases: the frequency of the *PON2*S* allele was significantly lower in the PON1/AA genotype (.51) than in the PON1/AB genotype (.76; $P = .0003$) and the PON1/BB genotype (.84; $P = .0001$) (fig. 2). This increase is also seen in the age- and sex-adjusted ORs for the PON2 dominant model: OR = 1.1 (95% CI = 0.1–2.1; $P = .87$) within the PON1/AA genotype, but OR = 3.6 (95% CI = 2.6–4.6; $P = .011$) within the PON1/AB and BB genotypes.

These, as well as the earlier results regarding the distribution of PON1 within the PON2 genotypes (table 5), suggest an interaction between these two loci in the determination of CHD risk. Indeed, if both the PON1 and the PON2 genotypes are included in the model, then their ORs are 2.4 (95% CI = 1.9–2.9; $P = .0007$) and 2.1 (95% CI = 1.4–2.8; $P = .040$), respectively, both of which are decreased from their univariate values (table

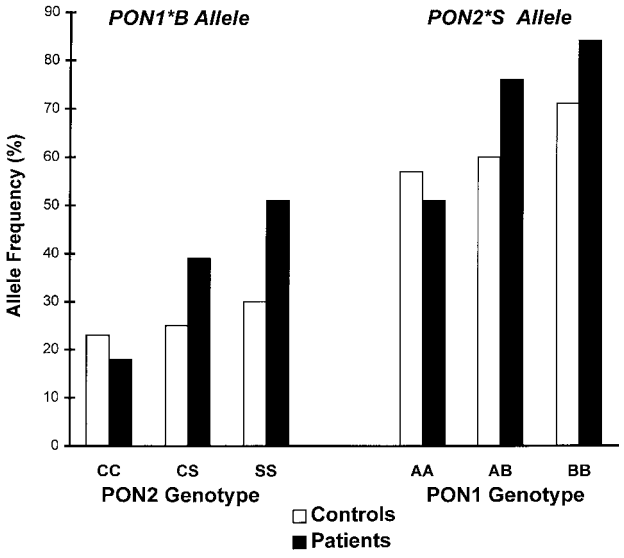

Figure 2 Distribution of the *PON1*B* allele among PON2 genotypes (CC, CS, and SS) and of the *PON2*S* allele among PON1 genotypes (AA, AB, and BB), in controls and cases. Note that both the frequency of the *PON1*B* alleles in non–*PON2*S* allele carriers (CC genotype) and the frequency of the *PON2*S* alleles in non-*PON1*B* carriers (AA genotype) are comparable between cases and controls; however, the frequency of the *PON1*B* allele increases among *PON2*S* carriers, and the frequency of the *PON2*S* allele increases among *PON1*B* carriers, in a dose-related manner, in cases compared with controls.

Table 5

Distribution of PON2 Genotypes, Stratified by Non-*PON1*B* Carriers and *PON1*B* Carriers, and Distribution of PON1 Genotypes, Stratified by Non-*PON2*S* Carriers and *PON2*S* Carriers

	Non-*PON1*B* Carriers		*PON1*B* Carriers	
	Controls (n = 87)	Cases (n = 35)	Controls (n = 93)	Cases (n = 92)
PON2:				
Genotype:				
CC[a]	17 (19.5%)	10 (28.6%)	12 (12.9%)	4 (4.3%)
CS	36 (41.4%)	14 (40.0%)	42 (45.2%)	33 (35.9%)
SS[b]	34 (39.1%)	11 (31.4%)	39 (41.9%)	55 (59.8%)
Allele:				
S (Ser)[c]	.60	.51	.64	.78
C (Cys)	.40	.49	.35	.22
	Non-*PON2*S* Carriers		*PON2*S* Carriers	
	Controls (n = 29)	Cases (n = 14)	Controls (n =151)	Cases (n = 113)
PON1:				
Genotype:				
AA[d]	17 (58.6%)	10 (71.4%)	70 (46.4%)	25 (22.1%)
AB[d]	9 (31.0%)	3 (21.4%)	61 (40.4%)	70 (62.0%)
BB	3 (10.3%)	1 (7.1%)	20 (13.2%)	18 (15.9%)
Allele				
A (Gln)[e]	.74	.82	.67	.53
B (Arg)	.26	.18	.33	.47

[a] Significant difference between cases and controls, among *PON1*B* carriers: $P < .03$.
[b] Significant difference between cases and controls, among *PON1*B* carriers: $P < .01$.
[c] Significant difference between cases and controls, among *PON1*B* carriers: $P < .003$.
[d] Significant difference between cases and controls, among *PON2*S* carriers: $P < .0001$.
[e] Significant difference between cases and controls, among *PON2*S* carriers: $P < .001$.

4, "PON1 and PON2" model). However, a direct test of interaction (PON1[*dominant*] × *PON*2[*dominant*]) between PON1 and PON2 showed no significant effect in this sample (OR = 3.3; 95% CI = 1.9–4.7; P = .090; table 4, "Interaction" model).

To further investigate the interaction effects, a risk variable consisting of the number of high-risk carrier genotypes that an individual carries (0, 1, or 2, where 0 is [PON1/AA and PON2/CC], 1 is [PON1/AB, BB and PON2/CC] or [PON1/AA and PON2/CS, SS], and 2 is [PON1/AB,BB and PON2/CS,SS]) was fitted to the data. With the 0, or low-risk, carrier genotype as the referent group, then, for the 1 high-risk genotype the OR was 1.0 (95% CI = 0.1–2.0) and for the 2 high-risk genotype the OR was 3.2 (95% CI = 2.3–4.1), with overall $P < .0001$. The same result was obtained if this variable was fitted as a linear effect. However, as for the PON1 × PON2 interaction effect, the addition of an interaction effect (an indicator variable for the presence of the double-high-risk genotype) did not significantly improve the fit to the data (P = .73). Although the interaction effects were not significant, the OR estimates (table 4) were suggestive of an interaction between the PON1 and the PON2 polymorphisms in affecting CHD risk.

Given that the PON1 and the PON2 genes are linked on chromosome 7, an interaction effect may be due to linkage disequilibrium between these two loci. However, no significant linkage disequilibrium (P = .088) was seen in controls (tested by omitting the double heterozygotes) (table 6). There was, however, significant linkage disequilibrium in the cases (P = .0007), which may be a result of ascertainment with an artificial selection against the *PON1*A-PON2*C* low-risk combination. Linkage disequilibrium was estimated to be D = .023 for controls and D = .073 for cases (D is estimated as described by Hill [1974]).

Relationship between Genotypes and Lipid Variables

Table 7 shows the distribution of mean ± SE values for age, BMI, and quantitative lipid and apolipoprotein levels, among different PON2 genotypes, in CHD cases and in controls. No significant difference was present among the genotypes, for any of the traits examined.

Discussion

Three independent studies, including one from our laboratory, have indicated that the PON1 codon 192 polymorphism is a significant risk factor for CHD in

Table 6

PON1/PON2 Haplotype Frequencies

		Observed Frequency/Expected Frequency[a]	
PON1	PON2	Cases[b]	Controls[c]
A	C	.24/.16	.28/.26
A	S	.32/.38	.40/.42
B	C	.05/.14	.10/.12
B	S	.38/.32	.22/.20

[a] Observed haplotype frequencies were calculated with the double-heterozygote individuals (PON1/AB:PON2/CS) omitted; expected haplotype frequencies were calculated under the assumption of no linkage disequilibrium.

[b] $D = .073$, calculated by use of the iterative method of Hill (1995).

[c] $D = .023$, calculated by use of the iterative method of Hill (1995).

populations of Caucasian ancestry (Ruiz et al. 1995; Serrato and Marian 1995; Sanghera et al. 1997). However, this association was not confirmed in two Caucasian samples (Antikainen et al. 1996; Herrman et al. 1996) and one Chinese sample (Sanghera et al. 1997). A possible explanation for this inconsistent association is that the PON1 codon 192 polymorphism is not directly related to CHD risk but is in linkage disequilibrium with a functional mutation that is directly related to CHD risk and that is present elsewhere, either in the PON1 gene or in a nearby gene. Recently, two additional PON genes, PON2 and PON3, have been described that are tightly linked with the existing PON1 gene on chromosome 7 (Primo-Parmo et al. 1996*a*, 1996*b*). Possibly, one of these genes harbors a functional mutation, and the B allele of the PON1 polymorphism merely acts as a marker for this functional mutation. In their preliminary report, Primo-Parmo et al. (1996*b*) predicted a possible polymorphism at codon 311 in the PON2 gene. In the present investigation, we have confirmed the existence of a common polymorphism in the PON2 gene and have investigated its potential involvement in the susceptibility to CHD in an Asian Indian sample from Singapore, in which the *PON1*B* allele was a significant risk factor for CHD (Sanghera et al. 1997).

Our data indicate that the *PON2*S* allele is a significant risk factor for CHD and that its effect is dominant, with both the PON2/CS and the SS genotypes being associated with higher risk (OR = 2.5), compared with that associated with the CC genotype. However, the CHD risk associated with the *PON2*S* allele was confined to individuals who were also carriers of the *PON1*B* allele (OR = 3.6 in *PON1*B* carriers vs. OR = 1.1 in non-*PON1*B* carriers), suggesting the possibility of an interaction between the two polymorphisms. The *PON1*B* allele was not a risk factor among non-*PON2*S* carriers (OR = 0.9), and the same was true for the *PON2*S* allele among non-*PON1*B* carriers (OR = 1.1). Conversely, among *PON1*B* carriers, the *PON2*S* allele was associated with a significantly higher risk for development of CHD (OR = 3.6), and, among *PON2*S* carriers, the *PON1*B* allele was associated with a significant risk (OR = 2.9). Furthermore, the parameter estimates for the interaction model (table 4) and for the high-risk-genotype variable (see the Results section) suggest that the risk for development of CHD is present only in those individuals carrying both high-risk genotypes. However, direct tests for interaction between these loci show no statistically significant effect, nor is there any evidence of the significant linkage dis-

Table 7

Age, BMI, and Lipid and Apolipoprotein Concentrations, among PON2 Genotypes in Controls and Cases

	Controls				Cases			
	Mean ± SE				Mean ± SE			
	CC (n = 33)	CS (n = 80)	SS (n = 76)	P	CC (n = 14)	CS (n = 42)	SS (n = 59)	P
Age (years)	50.6 ± 1.8	48.4 ± 1.2	50.4 ± 1.4	.50	52.3 ± 2.4	57.1 ± 1.4	55.1 ± 1.2	.23
BMI (kg/m^2)	24.9 ± .7	25.1 ± .4	24.9 ± .5	.92	25.9 ± .8	25.0 ± .5	24.3 ± .4	.19
HDL cholesterol (mg/dl)	44.2 ± 3.1	39.2 ± 1.2	42.1 ± 1.5	.24	28.4 ± 2.3	27.4 ± 1.5	27.2 ± 1.0	.90
LDL cholesterol (mg/dl)	166.2 ± 6.2	155.1 ± 4.9	176.6 ± 8.9	.10	166.5 ± 10.8	161.6 ± 5.7	151 ± 5.9	.31
Total cholesterol (mg/dl)	242.2 ± 1.0	223.6 ± 1.0	239.9 ± 1.0	.12	219.5 ± 9.0	220.0 ± 5.8	208.8 ± 6.0	.40
Triglycerides (mg/dl)	154.4 ± 1.1	145.5 ± 1.1	148.4 ± 1.1	.87	126.5 ± 1.1	144.0 ± 1.1	142.6 ± 1.0	.51
Apo A-I (mg/dl)	149.3 ± 5.4	138.7 ± 2.9	141.1 ± 3.2	.21	89.1 ± 1.2	94.6 ± 1.1	86.5 ± 1.0	.70
Apo B (mg/dl)	145.7 ± 5.2	131.8 ± 3.5	136.3 ± 5.0	.18	108.3 ± 6.3	105.9 ± 4.8	102.3 ± 3.4	.74

equilibrium between the two loci that is seen in the controls. The acceptance of the "PON1 and PON2" model, as opposed to either "unigenotype" model—that is "PON1 Only" and "PON2 Only" (table 4)—indicates that, in this sample, both the *PON1*B* allele and the *PON2*S* allele contribute to an individual's susceptibility to CHD. The association of both polymorphisms with the CHD risk was independent of diabetic status, because removal of diabetic individuals from the cases and from the controls did not effect this association.

The physiological mechanism behind these associations is still not clear. However, our previous (Sanghera et al. 1997) and current findings on the lack of association of the PON1 and PON2 polymorphisms with plasma lipid and apolipoproteins suggest that CHD risk is not mediated through the conventional risk factors. However, in view of the accumulating evidence for PON-mediated protection of LDL against oxidative modifications, allelic variations at these loci may be involved in the determination of an individual's susceptibility to LDL oxidation. Possibly, the PON-associated protection against organophosphate poisoning and oxidative damage is mediated by completely different catalytic centers (La Du 1996). Unlike PON1, in which none of the three cysteine residues functions as an active-center cysteine (Sorenson et al. 1995), PON2 may utilize cysteine for its catalytic activity, as proposed by Augustinsson (1968). Therefore, the presence of cysteine at position 311 in the PON2 protein can be hypothesized as a potential activity center for hydrolysis of oxidized lipids, and its substitution by serine may inhibit its catalytic activity in the prevention of oxidative damage. Alternatively, if cysteine at position 311 is not a key component of the activity center for PON2, it may be involved in prevention of stability of the enzyme, by formation of a disulfide bond that could affect its catalytic efficiency. Additional functional studies are required in order to determine the role that cysteine at position 311 has in lipid peroxidation. It is possible that neither the *PON2*S* allele nor the *PON1*B* allele is functional but that, rather, they are in linkage disequilibrium with a yet to be determined functional mutation in the PON gene cluster on chromosome 7. The PON3 gene lies between the other two PON genes, and this may harbor the functional mutation. A recent report of a French diabetic sample has indicated that the codon 55 polymorphism in the PON1 gene is an independent risk factor for CHD and that it can significantly modulate enzyme concentrations (Blatter Garin et al. 1997). However, our data indicate that the codon 55 polymorphism is not associated with CHD (D. K. Sanghera, N. Saha, and M. I. Kamboh, unpublished data). Additional studies are needed in order to detect new mutations in this gene cluster and to identify the functional mutation in regard to the risk of CHD.

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