The PON1 M55L gene polymorphism is associated with reduced HDL-associated PAF-AH activity

Anna I. Kakafika,* Stavroula Xenofontos,[§] Vasilis Tsimihodimos,* Afroditi P. Tambaki,[†]
Evangelia S. Lourida,[†] Rigas Kalaitzidis,* Marios A. Cariolou,[§] Moses Elisaf,*
and Alexandros D. Tselepis[1,†]

Department of Internal Medicine,* Medical School and Laboratory of Biochemistry,[†] Department of
Chemistry, University of Ioannina, 45110 Ioannina, Greece; and The Cyprus Institute of Neurology and
Genetics,[§] Nicosia, Cyprus

Abstract The platelet-activating factor acetylhydrolase activity associated with high density lipoprotein (HDL-PAF-AH) may substantially contribute to the antioxidant, antiinflammatory, and overall antiatherogenic effects of HDL. Two enzymes associated with HDL express PAF-AH catalytic activity, PAF-AH itself and paraoxonase-1 (PON1). The relative contribution of these enzymes in the expression of PAF-AH activity on HDL remains to be established. We investigated whether the PON1 polymorphisms (M55L and Q192R) or the PAF-AH polymorphism V379A could affect the PAF-AH activity associated with HDL in both normolipidemic and dyslipidemic (type IIA and IIB) populations. We show for the first time that the PON1 M55L polymorphism significantly affects the HDL-PAF-AH activity in all studied groups, the PON1 L55L individuals having lower enzyme activity compared to those having 1 M and 2 M alleles. No differences in the HDL content concerning the major apolipoprotein and lipid constituents were observed between individuals carrying the PON1 L55L and those with the M55M polymorphism.█ Our results provide evidence that PON1 significantly contributes to the pool of HDL-PAF-AH activity in human plasma, and suggest that the low PAF-AH activity in HDL carrying the PON1 L alloenzyme may be an important factor contributing to the low efficiency of this HDL in protecting LDL against lipid peroxidation.—Kakafika, A. I., S. Xenofontos, V. Tsimihodimos, A. P. Tambaki, E. S. Lourida, R. Kalaitzidis, M. A. Cariolou, M. Elisaf, and A. D. Tselepis. **The PON1 M55L gene polymorphism is associated with reduced HDL-associated PAF-AH activity.** *J. Lipid Res.* **2003.** 44: 1919–1926.

Supplementary key words high density lipoprotein • platelet-activating factor acetylhydrolase • paraoxonase-1 • PON1 polymorphisms • platelet-activating factor acetylhydrolase polymorphisms

It is well established that HDL plays a protective role against atherogenesis and coronary heart disease (CHD) (1). This effect appears to be multifactorial and involves a number of mechanisms, including reverse cholesterol transport, as well as antiinflammatory and antioxidant activities. Potential roles in the HDL antiinflammatory and antioxidant properties are played by two enzymes associated with this lipoprotein, platelet-activating factor acetylhydrolase (PAF-AH) and paraoxonase-1 (PON1) (2).

PAF-AH exhibits a α/β hydrolase conformation and has broad substrate specificity toward lipid esters containing short acyl chains. It also displays phospholipase A_1 and A_2 activities as well as transacetylase activity. Of these, the Ca^{2+}-independent phospholipase A_2 activity of PAF-AH has been principally studied [reviewed in ref. (3)]. PAF-AH can hydrolyze and inactivate PAF, the potent lipid mediator involved in inflammatory diseases as well as in atherogenesis. PAF-AH can also effectively hydrolyze oxidized phospholipids that play key roles in several aspects of atherogenesis. In human plasma, PAF-AH is primarily associated with LDL particles, whereas a small proportion ($<20\%$ of total enzyme activity) is associated with HDL (3). The gene for the plasmatic PAF-AH has been mapped to chromosome 6q21.2-p12, comprises 12 exons, and spans at least 45 kb of DNA (4). Several genetic variants of this enzyme have been described. Two of them are situated in exon 9 (V279F and Q281R) and have been described only in Japanese populations. These mutations result in a complete loss of the enzyme activity and are associated with severe asthma, stroke, and cardiovascular disease (5, 6). Two other genetic variants, V379A (exon 11) and I198T (exon 7), have been described and are associated with atopy and asthma in European populations. Furthermore, in vitro studies have shown that these two polymorphisms are associated with a reduction in PAF-AH kinetic constants (7).

PON1 is an esterase that in plasma is exclusively associated with HDL. PON1 can hydrolyze oxidized phospholipids and cholesteryl ester hydroperoxides formed dur-

[1] To whom correspondence should be addressed.
 e-mail: atselep@cc.uoi.gr

ing lipoprotein peroxidation, and plays a protective role against the oxidative modification of plasma lipoproteins (8). The PON1 gene is located on the long arm of chromosome 7. Two genetic variants of this enzyme have been described, one at position 55 (methionine/leucine, M55L) and the other at position 192 (glutamine/arginine, Q192R). The substrates for PON1 that have been mostly used in assays in vitro are paraoxon and phenyl acetate. Several studies have shown that paraoxon hydrolytic activity using either HDL or purified PON1 is high in PON1 R192R and L55L individuals and low in PON1 Q192Q and M55M individuals. By contrast, both PON1 alloenzymes have a similar hydrolytic activity toward phenyl acetate (9). On the other hand, the capacity of PON1 alloenzymes to protect LDL from oxidation is distinguished from that of paraoxon hydrolytic activity. Thus, PON1 either purified or associated with HDL from MM/QQ individuals exhibits the greatest protective capacity toward LDL oxidation in vitro (10).

In addition to the above activities, PON1 expresses a PAF-AH catalytic activity (11). In this context, it has been suggested that the HDL-associated PAF-AH (HDL-PAF-AH) activity is mainly due to the PAF-AH catalytic activity of PON1, as no PAF-AH protein was found on this lipoprotein (11). By contrast, it was recently shown that the total PAF-AH activity associated with HDL is due to the PAF-AH protein (12). In the present study we investigated whether the PON1 polymorphisms (M55L and Q192R) or the PAF-AH polymorphism V379A could affect the PAF-AH activity in plasma and that associated with HDL in patients with dyslipidemia of type IIA or IIB, as well as in normolipidemic, apparently healthy volunteers.

METHODS

Patients

Two hundred and two unrelated dyslipidemic patients attending the Lipid Clinic of the University Hospital of Ioannina participated in the study. After the initial screening, patients were advised to follow the National Cholesterol Education Program step 1 diet (which limits dietary intake of cholesterol to 300 mg/day, that of saturated fats to 10% of total energy intake and total fats to 30% of total energy intake) for 3 months. At the end of this period, a complete laboratory baseline analysis was performed. According to their lipid levels, patients were divided into the primary hypercholesterolemia (dyslipidemia type IIA) group (LDL cholesterol >160 mg/dl) and the combined hyperlipidemia (dyslipidemia type IIB) group (LDL cholesterol >160 mg/dl and triglycerides >200 mg/dl). Among dyslipidemic IIA patients, 58 had a diagnosis of heterozygous familial hypercholesterolemia and 80 had a diagnosis of polygenic hypercholesterolemia.

A total of 122 healthy unrelated volunteers with no history of dyslipidemia or CHD also participated in the study. Volunteers were either staff of the University Hospital of Ioannina or attending for a routine health check at the Outpatient Clinic of this Hospital.

None of the participants were taking lipid-lowering drugs or any other medication known to affect lipid metabolism, including hormonal therapy, during the last 12 weeks. Individuals with hypertension (blood pressure, 140/90 mmHg on repeated measurements),

diabetes mellitus (fasting blood glucose, 126 mg/dl), obesity (body mass index, 30 kg/m^2) or thyroid, hepatic, or renal diseases, as well as subjects known to ingest more than two alcoholic drinks daily or who were taking vitamin supplements were excluded from the study. Finally, no patient had any clinical evidence of cardiovascular disease. The study was approved by the Ethics Committee of the University Hospital of Ioannina, and all study patients gave written informed consent for participation in the study.

Determination of PON1 and PAF-AH genotypes

Genomic DNA was obtained from leukocytes using standard procedures. The PON1 M55L and Q192R polymorphisms were detected using a previously reported protocol (13). To determine the variations of 279 (14) and 379 (7) of PAF-AH genes, DNA was amplified and analyzed by restriction isotyping. Briefly, primers for amplification of a 99 bp DNA that contains the coding sequence for position 192 were 5′TAT TGT TGC TGT GGG ACC TGA G3′ and 5′CAC GCT AAA CCC AAA TAC ATC TC3′. After an initial denaturation step of 5 min at 95°C, the PCR was carried out for 40 cycles, with each cycle consisting of 60 s of denaturation at 94°C, 45 s of annealing at 56°C, and 45 s of extension at 72°C. PCR product was digested with 5 units of AlwI restriction enzyme for 3 h at 37°C.

For genotyping the M55L polymorphism, the primers for amplification of 144 bp DNA encoding codon 55 were 5′GAG TGA TGT ATA GCC CCA GTT TC3′ and 5′AGT CCA TTA GGC AGT ATC TCC G3′. An initial incubation for 5 min at 95°C was followed by the step of amplification that was carried out for 40 cycles, with each cycle consisting of 1 min of denaturation at 94°C, 45 s of annealing at 61°C, and 45 s of extension at 72°C. PCR product was digested with 5 units of HinfI restriction enzyme for 24 h at 37°C.

The PAF-AH V379A polymorphism was detected using 5′GGG AGA CAT AGA TTC AAC TG3′ and 5′GGT CAT GAA AAA AAT AGT TT3′ primers. After an initial denaturation step of 5 min at 94°C, the PCR was carried out for 35 cycles, with each cycle consisting of 45 s of denaturation at 94°C, 50 s of annealing at 53°C, and 50 s of extension at 72°C. PCR product was digested with 5 units of PstI restriction enzyme for 24 h at 37°C.

Isolation of HDL

HDL (d = 1.063–1.210 g/ml) was isolated from the HDL-containing supernatant after treatment of serum with magnesium chloride/dextran sulfate to precipitate all apolipoprotein B (apoB)-containing lipoproteins (HDL-rich serum). HDL isolation was performed by sequential ultracentrifugation in a Beckman L7-65 ultracentrifuge at 40,000 rpm, 14°C, with a type NVT 65 rotor. The HDL preparation was dialyzed against 10 mM PBS (pH 7.4) for 24 h at 4°C. It was then filter sterilized and stored in the dark at 4°C under nitrogen for up to 2 weeks.

Measurement of PAF-AH and PON1 activities

PAF-AH activity in isolated HDL, in plasma, and in HDL-rich plasma (prepared as the HDL-rich serum) was determined by the trichloroacetic acid precipitation procedure using [^{3}H]PAF (100 mM final concentration) as a substrate (15). PON1 activity in serum and in HDL-rich serum was measured using paraoxon as a substrate, whereas PON1 activity in isolated HDL was measured using phenyl acetate as a substrate. Both PON1 activities were determined in the presence of 2 mM Ca^{+2} in 100 mM Tris-HCl buffer (pH 8.0) for paraoxon and in 20 mM Tris-HCl buffer (pH 8.0) for phenyl acetate (15).

Analytical methods

Serum total cholesterol and triglycerides were determined on the Olympus AU560 Clinical Chemistry analyzer (Hamburg, Ger-

many). Serum HDL cholesterol levels were measured with the above method in the HDL-rich serum. Serum LDL cholesterol levels were calculated using the Friedewald formula. Serum apoB, apoA-I, apoA-II, and apoE were measured by immuno-nephelometry with the aid of a Behring Nephelometer BN100 and reagents (antibodies and calibrators) from Behring Diagnostics GmbH (Liederbach, Germany). The total cholesterol and the phospholipid content of HDL were measured enzymatically using the Bio-Merieux kit (15).

Statistical analysis

One-way ANOVA followed by least significant difference test was used for comparisons among individual groups. Allele frequencies were estimated by the gene-counting method, and Hardy-Weinberg's equilibrium was tested by the χ^2 test. The same test was used to compare percentages. Mann-Whitney U test and Kruskal-Wallis test were used for comparisons among the paraoxonase activities. Student's *t*-test for independent samples was used to estimate the effect of the enzyme polymorphisms on lipid parameters and enzyme activities.

RESULTS

Clinical and biochemical characteristics of the study population

The clinical and biochemical characteristics of the populations participating in the study are shown in **Table 1**. As expected, the dyslipidemic populations had significantly higher levels of all lipidemic parameters compared with normolipidemic individuals, with the exception of HDL cholesterol levels in type IIB patients, which were lower compared with other groups. Plasma PAF-AH activity was higher in type IIA and IIB patients compared with controls, but there was no difference in enzyme activity between the two dyslipidemic populations. In accordance with our previously published results (15), the HDL-PAF-AH activity was significantly lower in type IIB patients com-

pared with the other studied groups ($P < 0.001$). Finally, no difference in serum PON1 activity toward paraoxon measured either in total serum or in HDL-rich serum (HDL-PON1) was observed among the studied groups (Table 1).

PON1 and PAF-AH genotyping analysis

The genotype and allele frequencies studied are shown in **Table 2**. Importantly, no difference in the distributions of both PON1 polymorphisms as well as in the allele frequencies was observed among the studied groups. Combined haplotypes (both haplotypes per subject) were constructed for PON1 polymorphisms and confirmed the well-known linkage disequilibria between M55L and R192Q (Table 2). All χ^2 tests to compare observed and expected genotype frequencies have been performed and all were in Hardy-Weinberg equilibrium.

No difference in the distributions of the PAF-AH V379A polymorphism and in the allele frequencies was observed among the studied groups. As expected, no one from the study populations exhibited the PAF-AH V279F mutation, which has been found only in the Japanese population (14).

Influence of PON1 and PAF-AH polymorphisms on lipid profile and enzyme activities

Individuals of each group were subgrouped as follows: *i*) according to PON1 192 genotype into homozygotes for the Q allele and those having one or two R alleles; *ii*) according to PON1 55 genotype into homozygotes for the L allele and those having one or two M alleles; *iii*) according to PAF-AH 379 genotype into homozygotes for the A allele and those having one or two V alleles. Comparisons between the above subgroups were performed within each studied group. These subgroups were matched for age, sex, body mass index, and smoking habits.

No difference in the serum lipid and apolipoprotein levels was observed among the above genotype subgroups

TABLE 1. Clinical and biochemical characteristics of the studied populations

	Normolipidemic Population	Type IIA Patients	Type IIB Patients
Number	122	138	64
Sex (males/females)	60/62	59/79	27/37
Age (years)	37 ± 10.5	52 ± 12.9[b]	54 ± 12.4[b]
Body mass index, kg/m^2	24 ± 3.3	27 ± 3.1[b]	28 ± 4.0[b]
Smokers/non smokers	42/80	38/100	16/48
Total cholesterol, mg/dl	202.1 ± 32.7	320.0 ± 58.4[b]	302.0 ± 37.7[b,c]
Triglycerides, mg/dl	104.2 ± 54.5	164.6 ± 86.5[b]	297.6 ± 84.5[b,d]
LDL cholesterol, mg/dl	135.2 ± 31.7	234.7 ± 59.2[b]	200.3 ± 39.4[b,c]
HDL cholesterol, mg/dl	46 ± 12.5	51.7 ± 15.2[b]	42.0 ± 6.2[a,d]
ApoB, mg/dl	96.3 ± 21.2	166.8 ± 33.5[b]	160.2 ± 20.3[b,c]
ApoA-I, mg/dl	143.7 ± 23.5	154.0 ± 32.5[a]	146.5 ± 17.4[a,c]
ApoE, mg/dl	36.3 ± 8.4	52.2 ± 13.1[b]	56.3 ± 16.6[b,c]
Plasma PAF-AH activity, nmol/ml/min	47.3 ± 11.7	74.6 ± 24.3[b]	71.0 ± 19.4[b]
HDL-PAF-AH activity, nmol/ml/min	3.3 ± 1.2	3.3 ± 1.4	2.4 ± 0.9[b,d]
Serum PON1 activity (paraoxon), U/l	75.3 ± 43.8	79.5 ± 48.2	70.3 ± 40.4
HDL-PON1 activity (paraoxon), U/l	72.4 ± 24.2	74.0 ± 32.6	67.9 ± 28.4

ApoB, apolipoprotein B; PAF-AH, platelet activating factor acetylhydrolase; PON1, paraoxonase-1. Values represent the mean ± SD. One-way ANOVA followed by least significant difference test was used for comparisons among studied groups.
[a] $P < 0.01$ compared with normolipidemic population.
[b] $P < 0.001$ compared with normolipidemic population.
[c] $P < 0.01$ compared with type IIA patients.
[d] $P < 0.001$ compared with type IIA patients.

TABLE 2. Genotype distribution and allele frequencies of PON1 and PAF-AH

	Normolipidemic Population n = 122	Type IIA Patients n = 138	Type IIB Patients n = 64	P
PON1 Q192R genotypes				
QQ	59 (48.4%)	43 (31.2%)	26 (40.6%)	
RR	12 (9.8%)	20 (14.5%)	9 (14.1%)	
QR	51 (41.8%)	75 (54.3%)	29 (45.3%)	
Alleles				
Q (Gln)	0.69	0.58	0.63	NS
R (Arg)	0.31	0.42	0.37	NS
PON1 M55L genotypes				
LL	46 (37.7%)	46 (33.3%)	18 (28.1%)	
MM	28 (23%)	21 (15.2%)	13 (20.3%)	
LM	48 (39.3%)	71 (51.5%)	33 (51.6%)	
Alleles				
L (Leu)	0.57	0.59	0.54	NS
M (Met)	0.43	0.41	0.46	NS
PON1 haplotypes				
QL/QL	11 (9%)	9 (6.5%)	3 (4.7%)	NS
QM/QM	23 (19%)	13 (9.4%)	6 (9.4%)	NS
RL/RL	10 (8.2%)	9 (6.5%)	2 (3%)	NS
RM/RM	0	2 (1.5%)	3 (4.7%)	NS
QL/RL	27 (22.2%)	26 (18.8%)	15 (23.4%)	NS
QL/QM	27 (22.2%)	22 (16%)	15 (23.4%)	NS
RL/RM	0	8 (5.8%)	3 (4.7%)	NS
QM/RM	5 (4.1%)	7 (5.1%)	3 (4.7%)	NS
QL/RM or QM/RL	19 (15.3%)	42 (30.4%)	14 (22%)	NS
PAF-AH V379A genotypes				
AA	78 (64%)	70 (50.7%)	38 (59.4%)	
VV	8 (6.5%)	5 (3.6%)	0 (0%)	
AV	36 (29.5%)	63 (45.7%)	26 (40.6%)	
Alleles				
A (Ala)	0.79	0.74	0.80	NS
V (Val)	0.21	0.26	0.20	NS

NS, nonsignificant. χ^2 test was used to compare percentages.

(data not shown), suggesting that none of the studied polymorphisms affect the lipid profile either in healthy volunteers or in any patient group.

As shown in **Table 3**, the PON1 Q192R polymorphism significantly influenced the serum PON1 activity toward paraoxon in each studied group, the Q192Q individuals having lower activity compared with the R carriers of the same group, an observation that accords with previously published results (16). The same phenomenon was observed when subjects of each group were subgrouped according to the PAF-AH V379A polymorphism (Table 3). It must be noted that when the R carriers were divided into homozygotes and heterozygotes, the Q192R individuals presented intermediate levels of enzyme activity between the Q and R homozygotes of the same group (data not shown). Furthermore, independent of the presence of the PAF-AH V379A polymorphism, the PON1 activity was significantly influenced by the M55L polymorphism, the L55L individuals having higher activity compared with the M-carriers in each studied group (Table 3), a finding that also accords with previously published data (16). When the M carriers were divided into homozygotes and heterozygotes, the M55L individuals presented intermediate levels of enzyme activity between the M and L homozygotes of the same group (data not shown). It must be noted that similar results were obtained for the PON1 activity measured in the HDL-rich serum (data not shown). The present study shows for the first

time that neither the Q192R nor the M55L polymorphism of PON1 influences the plasma PAF-AH activity. Furthermore, the plasma PAF-AH activity was not influenced by the PAF-AH V379A polymorphism (Table 3).

The most important finding of the present study is that the PON1 M55L polymorphism significantly affects the HDL-PAF-AH activity. As shown in Table 3, the PON1 LL homozygotes of each group exhibited significantly lower HDL-PAF-AH activity compared with M carriers of the same group. The same phenomenon was observed when subjects of each group were subgrouped according to the PAF-AH V379A polymorphism (Table 3). When the M carriers were divided into homozygotes and heterozygotes, the M55L individuals presented intermediate levels of enzyme activity between the M and L homozygotes of the same group (HDL-PAF-AH activity in nmol/ml/min; normolipidemic group: M55L = 3.2 ± 0.8 and M55M = 3.9 ± 0.9, $P < 0.05$; type IIA: M55L = 3.3 ± 0.7 and M55M = 4.0 ± 0.9, $P < 0.05$; type IIB: M55L = 2.5 ± 0.6 and M55M = 3.2 ± 0.7, $P < 0.05$). The above results are further supported by those obtained when the comparisons of the HDL-PAF-AH activity in each group were performed according to the PON1 M55L and Q192R haplotypes. Thus, in each studied group, subjects with the L allele had lower enzyme activity compared with those having the M allele independent of the existence of the Q or R allele. **Figure 1** presents the results for the type IIA dyslipidemic patients. Finally, there was no influence of the PON1 Q192R or the PAF-AH V379A polymorphism on the HDL-PAF-AH activity.

Compositional analysis of HDL

In an effort to provide a mechanistic explanation for the lower PAF-AH activity in HDL of subjects carrying the PON1 L55L polymorphism, we performed compositional analysis of HDL isolated from the HDL-rich serum of 10 subjects from each group carrying the PON1 L55L or the PON1 M55M polymorphism. ApoA-I, apoA-II, and apoE, the phospholipids, and the total cholesterol content of HDL, as well as the PAF-AH activity and the PON1 activity toward phenyl acetate, were determined. As shown in **Table 4**, the PAF-AH activity of the HDL isolated from the L55L individuals was significantly lower compared with the enzyme activity of the M55M individuals of the same group. By contrast, no differences in the other parameters of HDL were observed between individuals with the PON1 L55L and those with the PON1 M55M polymorphism within each studied group.

DISCUSSION

Several lines of evidence suggest that the HDL-PAF-AH activity may substantially contribute to the antioxidant and antiinflammatory effects of HDL, thus this activity may be an important component of the multiple mechanisms by which HDL slows the progression of atherosclerosis (17). The HDL-PAF-AH activity may represent a pool of similar catalytic activities expressed by three different enzymes, PAF-AH, PON1, and LCAT (3, 17). However, the relative

TABLE 3. Influence of paraoxonase 1 and platelet-activating factor polymorphisms on the enzymic activities

	Normolipidemic Population				Type IIA Patients				Type IIB Patients			
	QQ	QR+RR	LL	LM+MM	QQ	QR+RR	LL	LM+MM	QQ	QR+RR	LL	LM+MM
Plasma PAF-AH activity, nmol/ml/min												
All subjects	46.7 ± 11.1	49.2 ± 12.9	47.7 ± 11.1	47.9 ± 12.7	72.6 ± 21.7	75.6 ± 25.8	75.7 ± 24.2	74.4 ± 24.8	66.8 ± 13.2	74.4 ± 22.6	68.6 ± 20.6	72.4 ± 19.3
PAF-AH 379 (AA)	44.7 ± 9.2	50.6 ± 14.0	46.2 ± 9.3	48.4 ± 13.9	73.4 ± 26.9	78.1 ± 30.9	76.1 ± 26.7	77.2 ± 31.3	70.9 ± 11.9	69.5 ± 21.7	62.7 ± 19.2	72.6 ± 20.9
PAF-AH 379 (VA+VV)	50.6 ± 15.6	47.3 ± 11.9	48.7 ± 16.1	47.2 ± 11.5	71.7 ± 15.1	73.2 ± 19.4	75.1 ± 21.1	71.8 ± 16.9	68.1 ± 13.5	71.6 ± 18.8	77.1 ± 29.3	72.1 ± 17.2
HDL-PAF-AH activity, nmol/ml/min												
All subjects	3.3 ± 1.1	3.3 ± 0.9	2.9 ± 0.7[b]	3.4 ± 1.2	3.4 ± 1.0	3.3 ± 1.1	2.8 ± 0.7[d]	3.6 ± 1.2	2.6 ± 0.8	2.3 ± 0.9	1.6 ± 0.5[f]	2.7 ± 0.9
PAF-AH 379 (AA)	3.5 ± 1.6	3.2 ± 1.0	2.7 ± 0.8[b]	3.5 ± 1.2	3.5 ± 1.7	3.2 ± 1.2	2.8 ± 1.1[d]	3.5 ± 1.5	2.4 ± 0.4	2.3 ± 1.0	1.7 ± 0.5[f]	2.5 ± 0.9
PAF-AH 379 (VA+VV)	3.5 ± 1.1	3.4 ± 1.2	2.4 ± 0.7[b]	3.3 ± 1.0	3.3 ± 1.7	3.4 ± 1.3	2.6 ± 0.9[d]	3.6 ± 1.5	2.7 ± 1.1	2.2 ± 0.8	1.4 ± 0.4[f]	2.9 ± 0.8
Serum PON1 activity, (paraoxon), U/l												
All subjects	40.2 ± 23.0[a]	103.9 ± 31.5	79.5 ± 46.0[b]	64.8 ± 34.0	53.9 ± 33.7[c]	87.9 ± 44.9	107.1 ± 47.1[d]	64.1 ± 37.3	49.8 ± 24.1[e]	82.7 ± 44.5	102.6 ± 60.1[f]	62.9 ± 32.6
PAF-AH 379 (AA)	38.7 ± 15.3[a]	97.2 ± 28.7	75.6 ± 36.1[b]	59.7 ± 27.8	61.8 ± 41.4[c]	88.5 ± 45.5	107.2 ± 46.8[d]	64.2 ± 39.5	46.2 ± 17.1[e]	96.2 ± 36.7	98.7 ± 43.1[f]	64.6 ± 28.6
PAF-AH 379 (VA+VV)	47.2 ± 38.4[a]	111.8 ± 36.1	78.5 ± 24.4[b]	62.6 ± 23.4	43.1 ± 12.9[c]	87.4 ± 44.6	106.9 ± 49.1[d]	63.9 ± 35.6	41.6 ± 18.1[e]	110.1 ± 44.6	142.6 ± 46.2[f]	61.2 ± 37.1

PAF-AH, platelet activating factor acetylhydrolase; HDL-PAF-AH, PAF-AH activity associated with high density lipoprotein; PON1, paraoxonase-1. Values are means ± SD. Student's t-test for independent samples was used for comparisons between the genotype subgroups. A P value <0.05 was considered to be significant.

[a] $P < 0.05$ compared with QR+RR normolipidemic population.
[b] $P < 0.05$ compared with LM+MM normolipidemic population.
[c] $P < 0.05$ compared with QR+RR type IIA patients.
[d] $P < 0.05$ compared with LM+MM type IIA patients.
[e] $P < 0.05$ compared with QR+RR type IIB patients.
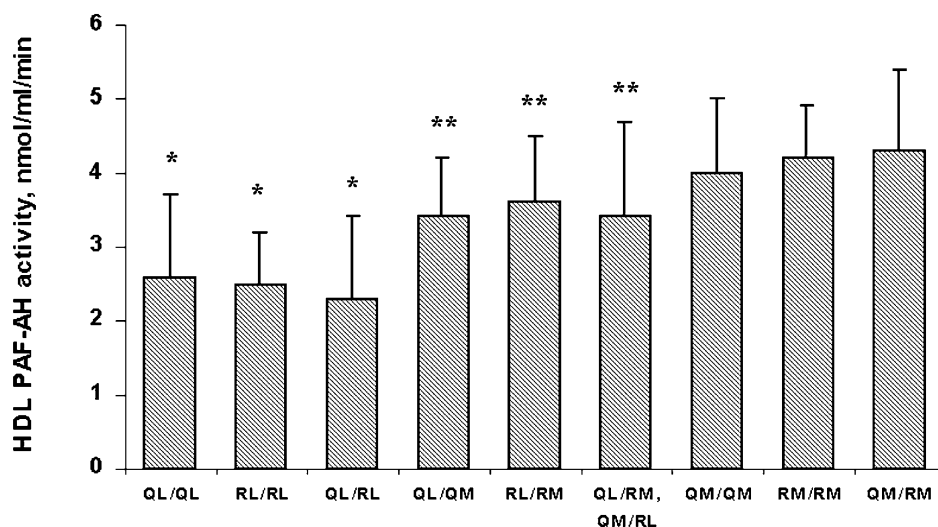[f] $P < 0.05$ compared with LM+MM type IIB patients.

Fig. 1. Influence of paraoxonase-1 haplotypes on platelet-activating factor acetylhydrolase activity associated with HDL in type IIA dyslipidemic patients. Values represent the mean ± SD. * $P < 0.03$ compared with the homozygote haplotypes for the M allele and ** $P < 0.05$ compared with the homozygote haplotypes for either the L or the M allele.

contribution of each of the above enzymes to the overall PAF-AH activity expressed by HDL, as well as the factors that regulate or influence the expression of this activity on HDL, is ill defined. In the present study, we show for the first time that the PON1 M55L polymorphism significantly affects the PAF-AH activity associated with HDL in dyslipidemic type IIA and IIB patients as well as in normolipidemic subjects, the PON1 L55L individuals having lower enzyme activity compared with those having 1 M and 2 M alleles. This phenomenon cannot be attributed to changes in the serum levels of HDL cholesterol, because according to our results, they are not affected by the PON1 M55L polymorphism in either studied group. Another factor that might have influenced the PAF-AH activity in L55L individ-

uals could be alterations in the apolipoprotein or lipid composition of HDL in these individuals. Indeed, it has been shown that alterations in lipid composition can influence PAF-AH activity transported on lipoproteins (18). However, according to our results, no alterations in the lipid composition of HDL or in the apoA-II/apoA-I ratio were observed between L55L and M55M individuals in any studied group. Furthermore, it has been shown that among the HDL particle populations, PAF-AH is mainly associated with the apoE-rich HDL particles (19). Thus, alterations in the apoE content of HDL might have influenced the association of PAF-AH with this lipoprotein. However, according to our results, there was not any difference in the HDL apoE/apoA-I ratio between L55L and M55M individuals. Consequently,

TABLE 4. Compositional analysis of HDL and HDL-associated activities of PON1 and PAF-AH in relation to PON1 L55M polymorphism

Parameters	Normolipidemic Population		Type IIA Patients		Type IIB Patients	
	L55L	M55M	L55L	M55M	L55L	M55M
PAF-AH activity/apoA-I, nmol/mg/min	1.20 ± 0.14^b	2.13 ± 0.22	1.34 ± 0.2^c	2.28 ± 0.30	1.02 ± 0.07^d	1.49 ± 0.10
PON1 activity[a]/apoA-I, U/mg	43.9 ± 12.3	47.6 ± 11.7	45.7 ± 10.2	48.1 ± 13.2	44.8 ± 13.3	47.5 ± 10.9
ApoA-II/apoA-I mass ratio	0.21 ± 0.07	0.20 ± 0.08	0.19 ± 0.06	0.18 ± 0.05	0.17 ± 0.03	0.18 ± 0.05
ApoE/apoA-I mass ratio	0.046 ± 0.014	0.049 ± 0.011	0.041 ± 0.02	0.044 ± 0.013	0.050 ± 0.021	0.048 ± 0.018
Phospholipids/apoA-I mass ratio	0.49 ± 0.11	0.48 ± 0.14	0.46 ± 0.13	0.47 ± 0.10	0.46 ± 0.12	0.45 ± 0.09
Cholesterol/apoA-I mass ratio	0.32 ± 0.06	0.34 ± 0.09	0.30 ± 0.04	0.31 ± 0.05	0.30 ± 0.07	0.29 ± 0.03

Values represent the mean ± SD from 10 HDL preparations for each PON1 genotype subgroup. Student's *t*-test for independent samples was used to estimate the effect of the enzyme polymorphisms on HDL-associated lipids, apolipoproteins, and enzyme activities.

[a] PON1 activity towards phenyl acetate.
[b] $P < 0.03$ compared with M55M of normolipidemic population.
[c] $P < 0.04$ compared with M55M of dyslipidemic type IIA patients.
[d] $P < 0.03$ compared with M55M of dyslipidemic type IIB patients.

due to the lack of differences in the HDL composition between L55L and M55M individuals, we may exclude the possibility that the low enzyme activity in HDL from carriers of the L allele can be attributed to any influence of the HDL composition either on the PAF-AH activity or on the enzyme affinity to HDL particles in this population.

It has been shown that PON1 exerts a PAF-AH activity (11), and at the same time, this enzyme is capable of oxidatively hydrolyzing fragmented phospholipids (20). These activities confer on PON1 the ability to retard LDL oxidation (21) and to reduce the proinflammatory effects of oxidized LDL (8). These PON1 activities are calcium independent and are distinguished from the PON1 hydrolytic activity against organophosphate substrates, which are highly calcium dependent (22). The contribution of PON1 to the PAF-AH activity expressed on HDL has recently been a subject for debate. Thus, it has been suggested that the HDL-PAF-AH activity is primarily due to PON1, although it was recently shown that the PAF-AH activity in HDL is exclusively due to the PAF-AH protein (12). Our finding that the PON1 M55L polymorphism significantly influences the HDL-PAF-AH activity rather favors the assumption that PON1 essentially contributes to the HDL-PAF-AH activity. We may exclude the possibility that the HDL of L carriers contains less PON1 protein compared with the M carriers, as we did not find any difference between L55L and M55M individuals in the HDL-associated PON1 activity toward phenyl acetate, which is not influenced by this polymorphism (9). Moreover, it has been shown that the PON1 allele is associated with higher serum concentrations of the enzyme compared with the M allele (23). Based on the above observations, we may suggest that the low PAF-AH activity in individuals carrying the L alloenzyme may be due to the replacement of 55-methionine by leucine, thus suggesting that the 55-methionine residue of PON1 is essential for the expression of the PAF-AH catalytic activity by this enzyme.

As expected, none of the subjects investigated exhibited the loss-of-function PAF-AH V279F mutation. On the other hand, one-third of our population exhibited the PAF-AH V379A polymorphism, which, however, did not affect either total plasma PAF-AH activity or the HDL-associated enzyme activity. It has been previously shown that this polymorphism significantly affects the enzyme kinetic constants (7); however, these experiments were performed using purified recombinant enzyme carrying the V379A variant. Thus, the difference between these previously published results and those of the present study could be due to other factors that influence the enzyme activity, such as the lipid environment of the lipoprotein particles that carry the enzyme in plasma (18).

The lower PAF-AH activity in L55L individuals compared with the carriers of the M allele found in the present study may explain, at least partially, the lower capacity of PON1 from the L55L individuals to protect LDL from oxidation compared with the carriers of the M allele (24). However, based on our results, we cannot comment on whether the low HDL-PAF-AH activity in L55L individuals may contribute to the association between the PON1 55L allele and atherosclerosis reported in previous studies (25, 26). On the other hand, other studies failed to show any association of this allele with the risk of CHD (27). Additionally, the PON1 Q192R polymorphism has been associated with the risk for CHD (28), and according to our results, this polymorphism does not affect the HDL-PAF-AH activity. Thus, it is profound that further studies involving CHD patients are required to show whether the results of the present study have any impact on the pathophysiology of CHD.

In conclusion, the present study shows for the first time that the PON1 M55L polymorphism influences the PAF-AH activity associated with HDL in both dyslipidemic and normolipidemic populations, and further suggests that PON1 significantly contributes to the pool of PAF-AH activity associated with HDL in human plasma.

REFERENCES

1. Gordon, T., W. P. Castelli, M. C. Hjortland, W. B. Kannel, and T. R. Dawber. 1977. High density lipoprotein as a protective factor against coronary heart disease: The Framingham Study. *Am. J. Med.* **62:** 707–714.
2. Navab, M., J. A. Berliner, G. Subbanagounder, S. Hama, A. J. Lusis, L. W. Castellani, S. Reddy, D. Shih, W. Shi, A. D. Watson, B. J. Van Lenten, D. Vora, and A. M. Fogelman. 2001. HDL and the inflammatory response induced by LDL-derived oxidized phospholipids. *Arterioscler. Thromb. Vasc. Biol.* **21:** 481–488.
3. Tselepis, A. D., and M. J. Chapman. 2002. Inflammation, bioactive lipids and atherosclerosis: potential roles of a lipoprotein-associated phospholipase A_2, platelet activating factor-acetylhydrolase. *Atherosclerosis.* **3(Suppl.):** 57–68.
4. Stafforini, D. M., K. Satoh, D. L. Atkinson, L. W. Tjoelker, C. Eberhardt, Y. Yoshida, and T. Imaizumi. 1996. Platelet-activating factor acetylhydrolase deficiency: a missense mutation near the active site of an anti-inflammatory phospholipase. *J. Clin. Invest.* **97:** 2784–2791.
5. Hiramoto, M., H. Yoshida, T. Imaizumi, N. Yoshimizu, and K. Satoh. 1997. A mutation in plasma platelet-activating factor acetylhydrolase (Val279Phe) is a genetic risk factor for stroke. *Stroke.* **28:** 2417–2420.
6. Yamada, Y., and M. Yokota. 1997. Loss of activity of plasma platelet-activating factor acetylhydrolase due to a novel Gln281Arg mutation. *Biochem. Biophys. Res. Commun.* **236:** 772–775.
7. Kruse, S., X. Q. Mao, A. Heinzmann, S. Blattmann, M. H. Roberts, S. Braun, P. Gao, J. Forster, J. Kuehr, J. M. Hopkin, T. Shirakawa, and K. A. Deichmann. 2000. The Ile198Thr and Ala 379Val variants of plasmatic PAF-acetylhydrolase impair catalytical activities and are associated with atopy and asthma. *Am. J. Hum. Genet.* **66:** 1522–1530.
8. Watson, A. D., J. A. Berliner, S. Y. Hama, D. N. La Du, K. F. Faull, A. M. Fogelman, and M. Navab. 1999. Protective effect of high density lipoprotein associated paraxonase. Inhibition of the biochemical activity of minimally oxidized low density lipoprotein. *J. Clin. Invest.* **96:** 2882–2891.
9. Mackness, B., M. I. Mackness, S. Arrol, W. Turkie, and P. N. Durrington. 1997. Effect of the molecular polymorphisms of human paraoxonase (PON), on the rate of hydrolysis of paraoxon. *Br. J. Pharmacol.* **112:** 265–268.
10. Mackness, M. I., B. Mackness, and P. N. Durrington. 1999. Polymorphisms of paraoxonase genes and low-density lipoprotein lipid peroxidation. *Lancet.* **353:** 468–469.
11. Rodrigo, L., B. Mackness, P. N. Durrington, A. Hernandez, and M. I. Mackness. 2001. Hydrolysis of platelet-activating factor by human serum paraoxonase. *Biochem. J.* **354:** 1–7.
12. Marathe, G. K., G. A. Zimmerman, and T. M. McIntyre. 2003. Platelet-activating factor acetylhydrolase, and not paraoxonase-1, is the oxidized phospholipid hydrolase of high density lipoprotein particles. *J. Biol. Chem.* **278:** 3937–3947.
13. Zama, T., M. Murata, Y. Matsubara, K. Kawano, N. Aoki, H. Yoshino, G. Watanabe, K. Ishikawa, and Y. Ikeda. 1997. A [191]Arg

variant of the human paraoxonase (HUMPONA) gene polymor-
phism is associated with an increased risk for coronary artery dis-
ease in the Japanese. *Arterioscler. Thromb. Vasc. Biol.* **17:** 3565–3569.

14. Yamada, Y., S. Ichihara, T. Fujimura, and M. Yokota. 1998. Identifi-
cation of the G994>T missense mutation in Exon 9 of the plasma
platelet-activating factor acetylhydrolase gene as an independent
risk factor for coronary artery disease in Japanese men. *Metabolism.*
47: 177–181.

15. Tsimihodimos, V., S-A. P. Karabina, A. P. Tambaki, E. Bairaktari, J. A.
Goudevenos, M. J. Chapman, M. Elisaf, and A. D. Tselepis. 2002.
Atorvastatin preferentially reduces LDL-associated platelet-activat-
ing factor acetylhydrolase activity in dyslipidemias of type IIA and
type IIB. *Arterioscler. Thromb. Vasc. Biol.* **22:** 306–311.

16. Ferre, N., M. Tous, A. Paul, A. Zamora, J. Vendrell, A. Bardaji, J.
Camps, C. Richart, and J. Joven. 2002. Paraoxonase Gln-Arg (192)
and Leu-Met (55) gene polymorphisms and enzyme activity in a
population with a low rate of coronary heart disease. *Clin. Biochem.*
35: 197–203.

17. Mertens, A., and P. Holvoet. 2001. Oxidized LDL and HDL: antag-
onists in atherothrombosis. *FASEB J.* **15:** 2073–2084.

18. Stafforini, D. M., M. Carter, G. A. Zimmerman, T. M. McIntyre,
and S. M. Prescott. 1989. Lipoproteins alter the catalytic behavior
of the platelet-activating factor acetylhydrolase in human plasma.
Proc. Natl. Acad. Sci. USA. **86:** 2393–2397.

19. Stafforini, D. M., T. M. McIntyre, M. E. Carter, and S. M. Prescott.
1987. Human plasma platelet-activating factor acetylhydrolase: as-
sociation with lipoprotein particles and role in the degradation of
platelet-activating factor. *J. Biol. Chem.* **262:** 4215–4222.

20. Ahmed, Z., A. Ravandi, G. F. Maguire, A. Emili, D. Draganov, B. N.
La Du, A. Kuksis, and P. W. Connely. 2001. Apolipoprotein A-I pro-
motes the formation of phosphatidylcholine core aldehydes that
are hydrolyzed by paraoxonase (PON-1) during high density lipo-
protein oxidation with a peroxynitrite donor. *J. Biol. Chem.* **276:**
24473–24481.

21. Mackness, M. I., S. Arrol, C. A. Abbott, and P. N. Durrington. 1993.
Protection of low density lipoprotein against oxidative modifica-

tion by high-density lipoprotein associated paraoxonase. *Atheroscle-
rosis.* **104:** 129–135.

22. Aviram, M., S. Billecke, R. Sorenson, C. Bisgaier, R. Newton, M.
Rosenblat, J. Erogul, C. Hsu, C. Dunlop, and B. La Du. 1998.
Paraoxonase active site required for protection against LDL oxida-
tion involves its free sulfhydryl group and is different from that re-
quired for its arylesterase/paraoxonase activities: selective action
of human paraoxonase alloenzymes Q and R. *Arterioscl. Thromb.
Vasc. Biol.* **10:** 1617–1624.

23. Garin, M-C. B., R. W. James, P. Dussoix, H. Blanche, P. Froguel,
and J. Ruiz. 1997. Paraoxonase polymorphism Met-Leu54 is associ-
ated with modified serum concentrations of the enzyme. *J. Clin.
Invest.* **99:** 62–66.

24. Mackness, B., M. I. Mackness, S. Arrol, W. Turkie, and P. N. Dur-
rington. 1998. Effect of the human serum paraoxonase 55 and 192
genetic polymorphisms on the protection by high density lipopro-
tein against low density lipoprotein oxidative modification. *FEBS
Lett.* **423:** 57–60.

25. Schmidt, H., R. Schmidt, K. Niederkorn, G. A. Gradert, M. Schu-
macher, N. Watzinger, H. P. Hartung, and G. M. Kostner. 1997.
Paraoxonase PON1 polymorphism Leu-Met 55 is associated with
carotid atherosclerosis: results of Austrian Stroke Prevention
Study. *Stroke.* **29:** 2043–2048.

26. Fortunato, G., P. Rubba, S. Panico, D. Trono, N. Tinto, C. Mazzaccara,
M. De Michele, A. Iannuzzi, D. Vitale, F. Salvatore, and L. Sacchetti.
2003. A paraoxonase gene polymorphism, PON1 (55), as an inde-
pendent risk factor for increased carotid intima-media thickness
in middle-aged women. *Atherosclerosis.* **167:** 141–148.

27. Gardemann, A., M. Philip, K. Heâ, N. Katz, H. Tillmanns, and W.
Haberbosch. 2000. The paraoxonase Leu-Met54 and Gln-Arg191
gene polymorphisms are not associated with the risk of coronary
heart disease. *Atherosclerosis.* **152:** 421–431.

28. Odawara, M., Y. Tachi, and K. Yamashita. 1997. Paraxonase poly-
morphism (Gln192-Arg) is associated with coronary artery disease
in Japanese non-insulin-dependent diabetes mellitus. *J. Clin. Endo-
crinol. Metab.* **82:** 2257–2260.