Allelic Variants of the Human Scavenger Receptor Class B Type 1 and Paraoxonase 1 on Coronary Heart Disease

Genotype–Phenotype Correlations

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Objective—The antioxidant properties of high-density lipoprotein (HDL) have been attributed to paraoxonase (PON) enzyme activity. Human scavenger receptor class B type 1 (SR-BI; CD36 and lysosomal integral membrane protein-II analogous-1 [CLA-1]) plays a central role in HDL-mediated native and oxidized cholesteryl ester uptake. We tested for a significant contribution of common variant of these genes to coronary heart disease (CHD) risk and hypothesized that genetic-mediated PON activity and CLA-1/SR-BI receptor functional properties jointly reduce plasma oxidation status.

Methods and Results—We studied 304 cases and 315 controls. Polymorphisms were analyzed by polymerase chain reaction–restriction fragment analysis. CLA-1/SR-BI–relative expression levels and mRNA stability were analyzed by the comparative threshold cycle method. There was a significant difference in the male genotype distribution of the CLA-1/SR-BI exon 8 (C_8/T_8) variant between groups with an odds ratio of 1.7 (95% CI, 1.16 to 2.51). This significant risk was restricted to those subject carriers of Arg (R) and Leu (L) allele of the PON1 192 and 55 variants and was confirmed in multiple logistic regression analysis. CLA-1/SR-BI mRNA expression levels differed according to CLA-1/SR-BI genotypes.

Conclusions—These data suggest a plausible genetic interaction between the CLA-1 exon 8 gene polymorphism and the risk of CHD in males. (***Arterioscler Thromb Vasc Biol*. 2005;25:1-7.)**

Key Words: paraoxonase ■ arylesterase ■ scavenger receptor class B type 1 ■ polymorphism ■ CD36 and lysosomal integral membrane protein-II analogous-1

Plasma levels of high-density lipoprotein (HDL) cholesterol are inversely related to coronary heart disease (CHD) risk.[1] Nascent HDL removes cholesterol from peripheral tissues by selective uptake.[2] This selective uptake has remained elusive until identification of the mouse scavenger receptor class B type 1 (SR-BI)[3] and its human homologue CD36 and lysosomal integral membrane protein-II analogous 1 (CLA-1).[4] The atheroprotective role of SR-BI has been well established in engineered mice.[5] Several studies demonstrated that CLA-1/SR-BI plays an important role in the bidirectional flux of free cholesterol (FC) and HDL–cholesteryl ester (CE) uptake.[6] Interestingly, in vitro studies have shown a preferential SR-BI–mediated selective uptake of CE hydroperoxides (CEOOHs) compared with unoxidized CE.[7] Several polymorphic variants have been described in the human CLA-1/SR-BI gene.[8] A C→T transition located at cDNA 1050 base position on exon 8 was associated in healthy women with lower low-density lipoprotein (LDL) concentrations and was found linked with a C to T variant at intron 5 of the gene. A glycine to serine substitution in exon 1 of the gene was described and associated with different HDL cholesterol concentrations in healthy men.[8]

It is known that HDL exerts other antiatherogenic properties such as preventing the oxidative modification of LDL.[9,10] These HDL antioxidant properties have been attributed to paraoxonase 1 (PON1)[11,12] and platelet-activating factor acetyl hydrolase (PAF-AH)[13] enzyme activities. PON is a serum esterase entirely complexed to HDL, whereas most of the PAF-AH enzyme is located on the LDLs.[14] PON has also been identified as a homocysteine thiolactonase[15] and possesses PAF-AH–like activity.[16] Although a recent study has shown that PON1 had no phospholipase A_2 activity,[17] conflicting results have been reported.[18] Therefore, the protecting role of PON is the subject of considerable debate.[19–21]

There are allelic variants in the human PON1 gene, a glutamine (Q allele) for arginine (R allele) at codon 192 and a methionine (M allele) to leucine (L allele) at codon 55, that have been studied and associated with susceptibility to

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developing vascular disease.[22,23] The R alloenzyme displays higher activity against paraoxon, whereas the Q alloenzyme displays low activity. Mackness et al[24] showed that the protective effect of HDL from individuals with the PON RR genotype against LDL oxidation was lower than that from subjects with the PON1 QQ genotype. Similar results were obtained by Aviram et al[25] using purified PON Q and R forms. The Met55Leu substitution modulates activity through an effect on PON1 concentration. Arylesterase activity lies on the same protein, correlated with the 55 variant, and is considered an index of protein concentration.[22]

Other studies have proved that the HDL isolated from QQ/MM homozygous subjects have lowest activity toward paraoxon[22,26] and greatest protective capacity toward LDL oxidation in vitro.[26]

Therefore, CLA-1 receptor plays a central role in FC and HDL-CE uptake but a preferential selective uptake of CEOOHs regarding unoxidized CE as described.[6,7] PON seems to prevent oxidation of LDL and HDL by hydrolyzing lipid hydroperoxides (LOOHs).[11,12] These findings prompted us to investigate the role of CLA-1 and the PON1 gene variants in CHD and whether polymorphism-related effects could explain changes in plasma LOOH concentrations and lipid profile.

Materials and Methods

Methods

Participants were selected from the PROCAGENE case-control study.[27] For details on subjects, laboratory procedures; CLA-1/SR-BI and PON1 genotyping, cell isolation and culture; CLA-1/SR-BI expression levels; and mRNA stability studies, please see the online supplement, available at http://atvb.ahajournals.org (file I).

Statistical Analysis

The SPSS statistical software version 11.0 was used for data analysis. Haplotype frequency estimation was evaluated by using Arlequin version 2.000 software.[28]

Results

Main Characteristics of Population Studied

The clinical characteristics are depicted in Table 1. A total of 304 cases (mean age 56±10 years; 22% females) and 315 randomly selected age- and gender-matched community controls (mean age 54.5±11 years; 26% females) were included. Patients showed a significant increase in plasma Lp(a) levels ($P<0.0001$), plasma CE content, and ester ratio ($P<0.0001$), whereas controls showed higher levels of HDL cholesterol ($P<0.001$) and arylesterase activity ($P<0.001$). However, values for diastolic blood pressure, total cholesterol, triglycerides, and LDL cholesterol were lower in cases than in controls.

We tested for significant correlations between enzyme activities and plasma lipid profile in controls because most of our study patients (58.2%) were pharmacologically treated before recruitment. There was a significant correlation between plasma arylesterase and PON activities and HDL levels ($\rho=0.199$; $P<0.001$; n=308 and $\rho=0.252$; $P<0.001$; n=303). PAF-AH activity correlated with LDL values ($\rho=0.568$; $P<0.001$; n=309) and HDL values ($\rho=-0.217$; $P<0.001$; n=311). There was a significant correlation between plasma levels of LOOH and HDL concentrations ($\rho=-0.156$; $P=0.006$; n=305) and PON and arylesterase activities ($\rho=-0.178$; $P=0.002$; n=300 and $\rho=-0.201$; $P=0.001$; n=293).

We performed logistic regression analyses with the main studied variables without inclusion of the PON, arylesterase, and PAF-AH activities. The same analysis was performed excluding LDL and HDL cholesterol values and including

TABLE 1. Main Characteristics of the Cases and Controls

	Cases	Controls	*P*
Sample size, n	304	315	—
Sex (male/female), n	237/67	232/83	0.211
Age, years	56±10	54.5±11	0.062
MI, %	60	—	—
Smoking status, %	50	27.3	<0.001
Diabetes, %	33.9	12.1	<0.001
BMI, Kg/m^2	27.2±3.7	27.3±3.8	0.693
SBP, mm Hg	135±24	136±26	0.954
DBP, mm Hg	76±13	84±12	<0.001
Alcohol, g/day	16±27	11±19	0.011
Total cholesterol, mmol/L	5.2±1.1	6.1±1.0	<0.001
LDL cholesterol, mmol/L	3.5±1.0	4±1.0	<0.001
HDL cholesterol, mmol/L	0.92±0.24	1.3±0.32	<0.001
Triglycerides, mmol/L	1.24±0.67	1.52±0.77	<0.001
Lp(a), mg/dL	53.5±68	36.8±43	<0.001
PON, U/mL	34.5±34	34±29.3	0.827
Arylesterase, U/mL	26.9±9	31±10	<0.001
PAF-AH, U/mL	476±115	529±115	<0.001
CE, mmol/L	3.9±0.9	2.5±1.8	<0.001
LOOH, mmol/L	1.78±1.0	5.1±5.6	<0.001

SBP indicates systolic blood pressure; DBP, diastolic blood pressure.

Values are means±SD except percentages of diabetics, patients with myocardial infarction (MI), and current smokers.

TABLE 2. Haplotypes Frequency Estimation

	Cases	Controls	*P*
PON1 Haplotypes			
Sample size, n	275	303	
RL	0.322	0.286	
RM	0.016	0.025	
QL	0.278	0.321	
QM	0.384	0.367	
			0.682
SR-BI Haplotypes			
Sample size, n	268	303	
C_8C_5	0.508	0.464	
C_8T_5	0.117	0.087	
T_8C_5	0.367	0.415	
T_8T_5	0.008	0.033	
			0.102

Subscript indicates the corresponding allele of the intron 5 or exon 8 variants.

TABLE 3. Multivariate Models for Total Population and Men

A. Total Population	Without Genotypes		CC*R Allele		CC*R and L Alleles	
	OR	95% CI	OR	95% CI	OR	95% CI
BMI	0.928	0.863–0.999	0.928	0.862–0.999	0.927	0.861–0.999
Tobacco	2.350	1.392–3.968	2.411	1.421–4.089	2.405	1.418–4.079
Alcohol	1.019	1.007–1.032	1.019	1.006–1.031	1.018	1.006–1.031
HDL cholesterol	0.001	0.000–0.004	0.001	0.000–0.004	0.001	0.000–0.004
Hypertension	2.680	1.583–4.538	2.553	1.500–4.344	2.559	1.503–4.354
Triglycerides	0.225	0.151–0.335	0.218	0.145–0.327	0.218	0.145–0.327
Lp(a)	1.007	1.002–1.013	1.007	1.002–1.013	1.007	1.002–1.013
Diabetes	3.759	2.034–6.948	3.657	1.975–6.772	3.636	1.963–6.734
CC*R allele			1.987	1.028–3.842		
CC*R and L alleles					2.081	1.068–4.053
Adjustment		84.3%		85%		85%

B. Men	Without Genotypes		CLA-1 CC		CC*R Allele		CC*R and L Alleles	
	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI
Tobacco	2.642	1.538–4.537	3.037	1.670–5.525	3.082	1.693–5.610	3.070	1.686–5.587
Alcohol	1.017	1.006–1.029	1.025	1.001–1.039	1.024	1.011–1.038	1.024	1.010–1.038
HDL cholesterol	0.001	0.000–0.003	0.001	0.000–0.003	0.001	0.000–0.003	0.001	0.000–0.003
Triglycerides	0.217	0.144–0.327	0.182	0.111–0.297	0.185	0.114–0.300	0.185	0.114–0.300
Lp(a)	1.007	1.001–1.013	1.008	1.002–1.014	1.008	1.002–1.014	1.008	1.002–1.014
Diabetes	2.254	1.176–4.319	2.431	1.209–4.889	2.289	1.134–4.620	2.276	1.128–4.595
CLA-1 CC			2.245	1.322–4.092				
CC*R allele					2.657	1.285–5.496		
CC*R and L alleles							2.729	1.314–5.672
Adjustment		82.9%		84.5%		84.7%	84.5%	

Data are for Wald's test.

CC indicates mean C_8C_8 homozygotes for the C1050T polymorphism of the CLA-1 gene; R allele mean, R allele carriers of the PON1 Gln192Arg variant; R and L alleles, mean carriers of the R and L alleles of PON1 Gln192Arg and Met55Leu polymorphisms.

PON, arylesterase, and PAF-AH activities Those significant variables in the first analysis remained significant in the second analysis. A marked protecting value was obtained for HDL cholesterol concentrations instead of arylesterase activity. We estimated that an increase from 0.9 to 1.25 mmol/L in HDL cholesterol causes a 16.7% reduction of coronary event.

Genotype Distribution

The genotype distribution of CLA-1–analyzed polymorphisms did not differ from that expected in Hardy–Weinberg equilibrium (HWE). Genotype distribution of the CLA-1 exon 8 variant was statistically different among patients and controls. Those CLA-1 C_8C_8 homozygote subjects had a significant CHD risk with an odds ratio (OR) of 1.47 (95% CI, 1.05 to 2.07). There were no differences in the genotype distribution of CLA-1 intron 5 and exon 1 variants between cases and controls. The exon 8 and intron 5 variants were in linkage disequilibrium ($P<0.001$).[8] Inferred haplotypes are depicted in Table 2. We observed gender-dependent differences regarding genotype distribution for the exon 8 variant, with a significant CHD risk for men C_8C_8 homozygotes with an OR of 1.705 (95% CI, 1.16 to 2.51) but not in women with an OR of 0.806 (95% CI, 0.38 to 1.69).

In multiple logistic regression analysis, we obtained an OR of 2.245 (95% CI, 1.32 to 4.09) for those male C_8C_8 homozygous (Table 3).

Genotype distribution of the PON1 Gln192Arg polymorphism was in HWE. A significant deviation from HWE for the PON1 Met55Leu genotypes was observed, probably because an excess of heterozygotes was obtained. Both variants were in linkage disequilibrium ($P<000.1$). No association between PON1 polymorphisms and CHD was found. The genotype distribution of subjects who were carriers of PON1 R and L alleles versus the remaining genotype combinations among cases and controls did not differ in the total population or after gender stratification.

Genotype–Genotype Interaction

We analyzed the genotype–genotype interaction, considering separately those male R allele carriers versus those QQ homozygotes and evaluating the genotype distribution of the CLA-1 exon 8 C1050T polymorphism and, conversely, the genotype distribution of the PON1 (Gln192Arg) variant, considering separately those CC homozygotes and T allele carriers of the exon 8 variant between cases and controls. Thus, the CHD risk associated to C_8C_8 homozygotes of the

CLA-1 gene was significantly different in those R allele carriers of the PON1 Gln192Arg gene polymorphism (χ_1^2=6.078; P=0.014) with an OR of 1.948 (95% CI, 1.143 to 3.32). A similar analysis was performed stratifying the PON1 Met55Leu as Leu allele carriers and MM homozygotes and evaluating the C1050T CLA-1 gene distribution and conversely evaluating the Met55Leu gene distribution according to C_8C_8 homozygotes and T_8 allele carriers of the exon 8 variant. We observed a significant difference in the genotype distribution of the CLA-1 exon 8 variant in those L allele carriers (χ_1^2=5.705; P=0.017) with an OR of 1.657 (95% CI, 1.093 to 2.511) but not in those MM homozygotes. On the other hand, no differences were observed between PON1 Met55Leu genotypes and CHD in either those T_8 allele carriers or C_8C_8 homozygotes. Because of the linkage disequilibrium between the PON1 gene variants, we analyzed the genotype distribution of the CLA-1 exon 8 C1050T gene variant, stratifying according to PON1 genotypes dichotomized as those R and L allele carriers and the remaining possible PON1 genetic combinations between groups. The CHD risk associated with the C_8C_8 genotype was 2.061 (95% CI, 1.198 to 3.545) and confined to those R and L allele carriers of the PON1 variants, whereas no statistical difference was obtained for those non-R, non-L allele carriers. A trend was observed only in the genotype distribution of those R and L allele carriers in the subgroup of C_8C_8 homozygotes for the C1050T gene polymorphism of the CLA-1 gene but not in those non-R non-L allele carriers. For more detailed data, see supplemental files II and III (available online at http://atvb.ahajournals.org).

Genotype–Phenotype Associations

Genotype–phenotype associations were evaluated in controls. Genotypes of the CLA-1 studied variants showed no differences when evaluated in relation to lipid profiles. We found significant differences regarding basal and after-copper sulfate treatment in LOOH levels for those C_5C_5 versus T_5 allele carriers of the intron 5 variant (P=0.008 and P=0.011), with lower levels for those C_5C_5 homozygote subjects. In addition, there was a graduation in plasma LOOH values according to haplotypes (Figure 1).

There were significant differences in PON and arylesterase activities according to PON1 Gln192Arg and Met55Leu polymorphisms. No differences regarding basal and after-copper sulfate treatment of LOOH levels between RRLL and QQMM subjects were observed. In addition, there were no differences according to PON1 haplotypes in plasma PAF-AH values or LOOH concentration values.

CLA-1/SR-BI Expression Levels and mRNA Stability

We analyzed the relative amount of CLA-1/SR-BI mRNA levels in peripheral blood mononuclear cells (PBMCs) isolated from subjects genotyped previously as T_8T_8/C_5C_5, C_8T_8/C_5T_5 and C_8C_8/C_5C_5 of exon 8 and intron 5 variants, respectively. CLA-1/SR-BI– and GAPDH-amplified products showed similar linearity and efficiency. Both parameters were assessed using standard curves generated by increasing amounts of total RNA ranging from 0.06 to 1 μg (Figure 2).

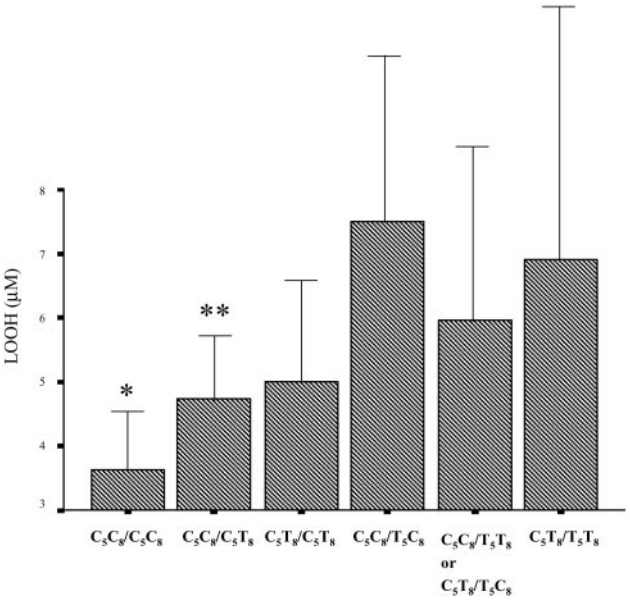


Figure 1. LOOH concentration values according to CLA-1/SR-BI haplotypes. Subscript indicates the corresponding allele of the intron 5 or exon 8 variants. Values are means±SD. *P=0.007; **P=0.038 for the Mann–Whitney U test comparing LOOH concentrations of the indicated haplotype vs C_5C_8/T_5C_8 haplotype.

Relative quantitation results are depicted in Figure 3. As shown, there was a significant difference in CLA-1/SR-BI basal expression levels between exon 8 C_8C_8 and T_8T_8 homozygous (3.3-fold). A similar difference was observed between exon 8 C_8T_8 heterozygous and T_8T_8 (5.9-fold) homozygous. The CLA-1/SR-BI mRNA stability was analyzed in cultured monocytes/macrophages. Experiments were performed in previously genotyped $T_8T_8C_5C_5$ and $C_8C_8C_5T_5$ cells. Total RNA was analyzed in control versus treated cells by relative quantitation. At the time point indicated, the relative expression of CLA-1/SR-BI mRNA did not reach statistical significance, but a trend was observed (P=0.06).

Discussion

We describe for the first time that the CLA-1 exon 8 (C1050T) gene polymorphism contributes per se to CHD risk in our male population.

Previous studies have reported different lipoprotein profiles and lipoprotein particle size associated with the 3 CLA-1/SR-BI–analyzed variants.[8,29] Acton et al[8] reported significant differences in LDL cholesterol and body mass index (BMI) according to the exon 8 and intron 5 variants in women. Our analysis did not reveal any sex-related difference in BMI and lipid profiles according to the CLA-1/SR-BI variants, probably because we analyzed an older population. Acton et al[8] reported that the association with BMI was more evident in premenopausal women, a finding that suggests hormonal regulation of the CLA-1/SR-BI gene.

We also found a significant difference in LOOH content according to CLA-1 genotypes and haplotypes. Because there is no amino acid change because of C_8 to T_8 substitution, it is possible that the C1050T polymorphism could constitute a marker of other functional polymorphisms. This possibility was also discussed by Acton et al.[8] These authors sequenced

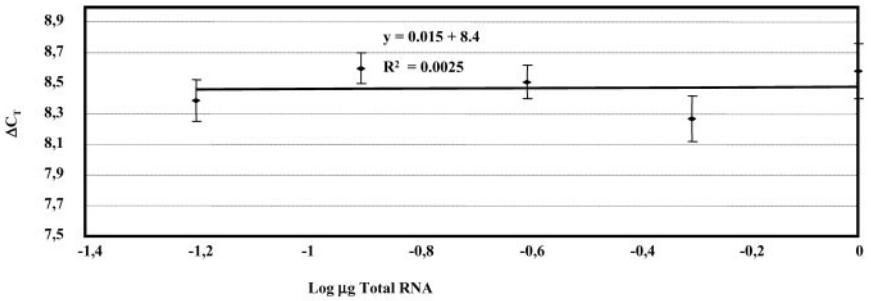
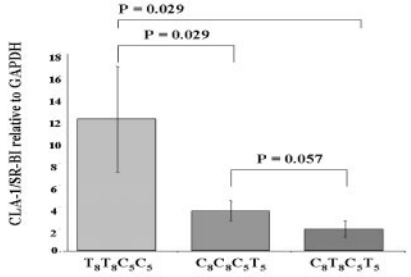


Figure 2. $\Delta\Delta C_T$ validation experiment. Plot of logarithm of total RNA amount vs ΔC_T. Slope and statistical value are assessed using LightCycler software. Data are means±SD of an experiment replicated 3×.

Total RNA (µg)	CLA-1/SR-BI Average C_T	GAPDH Average C_T	ΔC_T (CLA-1/SR-BI – GAPDH)
1.0	32.22 ± 0.17	23.64 ± 0.06	8.58 ± 0.18
0.5	32.66 ± 0.15	24.39 ± 0.03	8.27 ± 0.15
0.25	34.01 ± 0.11	25.5 ± 0.04	8.51 ± 0.11
0.12	34.78 ± 0.08	26.18 ± 0.06	8.6 ± 0.1
0.06	36.08 ± 0.13	27.69 ± 0.04	8.39 ± 0.136

the entering CLA-1 coding region in 3 individuals but did not find a functional mutation, and hypothesized that other genetic variants located at the 12q24 region could be linked with the phenotypic changes associated with polymorphisms. New variants have been characterized recently in the promoter region of the human CLA-1 gene.[30] An interesting 11-bp (−140 to −150) insertion/deletion promoter variant was described. Hsu et al[30] showed that this variant significantly influenced CLA-1 transcriptional gene activity. From our total population, 200 subjects were selected at random and genotyped for the 11-bp insertion/deletion. We found that the frequency of the deleted variant was low but similar to the 0.02 described previously. However, our analyses did not reveal any linkage with the exon 8 or intron 5 polymorphisms (data not shown). A larger population will be necessary to rule out this possibility. In addition, several other possibilities remain unexplored, including changes in the structure and stability of CLA-1 mRNA. Thus, we found in isolated PBMCs a significant difference in basal CLA-1/SR-BI mRNA expression levels according to CLA-1 genotypes. Those T_8T_8 and C_5C_5 homozygous subjects showed higher CLA-1/SR-BI mRNA expression levels than those C_8 and T_5 allele carriers. It was difficult for us to detect which of the linked studied variants was the main determinant of this difference because among all studied donors from which PBMCs were isolated, no T_5T_5 homozygous subjects of the intron 5 variant were found. Our findings concur with previous studies showing an atheroprotective role for CLA-1/SR-BI and could partially explain the CHD risk associated with the C1050T gene polymorphism. Total plasma LOOH



Genotypes	RNA (µg)	CLA-1/SR-BI Average C_T	GAPDH Average C_T	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
$T_8T_8C_5C_5$	**0.5**	28.81 ± 1.20	22.02 ± 1.2	6.79 ± 1.62	-3.22 ± 1.62	9.32 (7.7-10.94)
$T_8T_8C_5C_5$	**0.25**	29.24 ± 2.4	22.84 ± 1.5	6.4 ± 2.8	-3.52 ± 2.8	11.47 (8.67-14.27)
$T_8T_8C_5C_5$	**0.12**	27.69 ± 1.04	22.09 ± 0.7	5.6 ± 1.06	-4.32 ± 1.06	19.97 (18.91-21.03)
$T_8T_8C_5C_5$	**0.06**	29.38 ± 0.2	22.35 ± 0.06	7.03 ± 0.21	-2.89 ± 0.21	7.41 (7.2-7.62)
$C_8C_8C_5T_5$	**0.5**	30.29 ± 1.5	22.12 ± 1.1	8.17 ± 1.51	-1.75 ± 1.51	3.36 (1.85-4.87)
$C_8C_8C_5T_5$	**0.25**	31.97 ± 2.04	23.9 ± 0.36	8.07 ± 2.07	-1.85 ± 2.07	3.60 (1.53-5.67)
$C_8C_8C_5T_5$	**0.12**	33.68 ± 1.18	25.14 ± 0.91	8.54 ± 1.5	-1.38 ± 1.5	2.60 (1.1-4.1)
$C_8C_8C_5T_5$	**0.06**	34.52 ± 1.75	26.94 ± 0. 61	7.58 ± 1.85	-2.34 ± 1.85	5.06 (3.21-6.91)
$C_8T_8C_5T_5$	**0.5**	35.01 ± 2.09	26.66 ± 0.33	8.35 ± 2.12	-1.57 ± 2.11	2.97 (0.86-5.08)
$C_8T_8C_5T_5$	**0.25**	37.06 ± 1.11	27.14 ± 0.22	9.92 ± 1.13	0 ± 1.13	1 (-0.13-2.13)
$C_8T_8C_5T_5$	**0.12**	35.88 ± 0.12	27.23 ± 0.15	8.65 ± 0.19	-1.27 ± 0.19	2.41 (2.22-2.6)
$C_8T_8C_5T_5$	**0.06**	37.63 ± 0.13	28.44 ± 0.07	9.19 ± 0.15	-0.73 ± 0.15	1.66 (1.51-1.81)

Figure 3. Relative quantification results using the C_T method. Different RNA concentrations were used. Data are means±SD of $T_8T_8C_5C_5$ (n=5); $C_8C_8C_5T_5$ (n=5). $C_8T_8C_5T_5$ (n=5) samples replicated 2×.

content also differed according to CLA-1 genotypes, and higher levels were found in those T_8T_8 homozygous subjects. Thus, it seems that those subjects with higher CLA-1/SR-BI mRNA levels also presented high plasma levels of total plasma LOOHs. There are several reasons that could help to explain this paradox. First, we used a bulky method for detecting total plasma LOOHs. Also, there is considerable disagreement regarding LOOH levels and lipoprotein subfraction location, and it is difficult to explain this finding without measuring LOOHs in isolated lipoprotein subfraction.[31,32] Although CLA-1/SR-BI receptor has a broad substrate range, it shares a highly efficient selective uptake of CEOOHs regardless of CE. In our study, its activity was indirectly evaluated. It is known that human plasma lipoproteins are heterogenous in their CE and phospholipid content, and these molecules contain a large proportion of peroxidizable fatty acid. Finally, there are other determinants of total plasma LOOHs, including the genetic-determined PON enzyme activity.

In our study, there was no association of PON1 genotypes with CHD. However, we found that the CHD risk associated with C_8C_8 homozygosis of CLA-1 (C1050T) polymorphism was confined to the subset of individuals who were also carriers of R and L alleles of the PON1 variants. Conversely, the CHD risk associated with the PON1 gene variants was statistically different only in the subset of individual C_8C_8 homozygotes for the CLA-1 polymorphism.

Several studies have suggested that HDL-associated PON activity protects against atherosclerosis in part by inhibiting the oxidative modification of LDL.[11,12,14,24,26] However, PON1 variants have been inconsistently associated with CHD risk.[21,33,34] Thus, it seems that gene environment or gene–gene interactions modulate the CHD risk associated with the PON1 polymorphisms. Previously, the PON1 Met55Leu variant has been associated with reduced HDL-associated PAF-AH activity.[18] We did not obtain significant associations of the studied PON1 gene variant with plasma total LOOHs or PAF-AH enzyme activity. Most PAF-AH activity lies on the LDL particle, but HDL, to a lesser degree (<20%), also expresses PAF-AH activity.[13] Again, it is difficult to evaluate the biological meaning of PAF-AH measurements in serum samples without lipoprotein particle isolation. Nevertheless, we did not detect changes in PAF-AH activity not related to variations in LDL cholesterol values.

In summary, it has been demonstrated that CLA-1/SR-BI mediates the selective uptake of HDL-CE.[1,6] Furthermore, HDL containing oxidized CE may transfer it to the liver in an antiatherogenic pathway, and some studies showed a preferential CLA-1/SR-BI–selective uptake of oxidized CE regarding native CE.[7] PON1 gene variants have been associated with different degrees of protection against lipid peroxidation.[11] Thus, it is plausible that functional differences in CLA-1/SR-BI basal mRNA expression and activity linked to those reported differences associated with the PON1 gene–studied variants could explain the genetic interaction described here.

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Arteriosclerosis, Thrombosis, and Vascular Biology

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Allelic Variants of the Human Scavenger Receptor Class B Type 1 and Paraoxonase 1 on Coronary Heart Disease. Genotype-Phenotype Correlations

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METHODS

Subjects: All participants were selected from the PROCAGENE study, a case-control trial carried out in Gran Canaria between January, 1996 and December, 1998 to evaluate the role of gene variants on CHD risk. The controls sample design and cases inclusion selection criteria have been previously published. [1] Cases were recruited from Gran Canaria hospitals with a diagnosis of myocardial infarction (MI) or unstable angina and documented evidence of CHD by angiography. All consecutive incident cases admitted to the coronary unit were considered within the study period. A two-stage stratified selection process from the Gran Canaria population census randomly ascertained controls. Participants underwent a structured clinical interview and a physical exam as well as diagnostic tests (electrocardiogram and echocardiogram) to rule out cardiovascular disease. The study was approved by the Ethics Observational Studies Board at the Hospital *de Gran Canaria Dr. Negrín*. All participants provided written informed consent. The present study was conducted in subjects from which serum and DNA samples were available.

Laboratory Procedures: Total cholesterol, HDL-cholesterol, and triglycerides were measured by enzymatic-colorimetric methods. Plasma free cholesterol content was determined by an enzymatic-colorimetric method (Wako Chemicals USA, Inc). CE content was estimated as total cholesterol minus free–cholesterol. LDL-cholesterol was calculated according to the Friedewald formula when triglyceride levels were ≤ 4.52 mmol/L. Lipoprotein (a) [Lp(a)] was analyzed with an immunoturbidimetric method (Boehringer Mannheim Gmbh, Mannhein, Germany).

Total plasma lipid hydroperoxide (LOOH) content was calculated by the Xylenol Orange (FOX) assay as described. [2] Briefly, plasma samples stored at –70ºC, were analyzed under basal conditions or mixed with copper sulphate at a final concentration of 10μM at 37ºC for 24 h, using triphenyl phosphine (TPP) for signal authentication. The absorbance of the supernatants was monitored at 560 nm and the hydroperoxide content determined using a molar absorption coefficient of $4.3 \times 10^4\ M^{-1}.\ cm^{-1}$ or by reference to an H_2O_2 standard curve.

Paraoxonase and arylesterase activities were determined as previously described using molar absorption coefficients of $1.7 \times 10^4\ M^{-1}.\ cm^{-1}$ and $1310\ M^{-1}.\ cm^{-1}$ respectively. [3,4]

The Platelet-Activating Factor acetyl hydrolase activity (PAF-AH) was determined by the spectrophotometric assay developed by Kosaka, et al. [5] (Diagnostic Research & Development Department, R&D Division, Nesco Company, Aswell Inc., Osaka, Japan).

CLA-1/SR-BI and Paraoxonase-1 Single-Nucleotide Polymorphisms Genotyping: DNA was extracted from peripheral blood leukocytes by standard procedures. The following CLA-1 gene polymorphisms were genotyped, the G/A swap in exon 1 which causes a glycine to serine change, the C_8/T_8 transition located at 1050 cDNA base position in exon 8 and the C_5/T_5 transition located at intron 5 of the gene (Genbank mRNA accession number NM 005505; contig accession number NT 009755). [6] PON1 Gln192Arg and Met55Leu genotyping was performed using PCR-RFLP as described. [7,8] Genetic data were available for all tested polymorphisms in 268 cases and 297 controls.

Cell Isolation and Culture: Peripheral blood mononuclear cells (PBMCs) were isolated from normal donors by density-gradient centrifugation through Ficoll/Hypaque (Pharmacia). Furthermore, isolated cells were culture in 30-mm plates with 5 ml of RPMI 1640 medium containing 10% (vol/vol) normal heat inactivated calf serum and antibiotics. Cells were allowed to adhere overnight in the presence of 5% CO2 in a humidified incubator at 37^{o}C. No-adherent cells were washed away twice with phosphate-buffered saline (PBS). Typically 5 x 10^6 adherent cells were obtained.

CLA-1/SR-BI expression levels and mRNA Stability Studies: Total RNA was prepared from PBMCs and cultured monocytes/macrophages as described. [9] Cultures were treated with 5μg of actinomycin D or vehicle for 12h prior to RNA isolation. Reverse transcription of mRNA was carried out in 20 μl final volume using MMLV reverse transcriptase (Roche Applied Science), following the manufacter´s instructions. CLA-1/SR-BI mRNA expression levels were compared among C_8C_8 homozygous, C_8T_8 heterozygous and T_8T_8 homozygous subjects for the exon 8 variant by the comparative threshold cycle (C_T) quantitation method. We used a LightCycler and the LC Fast Start DNA Master SYBR Green Kit (Roche Applied Science). All C_8C_8 homozygous subjects for the exon 8 polymorphism were also T_5T_5 homozygous for the intron 5 variant whereas all C_8T_8 and T_8T_8 subjects for the exon 8 variant were C_5T_5 heterozygous for

the intron 5 variant because no T_5T_5 homozygous was found. For the comparative C_T method, a 251 base pair (bp) fragment of the CLA-1/SR-BI gene was amplified by PCR at 3mM Cl_2Mg concentration with the forward primer 5´GAAACTGCAGCTGAGCCTCT3´ and the reverse primer 5´ATTTCTCTTGGCTCCGGATT3´ (spanning CLA-1/SR-BI base pairs 1227-1477) [Genbank accession number NM_005505]. Normalization was performed against a 147 bp amplified fragment of the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) with the forward primer 5´GGCCTCCAAGGAGTAAGACC3´ and the reverse primer 5´AGGGGTCTACATGGCAACTG3´ at 2mM Cl_2Mg final concentration (spanning GAPDH 1031-1177 bp) [Genbank accession number BC020308]. PCR product specificities were analyzed by melting curve analysis and by standard agarose gel electrophoresis. The comparative C_T method was also used to measure CLA-1/SR-BI mRNA stability for a given genotype combination in control versus actinomycin D treated cells (12 hours). Experiments were three times replicated with pooled cDNA obtained by mixing four samples from each previously genotyped cells as $T_8T_8C_5C_5$ and $C_8C_8C_5T_5$ for the exon 8 and intron 5 variants respectively.

Statistical Analysis: The SPSS statistical software package, version 11.0 for Windows was used for data analysis. Haplotype frequencies estimation were evaluated by the Maximun Likelihood method using Arlequin version 2.000 software. [10] Quantitative variables are presented as mean ± standard deviation and qualitative variables as percentages. Assumption of normal distribution for continous variables was tested by Kolmogorov-Sminorv z statistics. Normally distributed continuous variables were compared by 1-way ANOVA whereas the Kruskal-Wallis test was used for comparisons of non-normally distributed variables. Means and pairwise comparisons were performed by unpaired Student´s t test.and the Whitney U-test when appropiate. Spearman rank correlations determined the association of continuous variables. Hardy-Weinberg equilibrium was assessed by chi-square analysis. Chi-square test and odds ratios (OR) with 95% of confidence interval (CI) analyses were carried out to estimate the risk of CHD associated with categorical variables and the analyzed polymorphisms. To assess the independent variables predictor ability for CHD, we performed logistic regression analysis

using the Wald Stepwise method. Two main groups of regression models were constructed. The first established model included the following independent categorical variables: habitat, smoking status, arterial hypertension (HT), diabetes and pharmacological treatment. Alcohol intake, triglycerides levels, Lp(a) levels, PAF-AH activity, paraoxonase and arylesterase activities and total plasma LOOH levels were introduced as continuous variables. To avoid co-linearity when HDL-cholesterol was included, arylesterase activity was excluded from the analysis. In a similar way, when LDL-cholesterol was introduced PAF-AH activity was excluded from the model. A second group of models was established with the genotypes for a given polymorphism, as a dummy variable and in combination with the genotypes of the other analyzed polymorphisms.

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II. (a) Male CLA-1 (C1050T) genotype distribution between cases and controls stratified by QQ homozygotes and R allele carriers of the PON1 (192) variant and (b) male distribution of PON1 (192) genotypes between cases and controls stratified by CC homozygotes and T allele carriers of CLA-1 (C1050T) variant

	C1050T		T allele carriers	CC
PON1 (192)	QQ homozygotes	Cases	54 (58.7%)	38 (41.3%)
		Controls	72 (67.9%)	34 (32.1%)
		$\chi_1^2 = 1.813$; P = 0.178		
	R allele carriers	Cases	59 (51.3%)	56 (48.7%)
		Controls	78 (67.2%)	38 (32.8%)
		$\chi_1^2 = 6.078$; P = 0.014; OR = 1.95 (1.14-3.32)		

	PON1 (192)		R allele carriers	QQ
CLA-1 (C1050T)	CC homozygotes	Cases	56 (59.6%)	38 (40.4%)
		Controls	38 (52.8%)	34 (47.2%)
		$\chi_1^2 = 0.767$; P = 0.381		
	T allele carriers	Cases	59 (52.2%)	54 (47.8%)
		Controls	78 (52.0%)	72 (48.0%)
		$\chi_1^2 = 0.001$; P = 0.973		

T allele mean T carriers (CT + TT) of the CLA-1 (C1050T) gene polymorphism. R allele mean R allele carriers (QR + RR) of the PON1 Gln192Arg at codon 192.