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Research article

The number of PON1 mutant alleles, but not PON1 phenotype, is associated with Gensini score of coronary damage

Numărul alelelor mutante ale PON1, dar nu şi fenotipul PON1, este asociat cu scorul Gensini de afectare coronariană

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Abstract

Objectives. The aim of this study was to examine the effects of single nucleotide polymorphisms (SNPs) of PON1 gene at the level of promoter region (−909 and −832) and of first exon (+575, A20352G, resulting Q192R substitution) on paraoxonase-1 (PON1) activities in 53 patients with angiographycally proven coronary heart disease (CHD) and 17 free-CHD subjects. Methods and Results. Serum PON1 arylesterase (Ar-ase) and salt-stimulated paraoxonase (ssPO-ase) activities were assessed with manual spectrophotometric methods, by using phenyl acetate and paraoxon as substrates. Common serum biochemical markers were assayed by enzymatic methods using commercial kits, on a Roche/Hitachi 912 Auto Analyzer. PON1 genotypes were determined by PCR and nucleotide sequencing of the amplicons with an ABI PRISMTM 310 Genetic Analyzer and a BigDye® Terminator v3.1 Cycle Sequencing Kit. The severity of coronary artery stenosis was assessed and classified using the Gensini score. We found no significant differences in the PON1 activities and -909(G→C), -832(G→A) and +575(A→G) PON1 polymorphisms between CHD and CHD-free groups. Considering all investigated subjects, we found that -909(G→C) and +575(A→G) SNPs had statistically significant effects on Ar-ase activity and PO-ase activity, respectively. In a multiple regression model we found that diabetes, LDL-cholesterol and the number of mutant alleles were significant independent determinants of the Gensini score. A significant positive correlation was observed only between the Gensini score and the number of mutant alleles. Conclusions. There are no differences between CHD and CHD-free groups regarding PON1 genotypes and phenotypes but the increasing number of PON1 mutant alleles is an important factor in determining the severity of coronary damage.

Keywords: *paraoxonase-1; arylesterase; atherosclerosis; Gensini score; genotyping*

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Rezumat

Obiective. Scopul acestui studiu a fost de a examina efectele polimorfismelor (SNPs) genei PON1 la nivelul regiunii promotor (-909 şi -832) şi a primului exon (+575, 20352A>G, rezultând substituirea Q192R) asupra activităţilor paraoxonazei-1 (PON1) într-un grup format din 53 de pacienţi cu boală coronariană (CHD) dovedită angiografic şi 17 subiecţi fără CHD. Metode şi rezultate. Activităţile PON1 din ser, respectiv arilesteraza (AR-ase) şi paraoxonaza NaCl-stimulată (ssPO-ase), au fost evaluate cu metode spectrofotometrice manuale, folosind acetat de fenil, respectiv paraoxon ca substrat. Markerii biochimici uzuali au fost analizaţi prin metode enzimatice, pe analizorul Roche/Hitachi 912, folosind kituri comerciale. Genotipurile PON1 au fost determinate folosind PCR, iar pentru secvenţierea ampliconilor s-a utilizat kitul BigDye® Terminator v3.1 şi analizorul ABI PRISM™ 310. Severitatea stenozei coronariane s-a evaluat şi clasificat folosind scorul Gensini. Nu s-au observat diferenţe semnificative statistic între grupurile cu şi fără CHD în ceea ce priveste activităţile PON1 şi polimorfismele -909G>C, -832G>A şi +575A>G. Dar, luând în considerare toţi subiecţii investigaţi, am constatat că SNPs -909G>C şi +575A>G au avut efecte semnificative statistic asupra AR-ase, respectiv PO-ase. Folosind modelul regresiei multiple am constatat că diabetul, LDL-colesterolul şi numărul de alele mutante au fost determinanţi semnificativi, independenţi, ai scorului Gensini. O corelaţie pozitivă semnificativă a fost observată numai între scorul Gensini şi numărul de alele mutante. Concluzii. Nu există diferenţe între subiecţi cu si fără CHD în ceea ce priveşte genotipurile şi fenotipurile PON1, dar creşterea numărului de alele PON1 mutante este un factor important în determinarea severităţii leziunilor coronariene.

Cuvinte-cheie: *paraoxonaza 1, arilesteraza, ateroscleroza, scorul Gensini, genotipare.*

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Introduction

Paraoxonase 1 (PON1) is considered to have the major role in the antiatherogenic activity of HDL (1). From a functional perspective, PON1 displays several activities. It can act as a phosphotriesterase (better known as paraoxonase), arylesterase, lactonase, peroxidase and A_2-like phospholipase (2).

To a considerable degree, PON1 level and/or catalytic efficiency are determined by genetic variants (single nucleotide polymorphisms: SNPs) in the regulatory and coding regions. PON1 serum concentration and the traditional assayed activities (paraoxonase and arylesterase) are mostly influenced by promoter polymorphisms -909G→C, -162A→G and -108C→T and exonic polymorphisms 11714T→A and 20352A→G, resulting in a Leu(L)/Met(M) and Gln(Q)/Arg(R) substitutions at codons 55 and 192 (+575, nucleotide position), respectively (3; 4).

Contradictory results have been reported regarding *PON1* polymorphisms, paraoxonase (PO-ase) and arylesterase (AR-ase) activities in the coronary heart disease (CHD) (5).

The aim of this study was to examine the effects of three *PON1* gene SNPs in the promoter region (−909 and −832) and first exon (+575, 20352A→G, resulting Q192R substitution) on salt-stimulated paraoxonase (ssPO-ase) and AR-ase activities, and also the distribution of these SNPs in a group with angiographycally proven coronary heart disease (CHD) and free-CHD subjects, addressing the question of whether PON1 genotypes and/or phenotypes are important in determining the severity of coronary atherosclerosis assessed and classified using the Gensini score.

Materials and methods

Subjects

The study populations consisted of 70 subjects (53 with CHD and 17 CHD-free) originating from Transylvania (Romania) who presented, between November 2008 and March

2009, at the Cardiology Department of Heart Institute "Niculae Stăncioiu" Cluj-Napoca, for coronary angiography (because of symptoms related to ischemic heart disease). Each angiogram was reviewed by two interventional cardiologists who had no access to clinical and biochemical measurements of the investigated subjects. The degree of stenosis was defined as the largest percentage of luminal diameter narrowing of the vessel, reported to the nearest segment that was considered normal as measured using specialized QCA software (Quantificative Coronary Angiography, Sanders Data Systems, LLC Palo Alto, California, USA). The severity of coronary artery stenosis was assessed and classified using the *Gensini Score* (GS = $n \times N$) (6), which allows severity scoring of each major epicardial coronary artery, depending on luminal stenosis degree and topographic importance of the question artery in myocardial vasculature. Luminal diameter reduction was assessed and to each reduction percentage (25%, 50%, 75%, 90%, 99% and 100%) a specific *initial score* (*n*) was attributed (1, 2, 4, 8, 16, and 32, respectively). Each vascular segment was then credited with a number (N) multiplied by *n*. The value of *N* is given in accordance with the functional importance of myocardial area served by the vascular segment, as follows: *5* for the left main trunk (LM), *2.5* for the proximal left anterior descending artery (LAD) and for the proximal circumflex artery (CX), *1.5* for the medium segment of LAD, *1* for the right coronary artery (CD), distal segment of LAD, medium and distal circumflex artery, diagonal artery (D1), the first obtuse marginal artery (OM1), and posterior interventricular artery (PIV) and *0.5* for other branches.

All subjects included in the study were evaluated by physical examination and a detailed questionnaire, concerning known CHD risk factors, cardiac history, and current medications. Weight and height were determined using the standard scale, and body mass index (BMI) was calculated by the formula: BMI (kg/m^2) = weight/height^2.

Patients older than 68 years, with associated neoplastic disease, other cardiomyopathies, chronic inflammatory diseases, liver and kidney diseases, major depression disorder personality, or history of surgical procedures performed in the last 30 days were excluded the study. For all performed measurements, patients' written consent and Ethics Committee approval were obtained.

Blood samples

Two venous blood samples were collected from each patient (before coronary angiography, after overnight fasting) on clot-activator and on EDTA, respectively. The serum, isolated by low speed centrifugation, was divided into 2 aliquots and stored at –20°C for common biochemical measurements and paraoxonase activities assay.

Common serum biochemical measurements

Total cholesterol (TC), triglycerides (TGs), HDL-cholesterol (HDL-C) and glucose, expressed in mg/dl, were assayed by enzymatic methods using commercial kits, on a Roche/Hitachi 912 Auto Analyzer.

LDL-cholesterol (LDL-C), expressed in mg/dl, was calculated using the Friedewald formula (LDL-C = TC – HDL-C – TG/5).

Analysis of serum PON1 activities

Salt stimulated paraoxonase (ssPO-ase) and arylesterase activities (AR-ase) were measured spectrophotometrically at 25°C, on a double beam UV-VIS spectrophotometer (ABLE&JASCO) with Peltier cooled cell holder with stirrer, according to Eckerson et al. (7) with minor modifications.

SsPO-ase activity was performed by using paraoxon (O,O-diethyl-O-*p*-nitrophenyl phosphate; Sigma Chemical Co., UK) as substrate. The assay mixture contained 1.0 mM paraoxon, 2M NaCl and 1 mM $CaCl_2$ in 50 mM glycine-NaOH buffer (pH 10.5). The reaction was started by the addition of the serum sample and the absorbance was monitored at 405 nm for 90 seconds.

AR-ase activity was measured using 1 mM phenyl acetate (Sigma Chemical Co.) in 20 mM Tris-HCl (pH 8) containing 1 mM $CaCl_2$. The rate of phenyl acetate hydrolysis was determined by monitoring the increase of absorbance at 270 nm over a 90-seconds period.

A blank sample, containing incubation mixture without serum, was run simultaneously to correct for spontaneous substrate breakdown, for both activity determinations. All samples were run in duplicate; the average value was used for activity calculation using a molar extinction coefficient of 18.290 $M^{-1}cm^{-1}$ at 412 nm for *p*-nitrophenol, and 1,310 $M^{-1}cm^{-1}$ at 270 nm for phenol. Results were expressed as U/L for ssPO-ase activity and as kU/L for AR-ase activity.

DNA Extraction and Genotyping

The whole blood (collected on EDTA) was used for genomic DNA extraction using a Wizard®Genomic DNA Purification Kit (Promega). The amplicons corresponding to *PON1* gene promoter SNPs (−909 and −832) and Q192R in the PON1 protein (respectively +575 in the *PON1* mRNA) were obtained by PCR. The following primers were designed at the Molecular Biology Center according to sequences from NCBI database:
- FW primer: 5'-AGGACAGATATTGCAGAAGAG-3' and RV primer: 5'-GATTCAGTAGCTTGATCATGG-3', for SNPs −909 and −832;
- FW primer: 5'-CTTTAAGGATTGTATCGGCAG-3', and RV primer: 5'-CAAATCTTCATCACAGTTCCC-3', for SNP +575 (Q192R).

The nucleotide sequencing of the amplicons was performed with an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems) using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Statistical analysis

Qualitative data was presented as counts and percentages. The association between qualitative variables was assessed with χ^2 test (if the expected frequencies table had more than 80% of the cells with values greater than five) or Fisher exact test otherwise.

Quantitative data was presented by mean and standard deviation (for normally distributed data), and by median and interquartile range for not normally distributed data. To check for differences between two independent groups of quantitative data, Mann Whitney U test was used (for not normally distributed data). Normality of the data was checked with strip-chart, quantile-quantile plot, and Shapiro-Wilk test. Comparisons between three of more groups regarding quantitative variables were made with the Kruskal Wallis test for not normally distributed data.

Association between quantitative data was evaluated first graphically with a scatter chart, then using Spearman correlation coefficient (for data not following the normal distribution) with 95% confidence intervals (calculated by bootstrapping), along with the test for its statistical significance. For some of the variables, simple and multiple linear regression, was performed. The regression assumptions (normality of residuals, heteroscedasticity and so on) were checked and the regression coefficients with 95% confidence intervals were presented.

For all statistical tests used, the significance level alpha was 0.05, and the two-tailed *p* value was computed. The statistical analysis was made in R environment for statistical computing and graphics, version 1.15. (8).

Results

Demographic data as well as clinical and laboratory findings in CHD and CHD-free patients are presented in *Table 1*. The statistical analysis of the groups did not show significant difference regarding age, gender distribution, and incidence of coronary risk factors such as hypertension, smoking, body mass index. There was a significant difference between CHD and CHD-free groups concerning the presence of diabetes or impaired glucose tolerance (IGT). In the CHD group there were 35.85% subjects with IGT and 32.08% patients with type II diabetes,

Table 1. Demographic as well as clinical and laboratory data of the investigated subjects

Characteristics	CHD (n = 53)	CHD-free (n = 17)	*p*
*Age, y	53 [47–56]	55 [50–57]	0.597
Men, n (%)	42 (79.25)	13 (76.47)	1
*BMI, kg/m^2	28.3 [26–31.29]	27.9 [25.6–30.69]	0.886
Hypertension, n (%)	30 (56.6)	9 (52.94)	0.791
Smoking, n (%)	29 (54.72)	5 (29.41)	0.069
Diabetes, n (%)	IT = 7 (13.21) OAT = 10 (18.87) NT = 36 (67.92)	IT = 0 (0) OAT = 0 (0) IGT = 17 (100)	0.018
Acute coronary syndrome, n (%)	20 (37.74%)	1 (5.88%)	0.012
Previous MI, n (%)	21 (39.62%)	0 (0%)	0.009
Statines therapy, n (%)	28 (52.83)	8 (47.06)	0.678
*Total cholesterol mg/dL	173 [153–192]	143 [132–180]	0.013
*HDL-cholesterol mg/dL	34.2 [31.2–39.4]	36.1 [30–44.4]	0.962
*LDL-cholesterol mg/dL	130.6 [106.8–152]	105 [84.8–132.4]	0.022
*Triglyceride mg/dL	152 [110–200]	152 [123–200]	0.913
*AR-ase, kU/L	72.5 [60.62–79.06]	68.76 [58.76–85.02]	0.935
*PO-ase, U/L	250.4 [157.9–437.3]	312.5 [196–418.4]	0.481
–909G>C			
GG, n (%)	12 (22.64)	5 (29.41)	0.826
GC, n (%)	26 (49.06)	7 (41.18)	
CC, n (%)	15 (28.30)	5 (29.41)	
–832G>A			
GG, n (%)	24 (45.28)	11 (64.71)	0.368
GA, n (%)	26 (49.06)	6 (35.29)	
AA, n (%)	3 (5.66)	0 (0)	
+575A>G/Q192R			
AA→QQ, n (%)	26 (49.06)	8 (47.06)	0.691
AG→QR, n (%)	24 (45.28)	7 (41.18)	
GG→RR, n (%)	3 (5.66)	2 (11.76)	

IT= insulin therapy; OAT = oral antihyperglycemic therapy; NT = no therapy.
*For non-normal distribution, data are presented as median [lower/upper quartiles]

while in the CHD-free group there were only 29.41% with IGT and no diabetics. Higher values of LDL-cholesterol and total cholesterol were observed in the CHD group compared to CAD-free group. No significant differences between investigated groups regarding PON1 activities and the distribution of analyzed genotypes (−909G→C, −832G→A and +575A→G) were observed. Considering all investigated subjects, we found that −832G→A SNP had no effects on PON1 activities, but −909G→C and +575A→G SNPs had very significant effects on AR-ase activity and ssPO-ase activity, respectively (*Table 2*). In the investigated population we identified patients without mutant alleles (n=3) with one (n=13), two (n=29), three (n=19) and four mutant alleles (n=6). We did not identify patients with five or six mutant alleles. The mean values of Gensini score fluctuates between 14 (for subjects without mutant alleles) and 71 (for subjects with four mutant alleles). A significant positive correlation was observed between the Gensini score and the number of mutant alleles (*Figure 1*). In a multiple regression model, includ-

Table 2. PO-ase and AR-ase activities related to investigated genotypes

	–909G>C	–832G>A	+575A>G
AR-ase (kU/L)	GG 80.04 [74.94–83.09]	GG 65.32 [55.68–78.93]	AA 75.26 [64.04–82.99]
	GC 72.65 [63.03–79.31]	GA 73.57 [64.01–79.53]	AG 68.47 [58.48–75.43]
	CC 58.47 [48.98–65.37]	AA 80.04 [75.92–81.37]	GG 63.03 [58.76–74.67]
	$p < 0.001$	$p = 0.113$	$p = 0.181$
ssPO-ase (U/L)	GG 197.1 [179.4–391.5]	GG 196 [138.8–402.35]	AA 161.85 [126.33–189.75]
	GC 386.3 [161–480.6]	GA 359.55 [179.3–493.9]	AG 400.1 [316.85–494.1]
	CC 263.05 [122.95–357.22]	AA 197.1 [173.25–370.45]	GG 679.6 [669–915.2]
	$p = 0.299$	$p = 0.175$	$p = 0.001$

Table 3. Predictors of Gensini score identified by logistic regression

Variable	β	95% CI	*p*
Arylesterase activity (kU/L)	-0.14	(-0.85 - 0.58)	0.705
BMI (kg/m^2)	0.74	(-1.47 - 2.95)	0.504
Diabetes mellitus	29.24	(6.86 - 51.63)	0.011
Smoker status	5.78	(-15.68 - 27.25)	0.592
Gender	14.44	(-10.12 - 39)	0.244
HDL-cholesterol (mg/dL)	-0.08	(-1.13 - 0.97)	0.881
Hypertension	-4.15	(-25.06 - 16.75)	0.692
LDL-cholesterol (mg/dL)	0.28	(0.05 - 0.52)	0.018
Number of mutant alleles	13.53	(1.61 - 25.44)	0.027
Paraoxonase activity (U/L)	-0.02	(-0.08 - 0.03)	0.393
Triglycerides (mg/dL)	-0.01	(-0.08 - 0.08)	0.982
Age (years)	0.27	(-1.2 - 1.74)	0.717

β is standardized regression coefficient. CI is confidence interval

ing 12 explanatory variables for the Gensini score as a dependent parameter, we found that diabetes, LDL-cholesterol and the number of mutant alleles were significant independent determinants (*Table 3*). Age, smoking, hypertension, total cholesterol, HDL-cholesterol, triglycerides, and PON1 activities did not appear to be related to the Gensini score. The gender influence was close to the limit of significance.

Discussion

In the present study we examined three *PON1* gene SNPs: two located in the promoter region (−832 and −909) and one in the coding region (+575). Earlier reports (9; 10) have shown a significant effect of the PON1 −108 and −909 promoter region SNPs of both PON1 activities. Recently, the results of Mackness et al. (11), using paraoxon as a substrate, validate the effect of the −108 SNP on PON1 activity in control group (CHD-free) as well as in the CHD group, on one hand, and the effect of the −909 SNP on PON1 activity only in the CHD group, on the other hand. Also, both −108 and −909 SNPs were not related to the presence of CHD. In this paper, by evaluating the PON1 activities and the distributions of two SNPs in the promoter region in each investi-

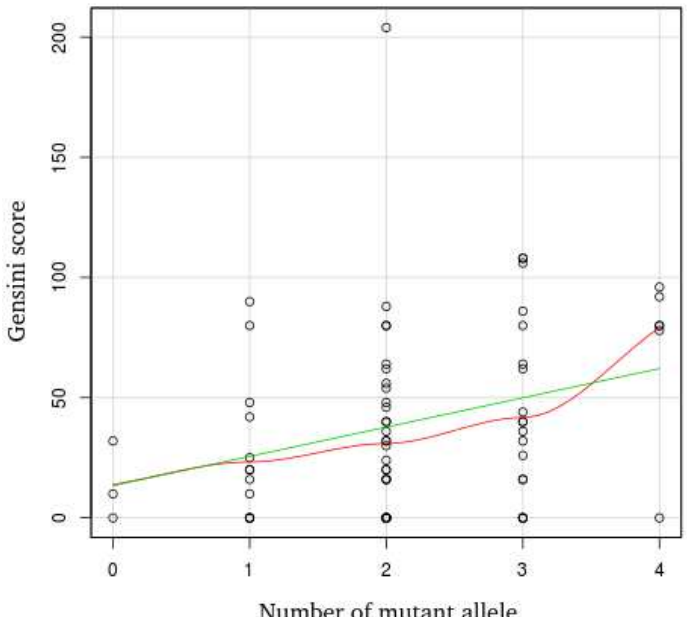

Figure 1. Positive correlation between the number of mutant alleles and Gensini score.

gated groups we obtained no significant differences. However, considering all investigated subjects we found that the PON1 −832G→A indeed had no effect on PON1 activities, but −909G→C had statistically significant effect on AR-ase activity (using phenyl acetate as a substrate), and +575A→G (resulting a Q192R substitution at the protein level) on ssPO-ase activity (using paraoxon as a substrate). The causes of these differences are still ambiguous. Discrepancies in the results described here and those of other investigators could be due to distinct methodologies used between studies. Furlong, in his paper (12), pointed out that the differences in PON1 activity, concentration and genotype distribution occur between different world populations. Previously, Wheeler et al. (13) in a meta-analysis of 43 studies, showed a small but significantly odds ratio for the association of *PON1*-192R allele and CHD. For that reason, differences between populations from different geographical areas may be the main cause for any difference between our study and other reports. It is also expected that the PON1 SNPs only have a minor impact on CHD progression, which may be overawed by competing effects of dietary and environmental factors affecting CHD and PON1 activity in some populations more than in others (11). Remarkably is that our study shows a positive correlation between Gensini score and the number of mutant alleles. Also, a multiple regression model including 12 explanatory variables for the Gensini score as a dependent parameter indicates that diabetes, LDL-cholesterol and the number of mutant alleles are meaningful independent determinants.

In conclusion, regarding the PON1 genotypes and phenotypes, there are no significant differences between CHD and CHD-free groups, but the increasing number of *PON1* mutant alleles is an important factor controlling the severity of coronary damage. More large prospective studies will better clarify the correlations between *PON1* SNPs and CHD in the future.

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