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 variants 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/T) 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;25:854-860.)

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. Nascent HDL removes cholesterol from peripheral tissues by selective uptake. This selective uptake has remained elusive until identification of the mouse scavenger receptor class B type 1 (SR-BI) and its human homologue CD36 and lysosomal integral membrane protein-II analogous 1 (CLA-1). The atheroprotective role of SR-BI has been well established in engineered mice. 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. Interestingly, in vitro studies have shown a preferential SR-BI–mediated selective uptake of CE hydroperoxides (CEOOHs) compared with unoxidized CE. Several polymorphic variants have been described in the human CLA-1/SR-BI gene. 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. It is known that HDL exerts other antiatherogenic properties such as preventing the oxidative modification of LDL. These HDL antioxidant properties have been attributed to paroxonase 1 (PON1) and platelet-activating factor acetyl hydrolase (PAF-AH) enzyme activities. PON is a serum esterase entirely complexed to HDL, whereas most of the PAF-AH enzyme is located on the LDLs. PON has also been identified as a homocysteine thiolactonase and possesses PAF-AH–like activity. Although a recent study has shown that PON1 had no phospholipase A2 activity, conflicting results have been reported. Therefore, the protecting role of PON is the subject of considerable debate. 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...
developing vascular disease.\textsuperscript{22,23} The R alloenzyme displays higher activity against paraoxon, whereas the Q alloenzyme displays low activity. Mackness et al\textsuperscript{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\textsuperscript{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.\textsuperscript{22}

Other studies have proved that the HDL isolated from QQ/MM homozygous subjects have lowest activity toward paraoxon\textsuperscript{22,26} and greatest protective capacity toward LDL oxidation in vitro.\textsuperscript{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.\textsuperscript{5,7} PON seems to prevent oxidation of LDL and HDL by hydrolyzing lipid hydroperoxides (LOOHs).\textsuperscript{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.

\section*{Materials and Methods}

\subsection*{Methods}

Participants were selected from the PROCAGENE case-control study.\textsuperscript{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).

\subsection*{Statistical Analysis}

The SPSS statistical software version 11.0 was used for data analysis. Haploview frequency estimation was evaluated by using Arlequin version 2.00 software.\textsuperscript{28}

\section*{Results}

\subsection*{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 LOOH, mmol/L 1.78 ± 1.0 5.1 ± 5.6 < 0.001

\begin{table}
\centering
\caption{Main Characteristics of the Cases and Controls}
\begin{tabular}{lccc}
\hline
 & Cases & Controls & $P$ \\
\hline
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 ± 7 & 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 ± 5.6 & < 0.001 \\
\hline
\end{tabular}
\end{table}

\begin{table}
\centering
\caption{Haplotypes Frequency Estimation}
\begin{tabular}{lccc}
\hline
 & Cases & Controls & $P$ \\
\hline
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 \\
\hline
SR-BI Haplotypes & & & \\
Sample size, n & 268 & 303 & \\
C5C5 & 0.508 & 0.464 & \\
C5T5 & 0.117 & 0.087 & 0.102 \\
T5C5 & 0.367 & 0.415 & \\
T5T5 & 0.008 & 0.033 & \\
\hline
\end{tabular}
\end{table}

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.

\section*{Supplementary Table 2. Haplotypes Frequency Estimation}

This table shows the frequencies of different haplotypes for PON1 and SR-BI genes in both cases and controls. The table indicates the distribution of different combinations of alleles across the population, highlighting the significance of certain haplotypes in relation to specific traits or conditions.
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 C8C8 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 C8C8 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 C8C8 homozygous (Table 3).

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 C8C8 homozygous

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**Table 3. Multivariate Models for Total Population and Men**

<table>
<thead>
<tr>
<th>A. Total Population</th>
<th>Without Genotypes</th>
<th>CC*R Allele</th>
<th>CC*R and L Alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>OR</td>
<td>95% CI</td>
<td>OR</td>
<td>95% CI</td>
</tr>
<tr>
<td>BMI</td>
<td>0.928</td>
<td>0.863–0.999</td>
<td>0.928</td>
</tr>
<tr>
<td>Alcohol</td>
<td>1.019</td>
<td>1.007–1.032</td>
<td>1.019</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>0.001</td>
<td>0.000–0.004</td>
<td>0.001</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.225</td>
<td>0.151–0.335</td>
<td>0.218</td>
</tr>
<tr>
<td>Lp(a)</td>
<td>1.007</td>
<td>1.002–1.013</td>
<td>1.007</td>
</tr>
<tr>
<td>CC*R allele</td>
<td>1.987</td>
<td>1.028–3.842</td>
<td>2.081</td>
</tr>
<tr>
<td>CC*R and L alleles</td>
<td>84.3%</td>
<td>85%</td>
<td>2.081</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B. Men</th>
<th>Without Genotypes</th>
<th>CLA-1 CC</th>
<th>CC*R Allele</th>
<th>CC*R and L Alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>OR</td>
<td>95% CI</td>
<td>OR</td>
<td>95% CI</td>
<td>OR</td>
</tr>
<tr>
<td>Alcohol</td>
<td>1.017</td>
<td>1.006–1.029</td>
<td>1.025</td>
<td>1.001–1.039</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>0.001</td>
<td>0.000–0.003</td>
<td>0.001</td>
<td>0.000–0.003</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.217</td>
<td>0.144–0.327</td>
<td>0.182</td>
<td>0.111–0.297</td>
</tr>
<tr>
<td>Lp(a)</td>
<td>1.007</td>
<td>1.001–1.013</td>
<td>1.008</td>
<td>1.002–1.014</td>
</tr>
<tr>
<td>CLA-1 CC</td>
<td>2.245</td>
<td>1.322–4.092</td>
<td></td>
<td>2.276</td>
</tr>
<tr>
<td>CC*R and L alleles</td>
<td>82.9%</td>
<td>84.5%</td>
<td></td>
<td>84.7%</td>
</tr>
</tbody>
</table>

Data are for Wald’s test.
CC indicates C8C8 homozygotes for the C1050T polymorphism of the CLA-1 gene; R allele, 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.

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CLA-1 gene was significantly different in those R allele carriers of the PON1 Gln192Arg gene polymorphism \((\chi^2=6.078; P=0.014)\) 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\(_6\)C\(_8\) 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 \((\chi^2=5.705; P=0.017)\) 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\(_6\)C\(_8\) 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\(_6\)C\(_8\) 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\(_6\)C\(_8\) 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\(_6\)C\(_8\) versus T\(_8\) allele carriers of the intron 5 variant \((P=0.008\) and \(P=0.011)\), with lower levels for those C\(_6\)C\(_8\) homozygous 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 RRL and QMM 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\(_8\)T\(_8\)/C\(_6\)C\(_8\), C\(_6\)T\(_8\)/C\(_6\)T\(_8\), and C\(_6\)C\(_8\)/C\(_6\)C\(_8\) 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 \(\mu\)g (Figure 2).

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\(_6\)C\(_8\) and T\(_8\)T\(_8\) homozygous (3.3-fold). A similar difference was observed between exon 8 C\(_6\)T\(_8\) heterozygous and T\(_8\)T\(_8\) (5.9-fold) homozygous. The CLA-1/SR-BI mRNA stability was analyzed in cultured monocytes/macrophages. Experiments were performed in previously genotyped T\(_8\)T\(_8\)C\(_6\)C\(_8\) and C\(_6\)C\(_8\)/C\(_6\)C\(_8\) 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.\(^8,20\) 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\(_6\) 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...
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 pro-
moter region of the human CLA-1 gene.30 An interesting
11-bp (140 to 150) insertion/deletion promoter variant
was described. Hsu et al30 showed that this variant signifi-
cantly 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 T8T8 and C5C5 homozygous subjects showed higher
CLA-1/SR-BI mRNA expression levels than those C8 and T5
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 T5T5 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

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>RNA (µg)</th>
<th>CLA-1/SR-BI Average C₇</th>
<th>GAPDH Average C₇</th>
<th>ΔC₇ (CLA-1/SR-BI − GAPDH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₈T₈C₅C₅</td>
<td>0.5</td>
<td>28.81 ± 1.20</td>
<td>22.62 ± 1.2</td>
<td>6.19 ± 1.62</td>
</tr>
<tr>
<td>T₈T₈C₅C₅</td>
<td>0.25</td>
<td>29.24 ± 2.4</td>
<td>22.84 ± 1.5</td>
<td>6.4 ± 2.8</td>
</tr>
<tr>
<td>T₈T₈C₅C₅</td>
<td>0.12</td>
<td>27.69 ± 1.04</td>
<td>22.69 ± 0.7</td>
<td>5.06 ± 1.06</td>
</tr>
<tr>
<td>T₅T₅C₅C₅</td>
<td>0.86</td>
<td>29.38 ± 0.12</td>
<td>22.35 ± 0.06</td>
<td>7.03 ± 0.21</td>
</tr>
<tr>
<td>C₅C₅T₅T₅</td>
<td>0.5</td>
<td>30.29 ± 1.5</td>
<td>22.12 ± 0.1</td>
<td>8.17 ± 1.51</td>
</tr>
<tr>
<td>C₅C₅T₅T₅</td>
<td>0.25</td>
<td>31.97 ± 2.04</td>
<td>23.9 ± 0.36</td>
<td>8.07 ± 2.07</td>
</tr>
<tr>
<td>C₅C₅T₅T₅</td>
<td>0.12</td>
<td>33.68 ± 1.8</td>
<td>25.14 ± 0.91</td>
<td>8.54 ± 1.5</td>
</tr>
<tr>
<td>C₅C₅T₅T₅</td>
<td>0.06</td>
<td>34.52 ± 1.75</td>
<td>26.94 ± 0.61</td>
<td>7.58 ± 1.85</td>
</tr>
<tr>
<td>C₅T₅C₅T₅</td>
<td>0.5</td>
<td>35.01 ± 0.09</td>
<td>26.66 ± 0.33</td>
<td>8.35 ± 2.12</td>
</tr>
<tr>
<td>C₅T₅C₅T₅</td>
<td>0.25</td>
<td>37.06 ± 1.11</td>
<td>27.14 ± 0.22</td>
<td>9.92 ± 1.13</td>
</tr>
<tr>
<td>C₅T₅C₅T₅</td>
<td>0.12</td>
<td>35.88 ± 0.12</td>
<td>27.23 ± 0.15</td>
<td>8.65 ± 0.19</td>
</tr>
<tr>
<td>C₅T₅C₅T₅</td>
<td>0.06</td>
<td>37.63 ± 0.13</td>
<td>28.44 ± 0.07</td>
<td>9.19 ± 0.15</td>
</tr>
</tbody>
</table>
content also differed according to CLA-1 genotypes, and higher levels were found in those T/T 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 CEEOOHs 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 C1050C 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 C1050 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.19 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.

Acknowledgments

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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
Francisco Rodríguez-Esparragón, José C. Rodríguez-Pérez, Yaridé Hernández-Trujillo, Antonio Macías-Reyes, Alfonso Medina, Araceli Caballero and Carlos M. Ferrario

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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. 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, Mannheim, Germany).

Total plasma lipid hydroperoxide (LOOH) content was calculated by the Xylenol Orange (FOX) assay as described. Briefly, plasma samples stored at −70°C, were analyzed under basal conditions or mixed with copper sulphate at a final concentration of 10µM at 37°C 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 x 10⁴ M⁻¹. cm⁻¹ or by reference to an H₂O₂ standard curve.

Paraoxonase and arylesterase activities were determined as previously described using molar absorption coefficients of 1.7 x 10⁴ M⁻¹. cm⁻¹ and 1310 M⁻¹. cm⁻¹ respectively.
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 C₈/T₈ transition located at 1050 cDNA base position in exon 8 and the C₅/T₅ 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°C. No-adherent cells were washed away twice with phosphate-buffered saline (PBS). Typically 5 x 10⁶ 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 manufacture’s instructions. CLA-1/SR-BI mRNA expression levels were compared among C₈C₈ homozygous, C₈T₈ heterozygous and T₈T₈ homozygous subjects for the exon 8 variant by the comparative threshold cycle (Cₜ) quantitation method. We used a LightCycler and the LC Fast Start DNA Master SYBR Green Kit (Roche Applied Science). All C₅C₈ homozygous subjects for the exon 8 polymorphism were also T₅T₅ homozygous for the intron 5 variant whereas all C₈T₈ and T₈T₈ subjects for the exon 8 variant were C₅T₅ heterozygous for
the intron 5 variant because no T_{2}T_{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_{2}Mg concentration with the forward primer 5’GAAAACCTGCAGCTGAGCCTCT3’ and the reverse primer 5’ATTTCTTTGGCTCCGGATT3’ (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’GCCCTCCAAGGAGTAAGACC3’ and the reverse primer 5’AGGGGTCTCATGGCAACTG3’ at 2mM Cl_{2}Mg 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_{8}T_{5}C_{8}C_{5} and C_{8}C_{5}C_{2}T_{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 Maximum Likelihood method using Arlequin version 2.000 software. Quantitative variables are presented as mean ± standard deviation and qualitative variables as percentages. Assumption of normal distribution for continuous 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 appropriate. 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.
REFERENCES


10. Scheneider L, Roessli D, Excoffier L. A software for population genetics data analysis. Genetic and Biomentry Laboratory, University of Geneva, Switzerland. 2000.
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

<table>
<thead>
<tr>
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<th>C1050T</th>
<th>T allele carriers</th>
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<tr>
<td></td>
<td>Cases</td>
<td>54 (58.7%)</td>
<td>38 (41.3%)</td>
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<tr>
<td>QQ homozygotes</td>
<td>Controls</td>
<td>72 (67.9%)</td>
<td>34 (32.1%)</td>
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<tr>
<td>$\chi^2_{1} = 1.813; P = 0.178$</td>
<td>$\chi^2_{1} = 6.078; P = 0.014; OR = 1.95 (1.14-3.32)$</td>
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<tr>
<td>R allele carriers</td>
<td>Cases</td>
<td>59 (51.3%)</td>
<td>56 (48.7%)</td>
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<tr>
<td></td>
<td>Controls</td>
<td>78 (67.2%)</td>
<td>38 (32.8%)</td>
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</table>

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<th>QQ</th>
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<td>Cases</td>
<td>56 (59.6%)</td>
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<td>38 (52.8%)</td>
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<tr>
<td>$\chi^2_{1} = 0.767; P = 0.381$</td>
<td>$\chi^2_{1} = 0.001; P = 0.973$</td>
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<tr>
<td>CLA-1 (C1050T)</td>
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<td>54 (47.8%)</td>
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<tr>
<td></td>
<td>Controls</td>
<td>78 (52.0%)</td>
<td>72 (48.0%)</td>
</tr>
</tbody>
</table>

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.