

Protective effect of R allele of *PON1* gene on the coronary artery disease in the presence of specific genetic background¹

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Abstract. *Background:* Genetic susceptibility to CAD may be determined by polymorphic variants of genes encoding isoforms involved in the processes important in the pathogenesis of atherosclerosis, including lipids disorders. Participation of single polymorphic variants is relatively small, however its significance may increase in the presence of specific genetic or environmental background.

Aim: The aim of the study was an evaluation a possible association between single polymorphic variants of *PON1*, *APOE*, *ABCA1* and *PPARA* genes and CAD and looking for specific multigene genotype patterns which differentiate study groups.

Materials and methods: We studied 358 subjects: 178 patients with angiographically confirmed CAD and 180 blood donors without history of CAD. Polymorphisms were genotyped using PCR-RFLP method.

Results: We observed statistically significant differences in the frequencies of R allele and R allele carriers of *PON1* gene between CAD and controls. The distribution of genotypes and alleles of other analyzed genes did not differentiate the study groups, however the presence of specific genotypes (*APOE* – $\epsilon 3\epsilon 3$, $\epsilon 3\epsilon 2$, *ABCA1* – AG, *PPARA* – GG) increased the protective effect of R allele.

Conclusion: The present study revealed an independent protective association between carrier-state of *PON1* R allele and CAD. This protective effect was especially strong in the presence of specific genotype arrangements of other analyzed genes.

Keywords: Coronary artery disease, apolipoprotein E, paraoxonase 1, ATP-binding cassette transporter 1, peroxisome proliferator-activated receptor α

1. Introduction

Coronary artery disease (CAD) is a common, multifactorial disease which phenotype results from progressive, atherosclerotic changes in the coronary arteries. Genetic susceptibility to CAD may be determined by specific polymorphic variants of genes en-

coding isoforms involved in processes important in the pathogenesis of atherosclerosis. Among the most important factors responsible for initiation and progression of atherosclerosis are lipids disorders. An elevated level of serum low density lipoproteins (LDL) may leads to injury and dysfunction of endothelium. LDL stimulates expression of adhesion molecules increasing migration and proliferation of inflammatory cells. Oxidized LDL uptaken by monocytes and macrophages leads to foam cells formation [7,22,23]. This process is also dependent on high density lipoproteins (HDL) playing significant role in the reduction of cholesterol content in atherosclerotic plaques [9].

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Thus polymorphic variants of genes involved in lipids metabolism may genetically differentiate human population and determine a susceptibility to the disease. Previous studies showed that participation of single polymorphic variants in determining the CAD risk is relatively small [8,10,11,21]. However it seems that its significance may increase in the presence of specific genetic or environmental background.

There is a lot of genes encoding proteins involved in lipid metabolism but we have chosen four genes, which are, in our opinion, especially interesting. First they are crucial for the main processes important in the pathogenesis of atherosclerosis, such as: cholesterol transport to peripheral tissues, LDL oxidation and reverse cholesterol transport. All analyzed genes show also pleiotropic effect and are involved not only in lipid metabolism but also e.g. inflammatory processes. Additionally products of all genes are functionally connected (see Discussion). *PON1* gene encodes paraoxonase 1 – an enzyme attenuating the oxidative modification of LDL particles and maintaining integrity and functions of HDL [5,17]. *APOE* gene encodes apolipoprotein E, being a component of chylomicrons and very low density lipoproteins (VLDL). Since it is a ligand for LDL and LRP receptors, it regulates lipids transport and metabolism. Apo E takes also part in removing excess of cholesterol from macrophages [6]. *ABCA1* gene encodes ATP-binding cassette transporter 1. This protein is a membrane transporter utilizing energy derived from the hydrolysis of ATP to transport cholesterol and phospholipids across the membrane on HDL particles. Thus it plays a crucial role in reverse cholesterol transport [3]. *PPARA* gene encodes peroxisome proliferator-activated receptor α (PPAR α), a transcription factor regulating numerous genes involved in the lipids metabolism [20].

The aim of the present study was an evaluation a possible association between single polymorphic variants of *PON1*, *APOE*, *ABCA1* and *PPARA* genes and coronary artery disease and looking for specific multi-gene genotype patterns which differentiate patients with CAD from healthy controls.

2. Materials and methods

2.1. Materials

We studied 358 Caucasian subjects, aged 18–55, divided into two groups:

- Group 1 (CAD) – 178 subjects, including 61 women and 117 men, aged 25–55 (mean 43.81 ± 6.11). The group included patients with angiographically confirmed CAD with more than 50% diameter stenosis of at least one major coronary vessel. All patients were recruited from the 1st Clinic of Cardiology in the Silesian Center of Cardiology in Katowice (Poland). The coronary angiography was performed by Judkin's method. Myocardial infarction (MI) was diagnosed according to recommendations of the Joint European Society of Cardiology/American College of Cardiology Committee [2]. The exclusion criteria were: clinical diagnosis of cardiomyopathy, coagulopathy, collagenoses and acute poisoning (e.g. CO, amphetamine).
- Group 2 (Control) – 180 subjects, including 52 women and 128 men, aged 18–55 (mean 35.02 ± 10.4). The control group included blood donors without any signs of CAD, recruited from Regional Center of Blood Donor and Blood Treatment in Katowice. The exclusion criterion was familial history of CAD or stroke.

All patients were characterized on the basis of medical interview in respect of concomitant risk factors for atherosclerosis such as hypertension, cigarette smoking, overweight or obesity, diabetes mellitus, familial history of CAD or stroke.

The study protocol was approved by the Ethics Committee of the Medical University of Silesia in Katowice (Poland) and all subjects gave written informed consents.

2.2. Methods

2.2.1. Biochemical analyses

Total serum cholesterol, HDL-cholesterol and triacylglycerols were measured by enzymatic methods (commercial Analco Kit). LDL-cholesterol levels were calculated according to the Friedewald formula [12] in subjects with triacylglycerols levels below 4.4 mmol/l.

2.2.2. Analyses of polymorphisms

All genes polymorphisms were genotyped using PCR-RFLP (restriction fragment length polymorphism) analysis.

Genomic DNA was extracted from peripheral lymphocytes using MasterPure genomic DNA purification kit (Epicentre Technologies).

PON1 **Q192R** polymorphism was genotyped using a method described by Ombres et al. [18], with some

modifications. The amplification parameters were: initial 5 min. denaturation at 94°C, 30 cycles with 60 s at 93°C, 30 s at 61°C, 90 s at 72°C, and final extension of 5 min at 72°C. The PCR product was digested by *Bsp*PI restriction enzyme (Fermentas) generating fragments 135 bp and 64 bp for allele R and 199 bp for allele Q.

APOE epsilon polymorphism was genotyped using a method described by Hixon et al. [13], with some modifications. The amplification parameters were: initial 5 min. denaturation at 95°C, followed by 60 s at 95°C, 60 s at 64°C, 60 s at 72°C, steps 2, 3, 4 were repeated two times decreasing an annealing temperature at 1 degree in each cycles up to 61°C, then 20 cycles with 60 s at 95°C, 60 s at 60°C, 120 s at 72°C, and final extension of 30 min at 72°C. The PCR product was digested by *Hha*I restriction enzyme (Promega) generating fragments 91, 48, 38, 35 bp for ϵ 3 allele, 91, 83, 38 bp for ϵ 2 allele and 72, 48, 38, 35 bp for ϵ 4 allele.

ABCA1 R219K polymorphism was genotyped using a method described by Clee et al. [8], with some modifications. The amplification parameters were: initial 5 min. denaturation at 94°C, 30 cycles with 60 s at 93°C, 30 s at 61°C, 90 s at 72°C, and final extension of 5 min at 72°C. The PCR product was digested by *Eco*NI restriction enzyme (Fermentas) generating fragments 107 bp and 70 bp for allele A and 177 bp for allele G.

PPARA G>C polymorphism in intron 7 was genotyped using a method described by Flavell et al. [11], with some modifications. The amplification parameters were: initial 2 min denaturation at 94°C, 30 cycles with 30 s at 94°C, 30 s at 56°C, 1 min at 72°C, and final extension of 5 min at 72°C. The PCR product was digested by *Taq*I restriction enzyme (Fermentas), generating fragments 216 bp and 50 bp for allele C and 266 bp for allele G.

2.2.3. Statistical analysis

The data were analyzed using the *Epi*Info 6 (WHO) and *Statistica* 6.0 (STATSOFT) software. Normality of distribution for quantitative data was computed by W Shapiro-Wilk's test and then comparison was performed by T-Student test or U Mann-Whitney's test. Alleles frequencies were deduced from the genotype distribution. Hardy-Weinberg equilibrium was tested in all groups by a χ^2 test as well as comparisons of genotypes and alleles frequencies between cases and control subjects. When the number of subjects in the sample was lower than 10 the Fisher's correction was used. Statistical significance was accepted at $p < 0.05$. To assess an association between alleles or genotypes

and the disease odds ratios (OR) as well as their 95% CI were computed. An odds ratio greater than 1 indicates that the disorder in the group with analyzed factor is more likely than in the group without this factor. And an odds ratio less than 1 indicates that the disease is less likely in the group with analyzed factor than in the group without this factor. Association between polymorphic variants and the disease was calculated using univariate analysis as well as multiple logistic regression analysis after adjustment for traditional risk factors, such as: smoking, elevated level of total cholesterol, LDL, triacylglycerols, overweight or obesity.

3. Results

3.1. Characteristic of the study group

General characteristic and biochemical parameters like mean age, BMI, total serum cholesterol, HDL, LDL cholesterol and triacylglycerols, smoking of the study groups are shown in Table 1. There were significantly higher serum concentrations of total cholesterol, LDL, triacylglycerols as well as body mass index and smokers number in CAD group than in controls. Due to the age differences between patients and controls age-matched subgroups were separated from both study groups. The differences in described characters between these two subgroups were similar to observed in the entire groups.

3.2. Clinical characteristic of patients

There were 79.8% cases who had suffered from myocardial infarction in CAD group. More than half patients had critical stenosis (61.2%), multivessel disease (62.9%) or hypertension (57.3%). Relatively small percentage of CAD subjects suffered from diabetes mellitus (5.6%), peripheral arterial disease (9.6%) or stroke (1.7%).

3.3. Analysis of polymorphism

3.3.1. Analysis of single gene polymorphisms

All genotype frequencies were compatible with Hardy-Weinberg equilibrium. Subjects with at least one analyzed allele were called "carriers" in the entire article. The distribution of all genotypes and alleles is shown in Table 2.

We observed statistically significant differences in the frequencies of R allele and R allele carriers of *PON1*

Table 1
Clinical and biochemical characteristic of study groups

	CAD	Control	CAD age 31–55	Control age 31–55
n	178	180	140	107
Gender n (%)	♀ n = 61 (34.3%) ♂ n = 117 (65.7%)	♀ n = 52 (28.9%) ♂ n = 128 (71.1%)	♀ n = 61 (43.6%) ♂ n = 79 (56.4%)	♀ n = 20 (18.7%) ♂ n = 87 (81.3%)
Age (mean ± SD, min-max)	43.81 ¹ ± 6.11 (25–55)	35.02 ± 10.4 (18–55)	42.87 ± 5.46 (31–55)	41.79 ± 6.65 (31–55)
BMI (mean ± SD)	27.30 ± 7.21 ¹	24.56 ± 3.79	26.78 ± 4.42 ²	25.35 ± 3.67
TC (mmol/l ± SD)	5.75 ± 1.37 ¹	5.09 ± 1.30	5.82 ± 1.40 ²	5.27 ± 1.30
LDL (mmol/l ± SD)	3.83 ± 1.18 ¹	3.37 ± 1.14	3.92 ± 1.20 ²	3.48 ± 1.17
HDL (mmol/l ± SD)	1.14 ± 0.41	1.09 ± 0.39	1.15 ± 0.44	1.10 ± 0.38
TG (mmol/l ± SD)	1.88 ± 0.99 ¹	1.43 ± 0.68	1.83 ± 1.00 ²	1.47 ± 0.70
Smoking n (%)	n = 97 (54.5%) ¹	n = 52 (28.9%)	n = 64 (45.7%) ²	n = 34 (31.8%)

¹)Statistically significant differences comparing to control.

²)Statistically significant differences comparing to control age 31–55.

Table 2

Distribution of genotypes and alleles of *APOE*, *ABCA1*, *PPARA* and *PON1* genes

Gene	Genotype/ allele	CAD, n = 178		Control, n = 180	
		n	%	n	%
<i>APOE</i>	ε3ε3	130	73	132	73
	ε3ε4	33	18	29	16
	ε3ε2	12	7	17	9
	ε2ε4	1	1	2	1
	ε4ε4	2	1	0	0
	ε2ε2	0	0	0	0
	ε3	305	86	310	86
	ε4	38	11	31	9
	ε2	13	4	19	5
	<i>ABCA1</i>	GG	90	51	91
AG		68	38	78	43
AA		20	11	11	6
G		248	70	260	72
A		108	30	100	28
<i>PPARA</i>	GG	123	69	134	74
	GC	51	29	43	24
	CC	4	2	3	2
	G	297	83	311	86
<i>PON1</i>	C	59	17	49	14
	QQ	107	60	86	48
	QR	64	36	71	39
	RR	7	4	23	13
	Q	278	78	243	68
R	78	22	117	32	

gene (OR = 0.58 and 0.61, respectively) between CAD and control group (Table 3). The results of univariate analysis were confirmed using multivariate logistic regression after adjustment for traditional risk factors (elevated level of total cholesterol, LDL, triacylglycerols, smoking, overweight, OR = 0.48 for R allele carriers). Especially important differences were observed in the number of RR homozygotes (OR = 0.28) (Table 3). The differences in the frequencies of R allele and R allele carriers between age-matched subgroups were also statistically significant (OR = 0.61 and 0.63

respectively). Calculated odds ratios (OR) in all these cases were less than 1, what indicates that the R allele has protective effect on CAD. We did not observed statistically significant differences between study groups in genotypes and alleles distribution of *APOE*, *ABCA1* and *PPARA* genes. However there was tendency to higher prevalence of ε4 allele and carriers of this allele (*APOE* gene), AA homozygotes (*ABCA1* gene), C allele and carriers of this allele (*PPARA* gene) in CAD group comparing to controls (Table 2). The distribution of all genotypes and alleles were also compared between subgroups of women and men. There were no statistically important differences.

3.3.2. Multigene analysis

Since we observed the protective effect of PON1 R allele on CAD we concentrated a multigene analysis on assessing if specific genotypes of other study genes modulate an effect of R allele. For this reason we analyzed only genotypes which were less frequent in CAD group than in controls (*APOE* – ε3ε3, ε3ε2, *ABCA1* – AG, *PPARA* – GG) looking for 2, 3 or 4-gene combinations which differentiated the study groups. In two-gene analysis significantly less frequent were combinations: 1) ε3ε3+ε3ε2 (*APOE*) and QR+RR (*PON1*), (OR = 0.63), 2) QR+RR (*PON1*) and AG (*ABCA1*), (OR = 0.43), 3) QR+RR (*PON1*) and GG (*PPARA*), (OR = 0.62). Three-gene analysis showed statistically important differences in all analyzed combinations (OR = 0.34, OR = 0.44, OR = 0.60). Significant differences were also observed for four-gene combination (OR = 0.31). The results of univariate analysis in all cases were confirmed in logistic regression after adjustment for traditional risk factors (Table 4). Comparison of OR values, especially in multivariate analysis shows the tendency to decrease with increasing the number of

Table 3
The results of statistical analysis of *PON1* polymorphism

Genotype or allele (gene)	CAD frequency / n	Control frequency / n	OR (%CI), p, χ^2
QR, RR / QQ (<i>PON1</i>)	0.40/71	0.52/94	0.61 (0.39-0.94), 0.019, 5.48 ¹⁾ 0.48 (0.27-0.83), 0.008, 7.01 ²⁾
RR / QQ+QR (<i>PON1</i>)	0.04/7	0.13/23	0.28 (0.11-0.71), 0.002, 9.12 ¹⁾ p Yates 0.005 non significant p = 0.055 ²⁾
R / Q (<i>PON1</i>)	0.22/78	0.32/117	0.58 (0.41-0.83), 0.001, 10.13 ¹⁾

¹⁾Univariate analysis; ²⁾multivariate analysis.

Table 4
The results of statistical analysis of genotype patterns

Genotype pattern	CAD frequency / n	Control frequency / n	OR (%CI), p, χ^2
QR,RR + $\epsilon 3\epsilon 3$, $\epsilon 3\epsilon 2$ (<i>PON1</i>) (<i>APOE</i>)	0.31/56	0.42/76	0.63 (0.40-0.99), 0.035, 4.45 ¹⁾ 0.53 (0.30-0.93), 0.028, 4.85 ²⁾
QR,RR + AG (<i>PON1</i>) (<i>ABCA1</i>)	0.12/21	0.24/43	0.43 (0.23-0.78), 0.003, 8.91 ¹⁾ 0.36 (0.16-0.78), 0.010, 6.63 ²⁾
QR, RR + GG (<i>PON1</i>) (<i>PPARA</i>)	0.27/48	0.37/67	0.62 (0.39-1.0), 0.038, 4.32 ¹⁾ 0.42 (0.23-0.77), 0.005, 7.98 ²⁾
QR,RR + $\epsilon 3\epsilon 3$, $\epsilon 3\epsilon 2$ + AG (<i>PON1</i>) (<i>APOE</i>) (<i>ABCA1</i>)	0.08/15	0.21/38	0.34 (0.17-0.68), 0.0007, 11.42 ¹⁾ 0.27 (0.11-0.67), 0.004, 8.18 ²⁾
QR,RR + AG + GG (<i>PON1</i>) (<i>ABCA1</i>) (<i>PPARA</i>)	0.08/15	0.17/31	0.44 (0.22-0.89), 0.013, 6.18 ¹⁾ 0.32 (0.13-0.82), 0.017, 5.69 ²⁾
QR,RR + $\epsilon 3\epsilon 3$, $\epsilon 3\epsilon 2$ + GG (<i>PON1</i>) (<i>APOE</i>) (<i>PPARA</i>)	0.21/38	0.31/56	0.60 (0.36-1.00), 0.036, 4.41 ¹⁾ 0.39 (0.20-0.74), 0.004, 8.16 ²⁾
$\epsilon 3\epsilon 3$, $\epsilon 3\epsilon 2$ + QR,RR + AG + GG (<i>APOE</i>)(<i>PON1</i>)(<i>ABCA1</i>)(<i>PPARA</i>)	0.07/10	0.16/29	0.31 (0.14-0.69), 0.001, 10.15 ¹⁾ 0.20 (0.07-0.59), 0.003, 8.66 ²⁾

¹⁾Univariate analysis; ²⁾multivariate analysis.

genes in combination (except for *PON1*+*APOE*). Thus the carrier-state of R allele of *PON1* gene has the weakest protective effect and the presence of four-gene combination – the strongest effect (Fig. 1). A similar results were observed in the age-matched subgroups (except for *PON1*+*PPARA* and *PON1*+*ABCA1*+*PPARA* combinations).

4. Discussion

Among four analyzed polymorphisms statistically significant differences between patients and controls were observed only in the distribution of genotypes and alleles of *PON1* genes. There were significantly lower frequencies of R allele and R allele carriers in CAD group than in controls. These differences were also observed between age-matched subgroups. The differences in the distribution of R allele carriers in entire groups and RR homozygotes in age-matched subgroups obtained in univariate analysis were confirmed in multiple logistic regression. All described results

indicate the protective role of R allele in determining the risk of CAD.

An association between *PON1* polymorphism and CAD probably results from the impact of the polymorphism on the paraoxonase 1 activity. Many studies show that product of R allele has higher enzymatic activity towards paraoxon [16], but the influence of *PON1* polymorphism on ability of paraoxonase 1 to protect against LDL oxidation is not so clear [1,4,14]. OxLDL play significant role in initiation and progression of atherosclerosis so higher activity of R allele product against oxidized lipids might explain protective effect of R allele on CAD observed in the present study. Recent studies emphasize an association of anti-atherosclerotic action of paraoxonase 1 with its ability to homocysteine thiolactone detoxification. This the most reactive metabolite of homocysteine can damage proteins by homocysteinylolation of the lysine residues, thus leading to atherosclerosis. *PON1* R allele has higher thiolactonase activity comparing to Q allele [15] what may partially explain its protective role observed in the present study.

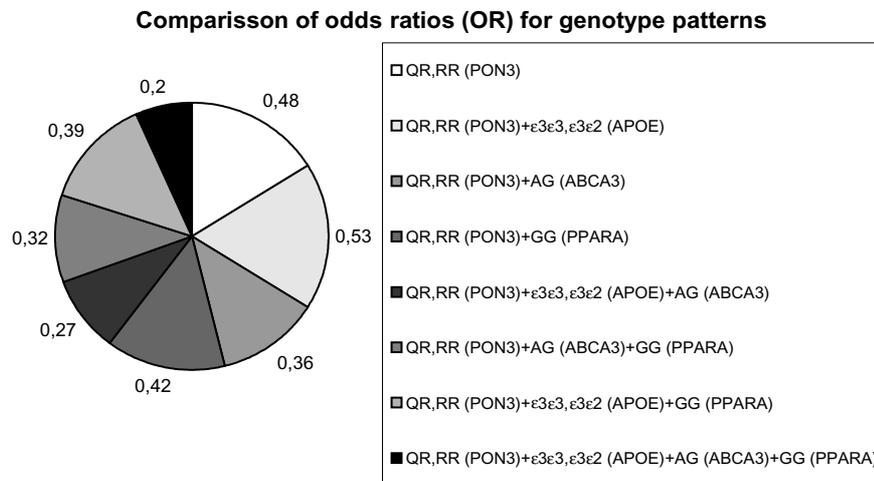


Fig. 1. The comparison of odds ratios (OR) for genotype patterns, the results of multivariate logistic regression.

The R192Q polymorphism of *PON1* gene was not previously analyzed in Polish population in a context of CAD and the results of studies performed in other populations are discrepant. The frequencies of genotypes and alleles observed in the present study are close to the values characteristic for Italian population, however the authors did not observe any association between R192Q polymorphism and CAD [18]. Some other authors described opposite relation, what means that R allele was associated with higher risk of CAD [19]. These discrepancies probably result from ethnic differences but also from differences in the definition of the disease phenotype and different inclusion criteria for control group. Although peptide products of all studied genes are associated with atherosclerotic processes in arterial walls, we did not find the relation between other analyzed polymorphisms and the disease. Due to multifactorial nature of arteriosclerosis participation of single genetic factor in determining the risk of CAD is relatively small and often undetectable. Additional difficulty in the association studies is heterogeneity of the disease, thus the different groups of patients may have different sets of genetic factors predisposing to the disease. Therefore some polymorphic variants may be strongly associated with the disease in one population whereas in another population the association may be weak due to the presence of other genetic factors. For this reason multigene analysis seems to be more powerful approach and may enable to study given polymorphic variant in the presence of specific genetic background. Our previously reported data revealed that some genotype patterns differentiate patients with CAD from healthy controls [24]. The present study

clearly showed that protective effect of *PON1* R allele was modulate by specific genotypes of other analyzed genes. Although an association between R allele carriers and CAD was relatively weak (OR = 0.48 in multivariate analysis), the effect was stronger in the presence of AG genotype of *ABCA1*, ε3ε3, ε3ε2 of *APOE* or GG of *PPARA* gene. The strongest protective effect in the entire groups and in the age-matched subgroups was observed for four-gene combination (OR = 0.2). The obtained results may be caused by functional connection between the products of analyzed genes. HDL-mediated macrophage cholesterol efflux via the *ABCA1* transporter is enhanced by paraoxonase 1 [25]. Thus specific polymorphic variants of both genes may act cumulatively or synergistically in the protection against atherosclerosis. *PPARα* regulates an expression of many genes involved not only in lipids metabolism but also in oxidative stress. It plays a physiologic role as radical scavenger [26] thus may also enhance the action of *PON1*. The only combination which had worse protective effect than R allele carrier-state of *PON1* gene was QR,RR (*PON1*) + ε3ε3, ε3ε2 (*APOE*) combination (OR = 0.53). What is interesting this combination together with GG (*PPARA*) or AG genotypes (*ABCA1*) had much better protective effect (OR = 0.42 and OR = 0.36, respectively). It may be caused by the possible synergistic effects between apo E and *ABCA1* or *PPARα* since apolipoprotein E is known to interact with *ABCA1* transporter in cholesterol efflux from human cells [27] and *PPARα* regulates the expression of *ABCA1* gene [28].

Observed in the present study OR values show that an association between some genetic factors and the

disease may be much stronger in the presence of specific genetic background. However it must be noticed that our study had focused only on the major polymorphisms of analyzed genes and it is possible that they are in a linkage disequilibrium with other mutations lying within the same screened genes. Therefore further analysis could be very interesting. Nevertheless it seems that establishing multigene genotype patterns associated with decreased or increased risk of the disease may have higher scientific and practical value than analysis of single genetic polymorphisms.

Concluding, the present study revealed an independent protective association between carrier-state of R allele of *PON1* gene and coronary artery disease. This protective effect is especially strong in the presence of specific genotype arrangements of other analyzed genes.

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