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A paraoxonase gene polymorphism, PON 1 (55), as an independent risk factor for increased carotid intima-media thickness in middle-aged women

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Abstract

Paraoxonase (PON) gene polymorphisms have been proposed as genetic markers of risk for cardiovascular disease (CVD). Sporadic results suggest they are correlated with intima-media thickness (IMT), an indicator of preclinical atherosclerotic disease. We have investigated whether polymorphisms PON 1 (M/L) 55, (Q/R) 192, PON 2 (S/C) 311 are related to site-specific carotid plaques in 310 middle-aged women. Subjects were also investigated for physical and biochemical parameters including oxidative markers to evaluate their effect on development of atherosclerotic plaques (IMT > 1.2 mm) identified by high resolution B-mode ultrasound. We demonstrate that PON 1 (LL+ML) 55 is associated with plaques both at the bifurcation (OR = 2.40; 95% CI 1.00–5.90) and at the common carotid artery (OR = 2.75; 95% CI 1.01–7.50), and to the total number of plaques at any site ($P < 0.05$). This polymorphism is an independent parameter with respect to other variables that are significantly associated with plaques, i.e. systolic blood pressure (OR = 2.06; 95% CI 1.11–3.81) and oxidized low-density lipoprotein (LDL) antibodies (OR = 1.96; 95% CI 1.05–3.69) in cases of common carotid plaques, and lipid peroxides (OR = 1.86; 95% CI 1.00–3.50) in cases of bifurcation plaques. In conclusion, PON 1 (LL+ML) 55 but not PON 1 (Q/R) 192 or PON 2 (S/C) 311, appears to be an independent risk factor for increased carotid IMT in middle-aged women.

Keywords: PON 1 and 2 polymorphisms; Carotid atherosclerosis; Oxidation markers; Intima-media thickness; Women

1. Introduction

Many genes, including the human paraoxonase gene (PON), have been implicated in cardiovascular disease (CVD) [1–3]. Three PON genes (PON 1, PON 2, PON 3) have been identified and mapped to chromosome 7 [4]. Polymorphisms have been detected at codons 192 (Gln → Arg, Q/R) and 55 (Met → Leu, M/L) in the PON 1 gene, and at codon 311 (Ser → Cys, S/C) in the PON 2 gene [5,6]. PON (E.C. 3.1.1.2) is a Ca^{2+}-dependent glycoprotein bound to high-density lipoproteins (HDL) and it may enhance their anti-atherosclerotic properties. In fact, PON prevents in vitro lipid peroxidation of low-density lipoproteins (LDL) [7] and PON 1 knockout mice have enhanced susceptibility to atherosclerosis [8]. Moreover, PON 1 hydrolyzes lipid peroxides in human atherosclerotic lesions [9]. The association between PON 1 and 2 gene polymorphisms and CVD has been investigated in Caucasian and non-Caucasian populations [6,10–14]. Unfortunately, the results have been discordant, probably due to the selection criteria of cases and controls.

High resolution B-mode ultrasound is a valid and reliable method to detect and monitor changes in carotid intima-media thickness (IMT), a marker of preclinical

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generalized atherosclerosis. Increased carotid IMT, i.e. thickenings and plaques, has been found in subjects with cardiovascular risk factors, and it is correlated with the presence of coronary atherosclerosis as an independent predictor of cardiovascular events [15–17]. The finding of genetic markers related to IMT, in addition to traditional risk factors and oxidation markers such as oxidized LDL antibodies, lipid peroxides, and 8-iso-$PGF_{2\alpha}$, might be useful to better identify people at high cardiovascular risk [14,15,18].

In this context, we studied PON 1 and 2 gene polymorphisms in relation to carotid plaque occurrence (evaluated with B-mode ultrasonography) and to other cardiovascular risk factors such as oxidation markers in 310 middle-aged women from South Italy enrolled in the Atena project [19].

2. Materials and methods

2.1. Subjects

The Atena project is a prospective study carried out in the Naples area (Italy). The total cohort, enrolled over 4 years, consisted of 5062 women aged between 30 and 71 years. Medical histories, including cardiovascular and metabolic disease and smoking habit, were collected from all subjects. Subjects with a previous diagnosis of myocardial infarction, stroke and major cancers were not included in the cohort. No selected women took hormone replacement therapy or were pharmacologically treated for diabetes or hypertension. Over 6 months, the older three of ten daily participants were offered a free vascular examination (high resolution ultrasound of the carotid arteries). The 310 women who accepted (77% response rate) underwent the PON genetic study described herein. There were no significant differences in the cardiovascular risk profile between the whole cohort and this subsample [15]. The study was approved by the Ethics Committee of our Medical School, and informed consent was obtained for each patient.

2.2. B-mode ultrasound

An internationally certified sonographer made the carotid B-mode ultrasound examinations using the Biosound 2000 II SA apparatus equipped with an 8 MHz anular array mechanical transducer. The system provides high-resolution ultrasonic images with 0.3-mm axial resolution and 256° of gray scale. Scans were performed according to a standardized protocol detailed elsewhere [15]. Briefly, the aim of the protocol was to measure IMT in the distal 1 cm of the common carotid artery and the carotid bifurcation; an IMT >1.2 mm was indicative of plaques (ultrasound end-point of the study). This cut-off point has been used in randomized clinical trials [20] and corresponds to the 90th percentile of the mean IMT of a random sample of 170 adult Neapolitan women. The sonographer looked for carotid plaques on the near and far walls of the common carotid arteries and carotid bifurcations at both sites. The total number of plaques at both sites is 'plaque score'. All IMT measurements were made by the sonographer at the time of the examination using an electronic caliper, as previously reported, the within-subject coefficient of variation was $<6\%$ [21].

2.3. DNA analysis

Genomic DNA was extracted from peripheral blood samples according to standard techniques [22]. DNA was available for 288 women (93% of total sample). The Gln-Arg 192 and Met-Leu 55 polymorphisms at PON 1 and the Ser-Cys 311 polymorphism at PON 2 were determined after polymerase chain reaction (PCR) amplification followed by digestion with Alw I, NlaIII and DdeI, for the three polymorphisms respectively [5,6]. The PCR products were resolved on a metaphore gel 4% and visualized by staining with ethidium bromide. PON genotypes were assessed independently by two observers. The identity of PCR products was verified by sequence analysis.

2.4. Anthropometry and laboratory measurements

The body mass index (BMI) was calculated for each subject as body weight (kg) divided by squared height (m^2). Venous blood was sampled from all subjects, after an overnight fast. Serum total cholesterol, triglycerides and glucose were evaluated enzymatically on an automated analyzer (Hitachi 747, Boehringer Mannheim, Germany). HDL cholesterol was determined enzymatically by measuring cholesterol in the supernatant after precipitation with phosphotungstate. LDL cholesterol levels were calculated according to the traditional Friedewald formula [23]. The serum IgG antibody titer against oxidized LDL was measured in duplicate with an enzyme-linked-immunosorbent-assay (ELISA) on microwells coated with Cu^{2+} oxidized-LDL (o-Lab-ELISA Kit, Biomedica, Vienna, Austria). The results were expressed as mU/ml. Imprecision coefficients (intra- and inter-assay), evaluated on control sera at low and high concentrations, were below 10%.

Total radical-trapping antioxidant plasma activity (TRAP) was measured with a spectrophotometric end-point method on a Cobas–Fara analyzer [24]. The synthetic water-soluble tocopherol analogue Trolox (Hoffman–La Roche) was used for calibration. The intra- and inter-assay imprecision coefficients, evaluated on a plasma pool, were 1.7 and 3.2%, respectively.

The concentration of thiobarbituric acid (TBA) reactive substances was measured with a modified TBA assay [25] and the results were quantified on the basis of a calibration curve obtained with the standard malondialdehyde, the main product of lipid peroxidation.

2.5. Statistical analysis

Allele frequencies were calculated by allele counting and the departure from Hardy–Weinberg expectation was evaluated by χ^2 analysis. The relationship of the PON gene polymorphisms with continuous variables was tested by one-way ANOVA. The relationship between PON gene polymorphisms and categorical variables was identified by the χ^2-test. The Bonferroni correction was used to make comparisons [26]. To compare groups with and without plaques at different sites, biochemical and physical parameters were divided into quartiles. PON gene polymorphisms were combined as follows: PON 1 MM (55) (group = 0) versus LL+ML (group = 1), PON 1 QQ (192) (group = 0) versus QR+RR (group = 1) and PON 2 SS (311) (group = 0) versus SC+CC (group = 1). Odds ratios (OR) of atherosclerotic plaques were determined by logistic regression analysis comparing group 0 versus group 1 for each polymorphism and quartile 4 versus quartiles 1–3 of biochemical and physical parameters; 95% confidence intervals (CI) were calculated from the β-coefficients and their standard errors after adjustment for age, smoking, LDL cholesterol and BMI.

The interaction between smoking habit, plaques and polymorphisms was also evaluated by logistic regression analysis.

Multiple linear regression analysis was used to investigate the relationship between the total number of plaques at both sites (plaque score) and each of the tested biochemical, physical and genetic (PON polymorphisms) characteristics. Statistical analyses were performed using the SPSS for WINDOWS software (Version 9.0).

3. Results

In a previous paper [15] concerning the Atena population we reported that there were no atherosclerotic plaques (IMT > 1.2 mm) at either the bifurcation or the common carotid artery in 105 women; in the remaining 205 subjects, plaques were found at the bifurcation ($n = 77$), at the common carotid artery ($n = 37$) or at both sites ($n = 91$). Table 1 summarizes the characteristics of the study sample. Most physical and biochemical variables did not differ significantly depending on the presence or absence of plaques. Differently, LDL-cholesterol, total cholesterol levels ($P < 0.01$) and lipid peroxides ($P < 0.05$) were associated with plaques at the bifurcation, and blood pressure ($P < 0.05$), BMI and oxidized LDL antibodies ($P < 0.01$) were associated with plaques at the common carotid artery.

Table 1
Physical and biochemical characteristics of the sub-sample of the Atena project population of middle-aged women ($n = 310$)

Parameter	Mean ± S.D.
Age (years)	55.0 ± 7.81
BMI(kg/m^2)	27.7 ± 4.57
Systolic blood pressure	140.2 ± 22.13
Diastolic blood pressure	83.3 ± 10.57
Smokers *n* (%)	71 (43.8)
Total cholesterol (mmol/l)	6.39 ± 1.35
LDL cholesterol (mmol/l)	4.08 ± 1.22
HDL cholesterol (mmol/l)	1.65 ± 0.41
Triglycerides (mmol/l)	1.38 ± 0.71
Glucose (mmol/l)	5.58 ± 1.09
Oxidized LDL antibodies (mU/ml)	330 ± 292.03
TRAP (mmol/l)	1.53 ± 0.11
Lipid peroxides (μmol/l)	0.46 ± 0.19

BMI, body mass index; TRAP, total radical-trapping antioxidant plasma activity; LDL, low-density lipoproteins; HDL, high-density lipoproteins.

Table 2 shows the distribution of the Gln-Arg (Q/R) 192 and Met-Leu (M/L) 55 polymorphisms at the PON 1 locus and the Ser-Cys (S/C) 311 polymorphism at the PON 2 locus, together with the corresponding allele frequencies. All three genotypes, at each locus, followed the Hardy–Weinberg equilibrium. The Q (192), L (55) and S (311) alleles were present in, respectively 68, 65 and 81% of the observed genotypes.

There were no significant differences (data not shown) between traditional cardiovascular risk factors, oxidation markers and Q/R (192), M/L (55) PON 1 or S/C (311) PON 2 polymorphisms with the only association with age for M (55) allele carriers ($P < 0.017$ by ANOVA).

The percentages of genotypes QQ (192), MM (55) PON 1 and SS (311) PON 2 were higher in the absence than in the presence of plaques (Table 3). However, only the MM (55) genotype was significantly associated with plaques at the bifurcation ($P = 0.05$) and at the common carotid ($P < 0.03$). In non smokers MM was significantly associated with less plaque formation ($P = 0.025$) at any site, whereas in smokers MM was associated with greater plaque formation, which suggests that the protective role of MM genotype is lost in these subjects.

We then used logistic regression analysis, after data adjustment for age, smoking, LDL cholesterol and BMI, to test the association between the site of atherosclerotic plaques and PON 1 and 2 genotypes, and physical and biochemical parameters (detailed in Table 1). As shown in Figs. 1 and 2, PON 1 (LL+ML) genotypes were significantly associated with plaques, both at the bifur-

Table 2
Genotype and allele distributions of Gln-Arg (Q/R) 192, Met-Leu (M/L) 55 PON 1 and Ser-Cys (S/C) 311 PON 2 polymorphisms in a subsample of middle-aged women of the Atena project

PON 1				PON 2	
Q/R 192 ($n=286$)		M/L 55 ($n=288$)		C/S 311 ($n=287$)	
Q/Q	140 (49%)	L/L	119 (41%)	S/S	188 (66%)
Q/R	111 (39%)	M/L	136 (47%)	S/C	90 (31%)
R/R	35 (12%)	M/M	33 (12%)	C/C	9 (3%)
Q allele	391 (68%)	L allele	374 (65%)	S allele	466 (81%)
R allele	181 (32%)	M allele	202 (35%)	C allele	108 (19%)

cation (OR 2.40; 95% CI 1.00–5.90) and at the common carotid (OR 2.75; 95% CI 1.01–7.50). Plaques at the bifurcation were also associated with lipid peroxides (OR 1.86; 95% CI 1.00–3.50), whereas common carotid plaques were associated with oxidized LDL antibodies (OR 1.96; 95% CI 1.05–3.69) and systolic blood pressure (OR 2.06; 95% CI 1.11–3.81). PON 1 (QR+RR) and PON 2 (SC+CC) genotypes did not differ in relation to plaques whatever the site. Finally, multiple linear regression analysis of physical and biochemical characteristics, and PON 1 and 2 polymorphisms (after adjustment for the confounding parameters age, smoking, LDL cholesterol and BMI) in relation to plaque score revealed that the latter was related to the PON 1 (LL+ML) genotypes and systolic blood pressure (Table 4). We investigated the effects of interaction between PON 1 and 2 polymorphisms versus plaques, but did not identify any significant risk carrier genotype.

4. Discussion

We have studied PON 1 and 2 polymorphisms, IMT and other traditional and non-traditional risk factors for CVD in a large sample of middle-aged clinically healthy women (310 subjects from more than 5000 of the Atena project). PON 1 activity has recently been reported to be a better predictor of CHD than PON polymorphisms [27]. The aim of our study was to explore the role of PON in plaque evolution, in the absence of cardiovascular events, in healthy women. To this aim we investigated PON genotypes as a predictor of the ability of PON to prevent LDL oxidation [7], rather than PON 1 activity, which reflects the enzyme's ability to hydrolyze organophosphate such as paraoxon and not its antioxidant capacity [2].

The results show that PON 1 genotypes play an important role in the early formation of atherosclerotic

Table 3
Allele distribution of Met-Leu (M/L) 55, Gln/Arg (Q/R) 192 PON 1 and Cys/Ser (C/S) 311 PON 2 in women of the Atena project, in presence (IMT >1.2 mm) and absence of plaques (IMT ≤ 1.2 mm) at any site, bifurcation and common carotid artery

	Any site		Bifurcation		Common carotid artery	
	IMT >1.2 mm	IMT ≤ 1.2 mm	IMT >1.2 mm	IMT ≤ 1.2 mm	IMT >1.2 mm	IMT ≤ 1.2 mm
PON 1 (55)	($n=192$)	($n=96$)	($n=153$)	($n=135$)	($n=120$)	($n=168$)
Genotype						
L/L	82 (43%)	37 (38%)	65 (43%)	54 (40%)	50 (42%)	69 (41%)
M/L	94 (49%)	42 (44%)	77 (50%)	59 (44%)	63 (52%)	73 (43%)
M/M	16 (8%)	17 (18%)	11 (7%)	22 (16%)	7 (6%)	26 (16%)
	$P=$ n.s		$P=0.05$		$P<0.03$	
PON 1 (192)	($n=190$)	($n=96$)	($n=151$)	($n=135$)	($n=118$)	($n=168$)
Genotype						
Q/Q	88 (46%)	52 (54%)	69 (46%)	71 (53%)	56 (47%)	84 (50%)
Q/R	76 (40%)	35 (37%)	58 (38%)	53 (39%)	47 (40%)	64 (38%)
R/R	26 (14%)	9 (9%)	24 (16%)	11 (8%)	15 (13%)	20 (12%)
	$P=$ n.s		$P=$ n.s		$P=$ n.s	
PON 2 (311)	($n=191$)	($n=96$)	($n=152$)	($n=135$)	($n=119$)	($n=168$)
Genotype						
C/C	7 (4%)	2 (2%)	5 (3%)	4 (3%)	6 (5%)	3 (2%)
C/S	63 (33%)	27 (28%)	51 (34%)	39 (29%)	43 (36%)	47 (28%)
S/S	121 (63%)	67 (70%)	96 (63%)	92 (68%)	70 (59%)	118 (70%)
	$P=$ n.s		$P=$ n.s		$P=$ n.s	

n.s = not significant.

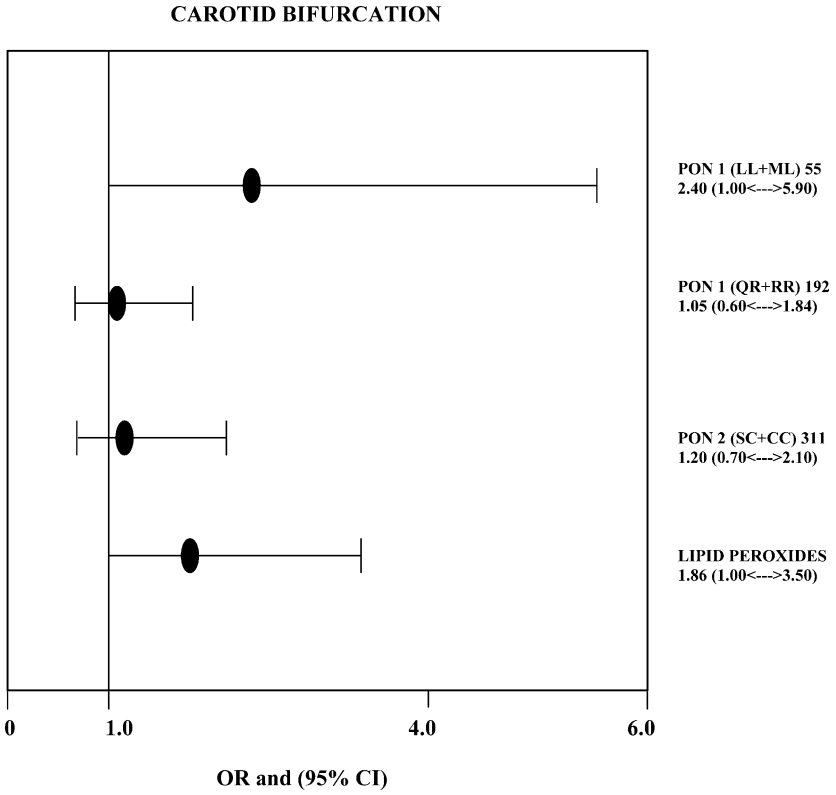

Fig. 1. Odds ratio and 95% CI obtained by logistic regression analysis of carotid bifurcation plaques, PON 1 (M/L, Q/R), PON 2 (S/C) polymorphisms, physical and biochemical parameters (quartile 4 versus quartiles 1–3) in the study sample. The data were adjusted for age, smoking, LDL cholesterol and BMI. Statistically significant association was found only among PON1 (LL+ML) genotypes and upper quartile 4 of values for lipid peroxides.

plaques. In fact, we demonstrate that PON 1 (LL+ML) 55 genotypes were associated with plaques both at the bifurcation and at the common carotid artery, and with plaque score. This polymorphism is an independent parameter with respect to other variables that are significantly associated with plaques, i.e. systolic blood pressure and oxidized LDL antibodies (plaques at the common carotid), and lipid peroxides (plaques at the bifurcation).

The frequencies of PON 1 M/L (55) genotypes were similar to those reported for other Caucasian populations [10,28–30] and differed from Asiatic [12] and Mestizos [31] populations whose MM genotype was respectively less (1%) and more (56%) frequent than in our sample (12%). Thus, our findings support the concept that PON 1 polymorphisms vary according to ethnic provenance. In about one-third of women free from plaques, the frequency of PON 1 MM (18% at any site, and 16% at both the bifurcation and common carotid) was higher than in the total population (12%), which agrees with the hypothesis that the MM genotype affords more protection against atherosclerosis than do the LL and LM genotypes. Accordingly, in in vitro experiments, PON 1 MM associated with HDL provided greater protection against LDL oxidative changes (49.5%) than LL and ML (respectively, 21.8 and 29.5%). These data support a similar susceptibility to lipid peroxidation of subjects bearing PON 1 ML or LL genotypes [7]. In addition, as indicated above, we found that the PON 1 (LL+ML) 55 genotypes were significantly associated with plaque score, thus providing direct evidence for an increased risk in women in the early phase of blood vessel atheroma formation.

Earlier studies of Caucasian populations established that PON 1 LL (55) alone or associated with PON 1 QQ (192) were risk factors for atherosclerosis evaluated by IMT [29,32,33]. However, studies on the role of the PON 1 M/L (55) polymorphism and CVD produced conflicting results [10,12,34,35]. Different inclusion criteria for CVD cases (i.e. myocardial infarction, $>$ 50% stenosis of major coronary arteries, familial hypercholesterolemia, etc.), or lack of appropriate control groups (i.e. selection based on normal ECG, absence of family history of CVD, medical history based on questionnaire, etc.), together with the specific ethnic characteristics of the populations examined may explain these discordant findings.

We previously found in the same Atena sample that common carotid artery plaques were significantly associated with higher systolic blood pressure, BMI and increased levels of antibodies to oxidized LDL, whereas higher LDL cholesterol and lipid peroxidation products

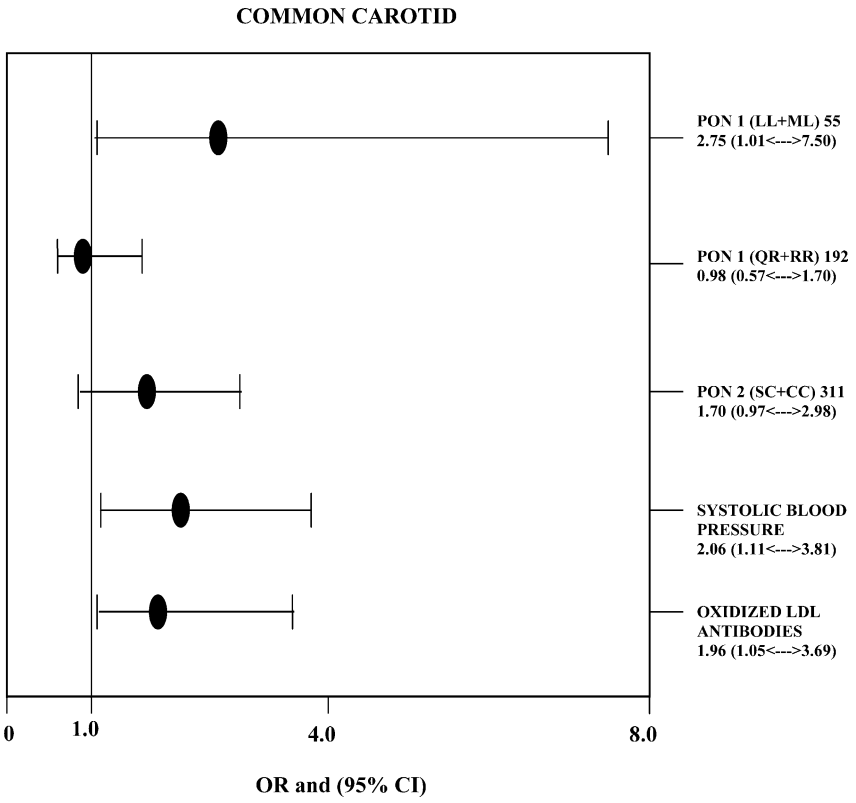

Fig. 2. Odds ratio and 95% CI obtained by logistic regression analysis of common carotid plaques, PON 1 (M/L, Q/R), PON 2 (S/C) polymorphisms, physical and biochemical parameters (quartile 4 versus quartiles 1–3) in the study sample. The data were adjusted for age, smoking, LDL cholesterol and BMI. Statistically significant association was found only among PON 1 (LL+ML) genotypes and upper quartile 4 of values for the parameters: systolic blood pressure and oxidized LDL antibodies.

Table 4
Multivariate analysis using 'Plaque score' as dependent variable and genetic polymorphisms of PON 1 (M/L, Q/R), PON 2 (S/C), physical and biochemical parameters* as independent variables in the study sample of middle-aged women of South Italy

	Coefficient 'β' ± ES	P
Independent variables[a]		
PON 1 (LL+ML) 55 genotypes	0.73 ± 0.29	< 0.05
Systolic blood pressure	0.46 ± 0.21	< 0.05
Dependent variable = 'Plaque score'		

*Quartile 4 versus Quartiles 1–3.
[a] Adjusted for age, smoking, LDL cholesterol and BMI.

were associated with plaques at the carotid bifurcation when compared with results from women without plaques [15]. These results suggest that different mechanisms underlie lesion development. In fact, the bifurcation segment, which contains more macrophages compared with contiguous segments, appears to be more susceptible to LDL oxidation and lipid accumulation as shown by high levels of lipid peroxidation. Although associated with medial hyperplasia due to hypertension, the common carotid artery is far from being susceptible to atherosclerosis. Therefore, common carotid plaques are likely to be related to atherosclerotic progression marked by high titers of oxidized LDL antibodies. These data, and the association of the PON 1 (55) polymorphism with plaques at both the bifurcation and common carotid and with plaque score reported herein, support the hypothesis that genetic PON predisposition, lipids and their oxidative status in blood, and hypertension are all involved in the development of atherosclerosis.

We found no association between the PON 1 (RR + RQ) 192 genotype and plaques even if in our patients with IMT > 1.2 mm the RR/RQ genotypes were more frequent than in patients with IMT < 1.2 mm. Similarly, two studies of a clinically healthy population failed to show an association between the PON 1 (192) polymorphism and carotid lesions [32,36]. On the other hand, a meta-analysis showed that PON 1 (RR or RQ) 192 genotypes were significantly increased if CVD was present [2]. Perhaps, as previously suggested [36], the effect of PON 1 Q/R (192) genotypes on plaque formation differs between the early and later stages of the atherosclerotic process.

Another result of our study concerns the PON 2 S/C (311) polymorphism that has been studied in conjunction with the presence of ultrasonographic plaques. PON 2 was unrelated to either the presence or absence of plaques. This result agrees with those reported for

African Caribbeans [14]. However, the SS genotype was more frequent in cases of IMT < 1.2 mm versus IMT > 1.2 mm, which is in accordance with data obtained in a Japanese population [12]. In contrast, another report suggested that PON 2 SS (311) contributes synergistically to the PON 1 RR (192) genotype as a risk factor for CVD [6]. It is noteworthy that in previous studies the PON 2 polymorphism was evaluated both in cardiovascular and control subjects in whom plaques were not measured [6,12]. Therefore, misclassification due to possible subclinical atherosclerosis cannot be excluded.

In conclusion, our data obtained in middle-aged women from South Italy, provide direct evidence that PON 1 (LL+ML) 55 genotypes play a role in carotid lesions at any site, independently of traditional and non-traditional risk factors. PON 1 Q/R (192) and/or PON 2 S/C (311) polymorphisms do not appear to be involved in the development of atherosclerotic plaques.

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