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Paraoxonase 1 L55M, Q192R and paraoxonase 2 S311C alleles in atherothrombosis

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Abstract Increased oxidative stress is known to play a role in the pathogenesis of atherosclerosis, and polymorphisms in genes encoding for enzymes involved in modulation of oxidant stress, such as paraoxonases (PONs), provide a potentially powerful approach to study the risk of disease susceptibility. Aim of our study is to investigate the possible association among PONs polymorphisms, clinical and metabolic factors, and atherothrombotic events in an Italian population. We evaluated in 105 subjects, with or without atherosclerotic risk factors, the presence of PON1 L55M, PON1 Q192R, and PON2 S311C genetic variants, as well as lipid profile, the concentration of aminothiols (blood reduced glutathione, plasma total glutathione, homocysteine, cysteine, cysteinyl glycine), and malondialdehyde as markers of lipid peroxidation. Clinical, biochemical, and genetic variables were correlated with a history of atherothrombosis. Previous atherothrombotic events were found in 42 patients (40 %): myocardial infarction in 24, stroke or transient ischemic attack in 18. By multiple logistic regression analysis, hypertension (OR = 5.538; 95 % CI 2.202–13.902, $P < 0.001$), HDL-cholesterol concentration (OR = 0.947; 95 % CI 0.910–0.985, $P = 0.007$), and the presence of C allele in PON2 gene (OR = 3.595; 95 % CI 1.247–10.361, $P = 0.018$) were independently associated with atherothrombotic events. Our study sheds light on the role of PON2 as a possible cofactor in determining the risk of events together with the well-known risk markers HDL-cholesterol and hypertension.

Keywords Paraoxonase · Oxidative stress · Aminothiols · Atherothrombosis

Introduction

Increased oxidative stress is known to play a role in the pathogenesis of the atherosclerosis. Oxidation of low density lipoproteins (LDL) and the subsequent production of lipid hydroperoxides accelerate the development of vascular disease by inducing endothelial cell injury, monocyte activation, smooth muscle cell proliferation, and foam cell formation. High density lipoprotein (HDL) concentrations are instead inversely correlated with the incidence of coronary heart disease [1].

High density lipoproteins exert their anti-atherogenic effect by different mechanisms: (1) the process of reverse cholesterol transport from peripheral tissues to liver; (2) the antioxidant action of paraoxonase (PON), an enzyme tightly associated with the HDL surface that decreases the peroxidation of LDL. The PON gene family has three known members, PON1, PON2, and PON3, located on the long arm of chromosome 7 between q21.3 and q22.1 in humans. PON1 is primarily associated with HDL particles and exerts a cardioprotective function through its hydrolyzing effect on LDL-oxidized phospholipids and also on homocysteine thiolactone [2, 3] which is responsible for homocysteine pro-atherogenic function [1]. Homocysteine, like the other metabolically correlated aminothiol cysteine,

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may produce free radical species responsible for the oxidative stress increment, which are known to impair endothelial function.

PON2 is an intracellular protein found in a large variety of tissues including the arterial wall [4] but it is not associated with HDL or LDL. In vitro, PON2 possesses antioxidant properties similar to that of PON1 in preventing LDL oxidation. The antioxidative and anti-atherogenic activities of PON2 have been well documented and recently reviewed [5]; reduced PON2 levels enhanced atherogenesis in mice [6, 7] and correlated with atherosclerosis progression in humans [8]. However, studies attempting to relate PON2S/C 311 polymorphism to the risk for CVD and for other diseases have led to equivocal conclusions [9]. These contradictory data may derive from ethnic backgrounds; however, they may also be related to exogenous modulation of PON2 expression by nutrition and drugs.

Because several evidences support the potential role of the PON gene family in atherogenesis [10–12], substantial research has looked for genetic association between PON variants and atherosclerosis; however, the results have often been conflicting [13].

Aims of our retrospective study were (a) to investigate the possible association among PON polymorphisms [PON1 L55M (rs854560) and Q192R (rs662), and PON2 S311C], clinical and metabolic factors, and a history of atherothrombotic events and (b) to verify the association between PON gene variants and metabolic patterns, assessed by aminothiols equilibrium, as an index of intracellular and extracellular redox state, in an Italian population.

Methods

Subjects

One hundred and five subjects with normal (<15 μmol/L) or moderate–intermediate (≥15 and <100 μmol/L) plasma homocysteine concentrations [14] were selected from a population referred to our Institution for suspected hyperhomocysteinemia.

Exclusion criteria were as follows: ≤19 and ≥75 years, previous history of immunologic or neoplastic diseases, ongoing infection, impaired renal function, surgery or trauma within the previous month, and vitamin supplements within 2 weeks before the study.

Forty-two subjects had a documented history of atherothrombotic events: 24 subjects had had a myocardial infarction [15] and 18 a stroke [16] or transient ischemic attack [17]; the remaining 63 subjects had no current symptoms or clinical/laboratory evidence of arterial vascular disease.

At the time of blood sampling, a complete clinical history was collected including the assessment of cardiovascular risk factors such as hypertension (systolic and diastolic blood pressure ≥140 and 90 mmHg, respectively, or on antihypertensive drugs [18]), dyslipidemia (LDL ≥ 160 mg/dL or current treatment with lipid-lowering drugs [19]), diabetes (fasting serum glucose ≥126 mg/dL in at least two separate measurements or on antidiabetic drugs [20]), moderate–intermediate hyperhomocysteinemia and smoking habits. Patients were taking recommended drugs for secondary or primary cardiovascular prevention, but omitted these medications for at least 24 h before sampling.

Written informed consent for genetic and biochemical analysis was obtained from all the patients.

Sample analysis

After an overnight fast, an antecubital vein was cannulated and blood was drawn into different pre-chilled Vacutainer tubes for genetic and biochemical determinations.

Genotyping

Genomic DNA was obtained from peripheral blood samples by the standard procedure. The analysis of PON1 L55M, Q192R, and the PON2 S311C polymorphisms was carried out by PCR amplification using a set of primers, designed as follows:

PON1 L55M	5′-GCTCTAGTCCATCAATTTAAAACAAA-3′ (upstream)
	5′-TGGGTATACAGAAAGCCTAAGTGA-3′ (downstream)
PON1 Q192R	5′-AGACAGTGAGGAATGCCAGTT-3′ (upstream)
	5′-CAGAGAGTTCACATACTTGCCATC-3′ (downstream)
PON2 S311C	5′-TTCAACAGCATGTCCCCTTA-3′ (upstream)
	5′-AGTGCCTATGAGCAGCTTCC-3′ (downstream)

PCR products for PON1 L55M and PON2 S311C were analyzed by restriction enzyme digestion, using *Nla*III and *Dde*I, respectively; DHPLC screening and sequence analysis of the samples with aberrant elution profiles were performed for PON1 Q192R.

Biochemistry

Immediately after blood collection, sample preparation and analysis of blood reduced glutathione (GSH) (index of

GSH concentrations in circulating cells), plasma total cysteine, homocysteine, and cysteinyl glycine were performed and their concentrations were determined by high pressure liquid chromatography (HPLC; ProStar, Varian, Surrey, UK), according to the method previously described [21]. MDA levels were determined in stored plasma by HPLC method using a commercial kit (Chromsystems). Values are expressed as μmol/L.

Total cholesterol, HDL, LDL, and triglycerides were determined using standard laboratory methods. Values are expressed as mg/dL.

Statistical analysis

Continuous variables are expressed as median and interquartile range (I, III). Allele distribution of different genetic variants were tested by χ^2 or Fisher exact test. Hardy–Weinberg equilibrium was assessed by calculating the expected genotype frequencies from the allele in our study population; deviation from the observed genotype frequencies was determined by χ^2. Genotype distributions in PON1 Q192R and PON2 S311C variants were compatible with Hardy–Weinberg equilibrium, but not those in PON1 L55M polymorphism, which were consequently dropped from further analysis. The independent relationship of vascular risk factors, biochemical parameters, genotype combinations of PON1 192 QQ versus QR + RR and PON2 311 SS versus SC + CC with events was tested by logistic regression analysis; results are expressed as odds ratio (OR) and their 95 % confidence intervals (CI). The same analysis was used to compare the genotype combinations reported above with aminothiol redox equilibrium in the overall population. Significant variables by univariable analysis ($P < 0.05$) were entered into a stepwise multivariable logistic regression model to identify those independently associated with atherothrombotic events. Diabetes was omitted from the analysis because of the scant number of subjects.

Statistical analysis was carried out using the Statistical Package for the Social Sciences (SPSS Inc., Chicago, Ill., U.S.) release 17.0 for Windows.

Results

Comparison of clinical, metabolic, and genetic factors with atherothrombotic events

We classified enrolled subjects in two groups, according to the absence (AE− group, $n = 63$) or presence (AE+ group, $n = 42$) of a history of atherothrombotic events. The distribution of PON1 and PON2 polymorphisms was assessed in AE+ and AE− groups. The allele frequency of PON1 Q192R variant was similar in both groups, while the allelic distribution of PON2 S311C significantly differed in AE+ and AE− groups (Table 1).

Baseline clinical, biochemical, and genetic characteristics of the enrolled subjects are reported in Table 2. Moderate/intermediate hyperhomocysteinemia (concentration range 15.4–74.8 μmol/L) was present in 45 % of enrolled subjects. Drug treatment was differently distributed between AE+ and AE− groups, in accordance with guideline recommendations for secondary and primary cardiovascular prevention: AE+ subjects took in a higher proportion of renin-angiotensin system inhibitors, statins, antiplatelet agents, and β-blockers than AE− subjects (Table 3). Because of this clinically consistent treatment bias, we did not include therapy variables in the logistic regression analysis.

By univariable logistic regression analysis, male gender, age, hypertension, dyslipidemia, total plasma GSH, and PON2 311C alleles were positively associated with atherothrombotic events, while HDL was inversely associated with AE+.

In the multivariable logistic regression model, only hypertension ($P < 0.001$, OR = 5.538; 95 % CI 2.202–13.902), HDL ($P = 0.007$, OR = 0.947; 95 % CI 0.910–0.985), and the presence of C allele in PON2 gene ($P = 0.02$, OR = 3.595; 95 % CI 1.247–10.361) were independently associated with previous atherothrombotic events.

Table 1 Genotype distribution and allelic frequencies of PONs polymorphisms in the study population

	Total ($n = 105$)	AE− group ($n = 63$)	AE+ group ($n = 42$)	*P*
PON1: Q192R				
Genotypes				
Q/Q	57 (54 %)	33 (52.4 %)	24 (57.1 %)	
Q/R	40 (38 %)	26 (41.3 %)	14 (33.3 %)	
R/R	8 (8 %)	4 (6.3 %)	4 (9.5 %)	
Alleles				1.00
Q	0.733	0.730	0.738	
R	0.267	0.270	0.262	
PON2: S311C				
Genotypes				
S/S	74 (70 %)	50 (79.4 %)	24 (57.1 %)	
S/C	26 (25 %)	10 (15.9 %)	16 (38.1 %)	
C/C	5 (5 %)	3 (4.8 %)	2 (4.8 %)	
Alleles				0.04
S	0.829	0.873	0.762	
C	0.171	0.127	0.238	

AE+ with atherothrombotic event, *AE−* without atherothrombotic events, *PON* paraoxonase

Table 2 Clinical, biochemical and genetic characteristic of the study population

Characteristic	Total ($n = 105$)	AE− group ($n = 63$)	AE+ group ($n = 42$)	*P*	OR	95 % CI
Age, years	50 [38, 60]	45 [31, 56]	53 [44, 63]	0.01	1.040	1.009–1.071
Male sex, *n* (%)	75 (71)	39 (62)	36 (86)	0.01	3.692	1.355–10.064
Hypertension, *n* (%)	42 (40)	15 (24)	27 (64)	<0.001	5.760	2.445–13.571
Hyperhomocysteinemia, *n* (%)	57 (45)	30 (48)	17 (41)	0.47	0.748	0.339–1.648
Smoking habit, *n* (%)	44 (42)	26 (41)	18 (43)	0.87	1.067	0.484–2.353
Dyslipidemia, *n* (%)	45 (43)	21 (33)	24 (57)	0.02	2.667	1.192–5.964
PON1 192 QR + RR, *n* (%)	48 (46)	30 (48)	18 (43)	0.63	0.825	0.376–1.811
PON2 311 SC + CC, *n* (%)	31 (30)	13 (21)	18 (43)	0.02	2.885	1.216–6.841
b-r-GSH, μmol/L	556 [388, 788]	599 [387, 789]	522 [355, 762]	0.35	0.999	0.998–1.001
p-t-GSH, μmol/L	5.47 [4.03, 7.35]	6.1 [4.8, 7.8]	4.6 [3.4, 5.9]	0.006	0.778	0.651–0.929
p-t-Cys, μmol/L	249 [198, 303]	252 [183, 302]	243 [207, 312]	0.71	1.001	0.996–1.006
p-t-Hcy, μmol/L	14.1 [8.9, 24.0]	14.1 [8.7, 24.2]	13.9 [9.5, 22.1]	0.50	0.990	0.960–1.020
p-t-CysGly, μmol/L	32 [23, 47]	31 [23, 48]	33 [23, 45]	0.27	0.988	0.968–1.009
MDA, μmol/L	0.75 [0.46, 1.45]	0.67 [0.38, 1.19]	0.94 [0.59, 1.54]	0.23	1.429	0.769–2.564
Total cholesterol, mg/dL	188 [158, 218]	189 [160, 222]	182 [152, 210]	0.32	0.995	0.986–1.005
HDL-cholesterol, mg/dL	52 [43, 62]	54 [45, 63]	46 [38, 59]	0.008	0.954	0.922–0.988
LDL-cholesterol, mg/dL	108 [88, 140]	111 [88, 147]	106 [83, 137]	0.62	0.997	0.987–1.008
Triglycerides, mg/dL	100 [70, 157]	95 [68, 144]	108 [79, 195]	0.35	1.002	0.997–1.008

Data are expressed as median [I, III] or number (%)

OR odds ratio, *CI* confidence interval, *PON* paraoxonase, *b* blood, *p* plasma, *r* reduced, *t* total, *GSH* glutathione, *Cys* cysteine, *Hcy* homocysteine, *CysGly* cysteinyl glycine, *MDA* malondialdehyde, *HDL* high density lipoproteins, *LDL* low density lipoproteins

Table 3 Drug treatment

	Total ($n = 105$)	AE− ($n = 63$)	AE+ ($n = 42$)	*P*
ACE-inhibitors/ ARBs	23 (22 %)	6 (10 %)	17 (41 %)	<0.001
Beta-blockers	15 (15 %)	3 (5 %)	12 (29 %)	0.001
Antiplatelet agents	45 (44 %)	12 (19 %)	33 (81 %)	<0.001
Statins	22 (21 %)	7 (11 %)	15 (37 %)	0.003
Calcium channel blockers	14 (14 %)	7 (11 %)	7 (17 %)	0.56

AE+ with atherothrombotic event, *AE−* without atherothrombotic events

Comparison between genetic variants and aminothiols status

A logistic regression analysis was also used to compare genotype combinations of PON1 192 QQ versus QR + RR and PON2 311 SS versus SC + CC with the aminothiols pattern in the overall population.

No association was found in these comparisons.

Discussion

Susceptibility to cardiovascular disease is increasingly demonstrated to be the result of an interaction among lifestyle, environment, and genetic factors. Our study found that PON2 S311C polymorphism, hypertension, and low levels of HDL are independently associated with atherothrombotic disease in an Italian subpopulation.

Several authors link oxidative stress to cardiovascular events [22] which are usually triggered by arterial thrombosis. Overproduction of reactive oxygen species (ROS) that outstrips antioxidant defenses can lead to endothelial cell injury, thus contributing to the pathophysiology of atherogenesis. Polymorphisms in genes encoding enzymes involved in oxidative stress modulation provide a potentially powerful approach to study disease susceptibility [23], and PON genes represent good candidates.

Although PONs were initially identified for their ability to metabolize pesticide-derived organophosphates like paraoxon, they have received increasing attention in the field of cardiovascular prevention for their presumed antioxidant and anti-inflammatory role.

PON1 genetic variants are known to have major influence on the serum concentration as well as on the catalytic activity of the enzyme. Indeed, the 192RR genotype is associated with the greatest hydrolytic activity against paraoxon; whereas, the 192QQ possesses the highest protective capacity against LDL oxidation [24]. In the coding region of PON2, the S311C polymorphism has been associated with variations in lipoprotein metabolism and plasma lipoprotein concentration [11].

PON2 is constitutively expressed in both primary and immortalized human endothelial cells and human aortic smooth muscle cells. This enzyme, absent in plasma, exerts antioxidant properties by reducing the production of intracellular hydroperoxides and/or cell-mediated LDL oxidation and inhibits the ability of oxidized LDL to induce monocyte chemotactic activity in human aortic endothelial cells. In vitro studies showed that PON2 overexpression lowers the intracellular oxidative state of cells treated with hydrogen peroxide or oxidized L-α-1-palmitoyl-2-arachi-donyl-sn-glycero-3-phosphorylcholine [4]. Moreover, recent studies suggest that PON2, in association with PON3, was found in subcellular mitochondrial fractions, which prevents the ubisemiquinone mediated-mitochondrial superoxide generation [5, 7]. As LDL oxidation is believed to be a major initiator of atherosclerotic lesions, PON2 expression in arterial cells is likely to play a key role in the body resistance to vascular disease. In our population, the presence of C allele in the PON2 gene was associated with previous vascular events. The PON2 311C allele showed a higher prevalence among Chinese Han patients [25], and was related to an increased risk of myocardial infarction in Italian [12] and Spanish populations [26]. Conversely, some studies indicated that PON2 311S allele frequency was significantly higher in coronary artery disease patients, both in Asian–Indian, than in Taiwan subjects [27].

However, the activity of PON2 enzyme is not as well characterized as that of PON1, and several studies showed that PON2 311C allele is linked to decreased lactonase [28] and LDL antioxidant activities [4].

We were not able to find any significant association between Q192R PON1 genetic variants and atherothrombotic events. Our results agree with other studies that failed to demonstrate a relation between PON1 gene variants and coronary artery disease risk [29, 30]. These conflicting results may arise from differences in the ethnic background, as well as in the selection criteria for cases and controls, and from heterogeneity of the clinical endpoints considered. Moreover, the lack of PON1 association with coronary artery disease may be due to the less specific action of this protein. In fact, PON1 is a plasma antioxidant enzyme, highly expressed in the liver where it is associated with the HDL particle. PON1, however, does not act in vascular endothelial cells where the early atherogenetic process starts after LDL accumulation and oxidation. Conversely, PON2 appears to be ubiquitously expressed in most human tissues, and particularly, in the arterial wall where this protein may be considered an additional element of antioxidant and anti-atherosclerotic vascular defense.

The protective role of HDL, as well as the negative contribution of hypertension, have been extensively studied and confirmed so far. In agreement with the literature [1, 31], we also found a strong independent relationship among low HDL levels, hypertension, and atherothrombotic events.

Study limitations

The relatively small sample size of our population represents a limit of our study; however, all data were in agreement with the distribution of PON polymorphisms in a large control population [32]. Furthermore, we have enrolled subjects who were clinically and biochemically well-defined.

We did not measure the enzymatic activity of PONs, but we have studied gene variations known to be associated with an altered antioxidant property.

Conclusions

Our study adds further knowledge on the role of PON2 as a possible cofactor in determining the risk of atherothrombotic events, through its cell-specific antioxidant property in the first step of lipid oxidation. Indeed, cell-associated PON2 could act, in concert with the serum-associated enzymes PON1, to decrease vascular ROS and contrast the development of atherosclerosis.

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