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Original Contribution

SERUM PARAOXONASE AND ARYLESTERASE ACTIVITY OF PON1 IN ACUTE CORONARY SYNDROME

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ABSTRACT

Serum paraoxonase (PON 1) is a HDL-associated enzyme that is believed to be a protective factor against cardiovascular disease, inhibiting oxidation of LDL complexes. PON1 is Ca^{2+} dependent hydrolase with two activities: Lactonase and 3 esterase, which are probably based on the properties of the enzyme to protect against oxidative changes in both LDL and HDL and thereby reduces the risk of cardiovascular disease. **Objective:** To determine serum paraoxonase and arylesterase activities of PON1 in patients with acute coronary syndrome, which can be compared with those in healthy subjects. **Material and methods:** Present study includes 42 patients with acute coronary syndrome, 36 with acute myocardial infarction with ST-segment elevation (STEMI), 6 with unstable angina (NAP) and 26 healthy subjects. **Results:** Serum paraoxonase activity of PON 1 in acute myocardial infarction and unstable angina was significantly lower compared with healthy controls ($p<0.005$, $p<0.008$). There was a weak trend ($R = 0.273$) of positive correlation between serum paraoxonase activity of PON1 and α-cholesterol in patients ($p=0.080$) and lack of that in controls ($p=0.89$). There was a weak trend ($R=-0.199$) of negative correlation between serum paraoxonase activity of PON1 and b-cholesterol in patients ($p = 0.213$). Normalized levels of paraoxonase and arylesterase activities of PON1 compared to levels of HDL-C were significantly lower in the group with acute myocardial infarction and unstable angina compared to controls ($p <0.001$, $p = 0.026$). PON-activity of PON1 in subjects with hypertension and those who experienced a cardiovascular event was significantly lower ($p = 0.03$, $p = 0.018$). **Conclusion:** Serum paraoxonase and arylesterase activities in STEMI and NAP were significantly lower compared with controls.

Key words: paraoxonase activity, arylesterase activity, PON1, control subjects

INTRODUCTION

Ischemic heart disease (IHD) is the leading cause of mortality and morbidity in both developed and developing countries. It is estimated that by 2020 ischemic heart disease will be become a major cause of death worldwide (1-3). Clinical manifestation of IHD included silent ischemia, stable angina, unstable angina, myocardial infarction, heart failure, sudden cardiac death.

Important risk factors for this multifactorial disease are low dense lipoprotein (LDL) and oxidation of LDL, which occupies a central role in atherogenesis (4, 5). High-density lipoprotein (HDL) is an independent protective factor against atherosclerosis, which underlies coronary heart disease. HDL complexes are found to contain the enzyme paraoxonase 1 (PON1), which is considered to have a protective effect

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against lipid peroxidation (6). Although many factors play a role in atherogenesis, the low PON1-activity can be an independent risk factor (6, 7). Originally paraoxonase is associated with its hydrolytic activity against organic phosphate compounds (7). The name of the enzyme is based on its ability to hydrolyze organic phosphate substrate paraoxon (paraoxonase activity EU 3.1.8.1), which is a toxic metabolite of insecticide parathion (8).

Biochemical functions of PON1

PON1 belongs to a family of paraoxonase consisting of three isoenzymes: PON1, PON2 and PON3. All three genes encoding these enzymes are localized in tandem on the long arm of chromosome 7 (7q21.3-q22.1) (9). PON1 and PON3 are expressed in liver and secreted into the bloodstream where they are associated with HDL (6, 7, 9). PON2 is not present in blood but is expressed in multiple tissues including liver, lung, brain and myocardium (7). PON1 is a glycoprotein with a molecular mass of 43 kDa and contains 354 amino acids. It is Ca^{2+} dependent hydrolase decomposing esters mainly of acetic acid (phenylacetate, tio-phenylacetate, 2-naphtylacetate) and toxic oxonium metabolites (paraoxon, diasoxon) of organic phosphate insecticides and nerve-paralyzing agents (zoman, sarin). Since compounds that are hydrolyzed by PON1 are non-physiological, the paraoxonase and arylesterase activities are clearly not physiological functions of the enzyme PON1. Just recently was established a new activity of PON1 - lactonase activity. The enzyme hydrolyzes a variety of aromatic and aliphatic lactones, dihydrocoumarine, γ-butyrolactone, homocysteine thiolactone. The enzyme also catalyses the opposite reaction of lactonisation γ- and δ-hydroxy carboxylic acids (10).

It has been proven that PON1 can also hydrolyze aromatic esters such as phenylacetate (arylesterase activity EU 3.1.1.2), thus the term "A-esterase" is given to the enzyme that hydrolyzes both substrates (11, 12). Subsequently, both enzymatic activities appear to be properties of the enzyme paraoxonase (PON1) (13). With in-vitro experiments has been shown that PON1 effectively metabolizes lactones of hydroxyl derivatives of polyunsaturated fatty acids arachidonic and docosahexaenoic acid. This enzyme hydrolyzes some phospholipid oxidation products such as isoprostane, carboxyl and aldehyde esters and hydroperoxides of phosphatidylcholine possessing phospholipase A2-like activity (14). Therefore, it is assumed that the lactones, hydroxyl and oxidation derivatives of polyunsaturated fatty acids (PUFAs) probably are the major endogenous substrates of the enzyme (15). More recently it was found that PON1 is active to other endogenous compounds such as estrogen esters, hydrolyzing esters in position 3 of the steroid A-ring (16).

Genetic variants of PON1

PON1 is an enzyme present in the form of options (alloenzyme forms) due to polymorphisms in the coding gene. Until now its described two single-nucleotide polymorphisms (SNPs) in coding sequences of genes: Gln (Q)/Arg (R) substitution at position 192 and Leu (L)/Met (M) substitution in position 55 (8). Amino acid substitutions Q / R at position 192 has a significant effect on catalytic activity of the enzyme by monitoring substrate-dependent variability in the activity of wild-type ($PON1_{Q192}$) and variant ($PON1_{R192}$) form of the enzyme: wild-type enzyme is more active in the hydrolysis of paraoxon and fenitrokson, hydrolyzed slower diazokson, sarin, zoman and has the same activity to phenylacetate in comparison with variant alloenzyme $PON1_{R192}$ (17). There are differences between enzyme isoforms within regard to their effectiveness in reducing oxidation of LDL: variant R allo-enzyme of the PON1 is less effective than allo-enzyme Q, due to lower activity of alloenzyme R to hydrolyse lipid peroxides (17, 18). Despite distinct differences in the activities of polymorphic variants, however, there is vast variation in enzyme activity between individuals of the same genotype (19).

The role of PON1 in cardiovascular diseases

Large amount of information clearly demonstrates that serum PON1 is the most important enzyme in HDL complexes responsible for their protective function for the oxidation of LDL. In addition, numerous epidemiological studies have found that polymorphisms in genes encoding PON1, responsible for the variation of enzyme activity and concentration also contribute to the variations in plasma levels of HDL-C (α-

cholesterol) (7). Because HDL has many atheroprotective functions, such as removal of excess cholesterol from tissues (reverse cholesterol transport) and inhibition of inflammation, protection of HDL may be the main role of PON1 in mammals and man (7).

The relationship between activity and concentration of PON1, and severity and level of coronary artery disease and its association with the global severity index ($p < 0.001$) was demonstrated by Garner and coworkers (20). They found lower PON1-activity and concentration respectively in severe and mild coronary artery disease ($p = 0.003$, $p = 0.016$). In the same study, Garner et al. found a significant association between activity and concentration of PON1, and coronary atherosclerosis, expressed as quantitative angiographic indexes based on the level and severity of atherosclerosis (20). The results of a meta-analysis based on 43 studies of PON1 polymorphisms, including 11 212 studied with coronary heart disease (CHD) and 12 786 controls showed that the relationship between PON1-polymorphisms and CHD is weak (21). Information about the relationship between directly measured PON1 activity, concentration and angiographically proven coronary artery disease is limited. Although not reached statistical reliability, Azarsiz et al. found that paraoxonase activity in patients with coronary artery disease is lower in patients than in control subjects (22). Mackness et al. prove that PON1 activity and concentration were lower in patients with CHD compared with the control group (17), the result is independent of genotype. Activity and concentration of PON1 can vary up to 40 folds in human populations (23, 24). Some of this variability is explained by polymorphism of the PON1 gene in replacement of amino group of 192 site (17, 25, 26). However, activity and concentration of PON1 is affected by various other factors as diet, lifestyle and environmental factors. Cooking oil with poor quality reduces serum levels of the enzyme (27). Polyphenols in the food and moderate use of alcohol increase PON1 activity (28, 29). Smoking also affects the activity of PON1 leading to its decrease (30). There are data describing an inhibition of PON1 in exposure to the environment with chemicals (31, 32). Low serum activities of PON1 have been reported in diseases associated with atherogenesis, such as diabetes, hypercholesterolemia, and renal failure (31, 33). In human serum, most paraoxonase activity is associated with HDL. Serum paraoxonase attend in a separate subspecies of PON1, containing apoA-1 and clusterin (or apo-J) (34). La Du et al. found that it is extremely difficult to remove ApoA-1 from PON1 during purification of human serum, led to the assumption that ApoA-1 and PON1 are closely related (35). A statistically significant association is proved between PON1 and HDL and between apoA-1 and apoA-2 (20, 33, 35).

Marit et al. reported that the activity of PON1 towards phenylacetate and its concentration is reduced in patients with coronary heart disease and there is a significant relationship between activity and concentration of the enzyme with the severity of coronary atherosclerosis (20). Significantly from the study is that the protective role of HDL is modulated by its components i.e. serum concentrations of HDL cannot be equated with an equivalent protective capacity (20). They found that low serum enzyme activity towards paraoxon is a predictive factor for risk of future coronary events, independent of other risk factors except HDL, which PON1 is a component (36). The concentration and activity of PON1 is very variable in the human population. The quality and quantity of the enzyme in serum may be important for the individual response to organophosphorus poisoning or the risk of developing cardiovascular disease (25).

Objective

1.To determine serum paraoxonase and arylesterase activities of PON1 in patients with ACS in comparison with those in healthy subjects among the Bulgarian population.

2. To seek relationship between PON1 activity with HDL-and LDL-cholesterol.

3. To identify any change in serum activities of PON according to the degree of coronary involvement.

4. To identify any difference in enzyme activity in patients experienced a cardiovascular event.

MATERIALS AND METHODS

Patients and biological material

Present study includes 42 patients with ACS (27 men and 15 women) aged between 42 and 85 years. Among them 36 patients with acute

myocardial infarction with ST-segment elevation (STEMI) and 6 patients with unstable angina (NAP) were hospitalized in emergency cardiac intensive sector with clinical, ECG and biochemical markers of ischemia. As a control group there were involved 26 healthy young men - medical students aged from 19 to 27 years, (10 male and 16 female).

Measurements of enzyme activities were performed in serum obtained from whole venous blood and stored within 2 weeks at -20^{0}C. Simultaneously, venous blood was taken with an anticoagulant (Na2EDTA) to obtain plasma that was also stored in -20^{0}C. Informed written consent was obtained from all individuals, patients and controls, enrolled in the current study.

METHOD

1. Paraoxonase activity

The determination of serum activity of PON1 against paraoxon substrate (paraoxonase activity) was held in the Laboratory "Biology of tumor growth," section of "Biochemistry", Department of "Chemistry and Biochemistry", Medical Faculty - Trakia University, Stara Zagora, adapted by our method published earlier by Tomas et al., 2000 (37). The method is kinetic and is based on determining the rate of hydrolysis of paraoxon, where is measured the change in absorbance of the obtained *p*-nitrophenol at 405 nm in 37°C for a certain time interval (5 min). The concentration of PON1 is presented in U/l serum as 1U paraoxonase (paraoxonase activity) is the enzyme that produces 1 μmol *p*-nitrophenol for 1 min. Extensional molar ratio of p-nitrophenol is 18 053 $(mol/l)^{-1}$ cm^{-1} at pH 8.5.

2. Arylesterase activity

The method for determining the activity of PON1 against the substrate phenylacetate was adapted from our method described by Tomas et al., 2000 (37). The method is based on spectrophotometry in the UV range: rate of hydrolysis of phenylacetate was measured, tracing the increase in absorbance of phenol at 270 nm, obtained by hydrolysis of phenylacetate in 37°C for a certain time interval (3 min). The concentration of of PON1 is presented in kU/l (or U/ml) plasma as a 1U PON1 (arylesterase activity) is the enzyme that degrades 1 μmol phenylacetate for 1 min. Molar extensional ratio of phenol is 1310 (mol / l) cm^{-1} at pH 8.0.

3. Determining the lipid status of the control study group.

Studies of serum levels of total cholesterol, HDL-C (α-cholesterol) and TAG were conducted in the Laboratory of Medical College, Trakia University, Stara Zagora using standard methods with an automatic analyzer.

4. Other data

Results of the angiographic findings, laboratory findings and information for accompanying diseases were taken from the history of the disease.

RESULTS

In this pilot study there were included 42 patients with ACS (27 men and 15 women) aged between 42 and 85 years. Among them 36 patients were with acute myocardial infarction with ST-segment elevation (STEMI) and 6 patients were with unstable angina (NAP). As controls there were enrolled 26 healthy young voluntaries - medical students aged from 19 to 27 years; 10 males (38.46%) and 16 females (61.54%). Smokers among the study control group were 7 (26.92%), non-smokers 17 (65.39%), ex-smokers 2 (7.69%).

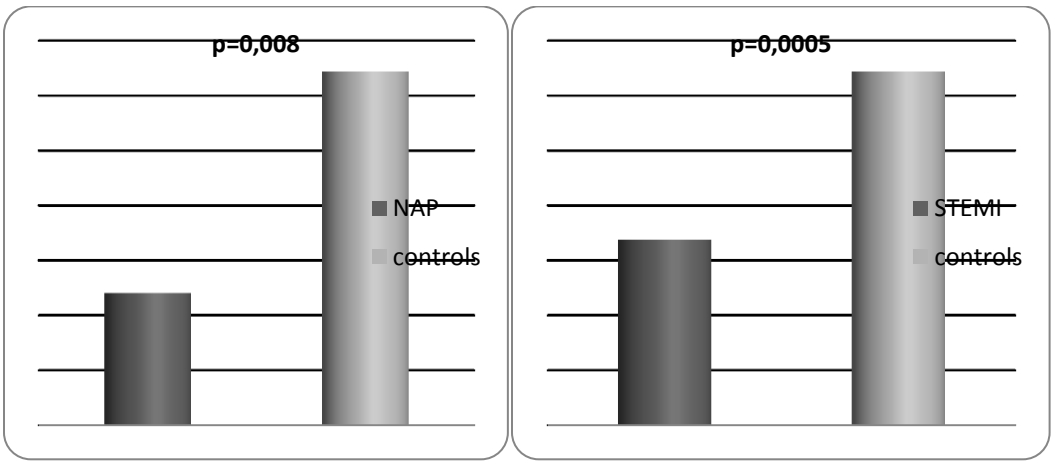
The obtained mean values±SD of PON activities in groups with STEMI and NAP are shown in **Table 1.**

Serum paraoxonase activities of PON1 in acute myocardial infarction with ST-segment elevation and unstable angina were significantly lower compared to the healthy controls ($p<0.005$, $p<0.008$) **(Figure 1)**

Serum arylesterase activity of PON1 in acute myocardial infarction with ST-segment elevation and unstable angina were significantly lower compared to the healthy controls ($p=0.002$, $p=0.008$) is presented **in Figure 2**.

Table 1. *Comparison of the main lipid serum characteristics and BMI between the studied groups.*

Research	NAP	STEMI	Controls	p-value
Number	6	36	26	
Total cholesterol (TC) (mmol/l, mean±SD	5,39±0,67	5,64±1,26	4,12±0,56	0,007; <0,0001
HDL-cholesterol (mmol/l, mean±SD	1,87±0,32	1,90±0,41	1,43±0,48	0,1; 0,003
TG(mmol/l, mean±SD)	1,15±0,59	1,07±0,49	1,11±0,65	
BMI kg/m (mean±SD))	26,62±4,40	26,25±3,80	22,76±4,6	0,047; 0,003
Smokers (N, %)	3 (50%)	17 (48,57%)	7 (26,92%)	



A B

Figure 1. Comparison of serum paraoxonase activity of PON1 of controls with that of acute myocardial infarction with ST-segment elevation (A) and unstable angina (B).

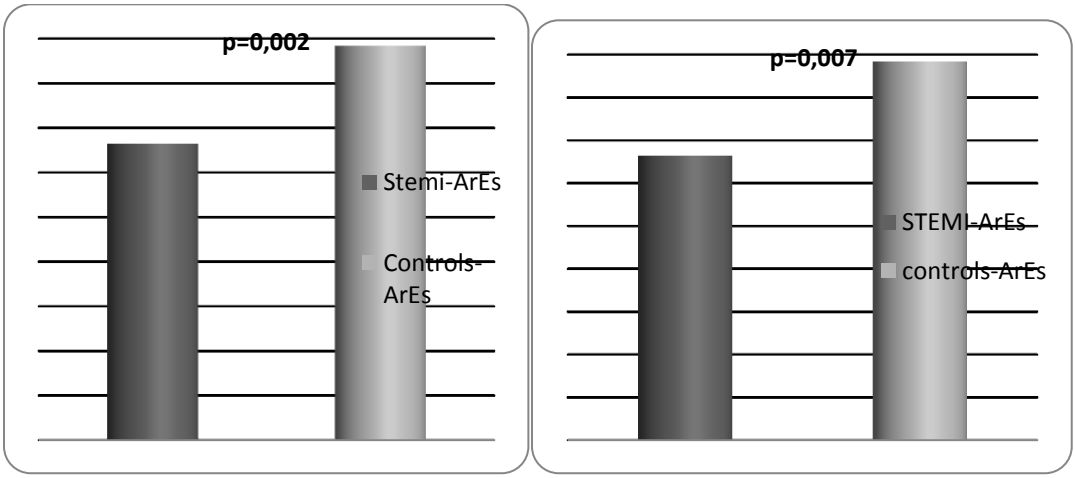


Figure 2. Comparison of serum arylesterase activity of PON1 of controls with that of acute myocardial infarction with ST-segment elevation (A) and unstable angina (B)

Normalized levels of paraoxonase activity of PON1 against the levels of HDL-C were significantly lower in the group with acute myocardial infarction with ST-segment elevation against controls (p<0.001), as shown in **Table 2**. Normalized levels of paraoxonase activity of PON1 against the levels of HDL-C were significantly lower in the group with unstable angina against controls (p=0.026) **(Table 2)**. Normalized levels of arylesterase activity of PON1 against the levels of HDL-C were significantly lower in the group with unstable angina and STEMI against controls (p<0.04, p=0.0008) **(Table 2)**. When comparing the paraoxonase activity of PON1 enzyme against the affect of the degree of coronary artery disease (single-branch, two-branches, three-branches and multi-branches) we have received a tendency for lower values in three-branch and multi-branch coronary artery disease (p=0.07). Normalized values of paraoxonase activity of PON1 to the levels of HDL-C in three-branch and multi-branch coronary disease are lower than single branch coronary artery disease without significant statistical reliability (p = 0.07). When comparing paraoxonase activity in subjects with hypertension and those without a history and instrumental data on hypertension we received a statistically significant lower values in patients presenting with hypertension (p=0.02). When comparing arylesterase activity in subjects with hypertension and no history and instrumental data for hypertension, we received statistically significant lower values in patients presenting with hypertension (p=0.03). Normalized values of PON-activity of PON1 with regard to levels of HDL-C (PON HDL-C) in patients with a history and instrumental data for hypertension are fairly lower than those without hypertension (p=0.03). We grouped patients into two groups: experienced and not experienced cardiovascular events (CVE) - NAP, NSTEMI, STEMI. Average paraoxonase activity of PON1 among people with experienced cardiovascular event was significantly lower than those without past CVE (p = 0.018). We have received significantly higher levels of total serum cholesterol in people with acute myocardial infarction and unstable angina, compared to the control group (p<0.001, p=0.0069); we did not receive significant differences in serum concentrations of TAG, and correlation with PON-activity.

Table 2. *Comparison of paraoxonase and arylesterase activities of PON1 in the studied groups.*

	Patients		**Controls**	**p-value**	
	NAP	**STEMI**		**NAP vs. Controls**	**STEMI vs. Controls**
PON-activity(U/L)	48,12± 25.6	67,45± 8,9	128,79± 15.1	0,008	0,0005
ArEs-activity(kU/L)	54.18 ± 12.8	66.34 ± 5.3	88.38 ± 3.0	0,008	0,002
PON1/HDL-C (U/mmol)	24,30±28,81	36,84±28,99	89,45±64,40	0,003	<0,0001
ArEs/HDL-C (kU/mmol)	37,21±11,29	36,91±11,38	60,83±24,9	0,037	0,0008

Patients with acute myocardial infarction and NAP have higher levels of body-mass index (BMI), compared to the control group (p=0.0027, p=0.046). In the group with AMI patients with diabetes are 6 (17.65%), in the NAP is 1 (16.68%). There is tendency to decrease in serum of PON activity in diabetes compared to subjects with ACS who do not have diabetes (p = 0.05). Arylesterase activity does not show significant variations in activity in diabetes in our group of patients (p=0.200). Considering that the PON1 in the serum is associated with HDL complexes, we have looked for correlation between paraoxonase activity of PON1 and levels of HDL- and LDL-cholesterol **(Figure 3).**

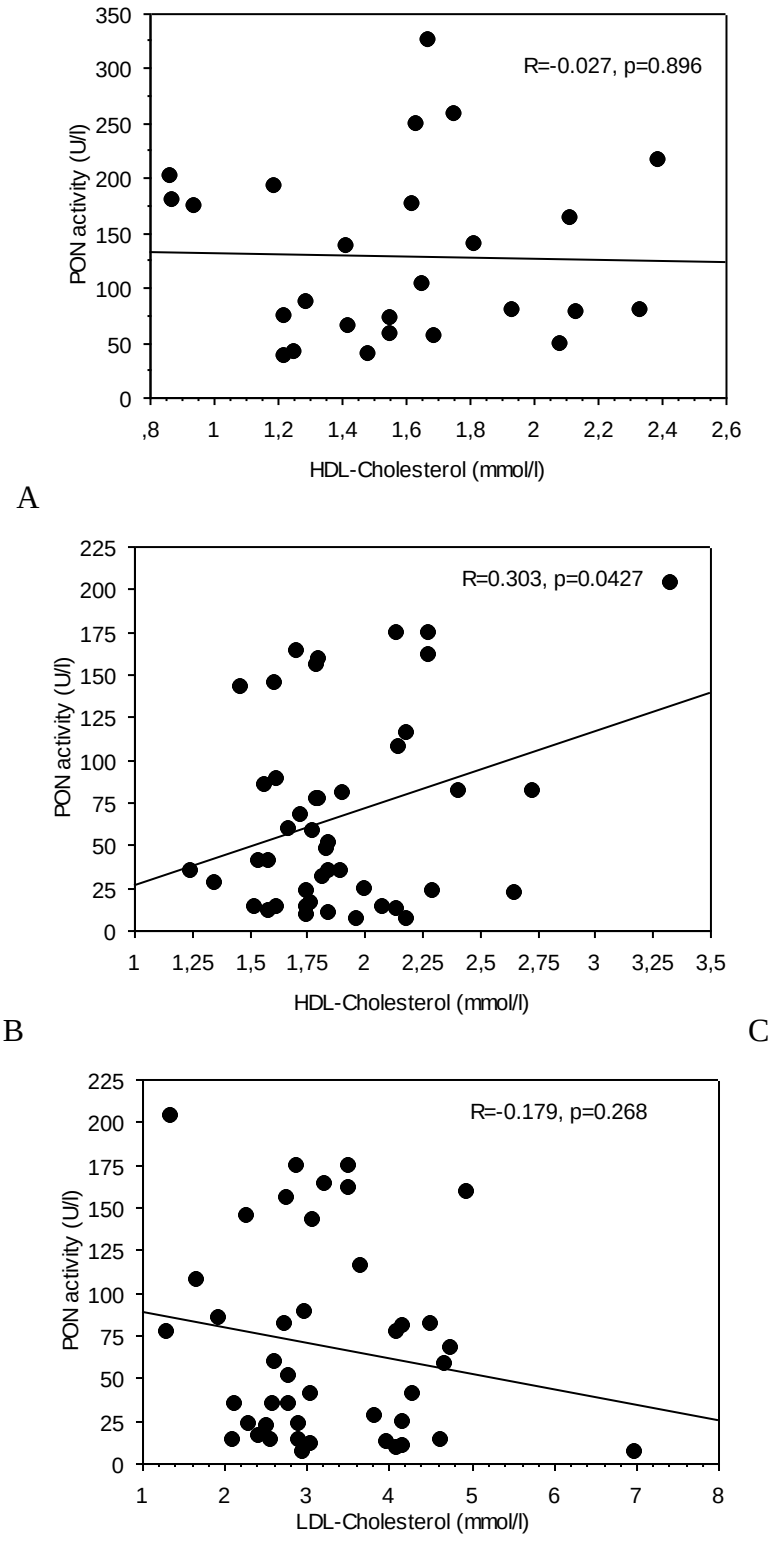


Figure 3. Correlations between the levels of serum PON activity of PON1 with HDL-cholesterol in controls (A), in patients (B) and the correlation of PON activity with LDL-cholesterol in patients (C).

We did not find a correlation between serum paraoxonase activity of PON1 and alpha-cholesterol in controls (p=0.895) **(Figure 3A),** however there was a significant positive correlation between serum paraoxonase activity of PON1 and α-cholesterol in patients (R=0.303,

p=0.043) **(Figure 3B)**. There was also a weak tendency for negative correlation between serum paraoxonase activity of PON1 and β-cholesterol in patients (R=-0.179, p=0.268).

DISCUSSION

These results showed heterogeneity in levels of both arylesterase and paraoxonase activity of PON1 among group of individuals included in our pilot study. Obtained great heterogeneity is in accordance with described up to 40-folds individual variation in PON1 activities, found in a large meta-analysis (18, 38, 39). The obtained values are close to those reported in research reports for other control groups and patients with acute coronary syndrome, which also differ considerably (37, 40, 41). **Table 3** shows the values of serum paraoxonase and arylesterase enzyme activity in various studies in healthy subjects and in coronary patients.

Table 3. *Serum paraoxonase enzyme activity in various studies in healthy subjects and in coronary patients*

Study	**Number of patients**	**PON activity, U/L**	**Controls**
Luo Yang-Ping et al.(42)	40	80±36	136±64
Kumar A. et al. (43)	165	69,66±9,99	98,42±6,15
Aksoy S. et al.(44)	30	142,4±119,1	
Lakshmy R et al.(45)	124	93.52 ± 59.14	90,37±65,38
Mackness B. Et al.(36)	163	132(28,6-622,7)	

We have received significantly lower values of the activities of PON1 in patients with acute myocardial infarction with ST-segment elevation and unstable angina compared to controls, confirming the results published in the literature (42-45, 36).

It is known that there is a close physiological and physical relationship between PON1 and HDL in the plasma. HDL complexes actually occurs as vector which facilitates the secretion of the enzyme from the liver (44), provides a hydrophobic environment for the signal peptide of PON1 and simultaneously stabilizes the enzyme and is essential for its function (45). In previous studies have shown that there is a positive association between arylesterase and paraoxonase activities of PON1 and the concentration of serum HDL-cholesterol (45). In studied group we did not get statistical correlation between arylesterase and paraoxonase activity of PON1 and serum concentration of alpha-cholesterol in controls, but there was observed a weak positive correlation between serum paraoxonase activity of PON1 and a-cholesterol in patients with ACS (p=0.080). The obtained by us tendency for lower values of enzyme activity in three-branches and multi-branches coronary artery disease and hypertension confirms the results of other studies (20, 22, 46). In the Caerphilly prospective study there was investigated the enzyme activity in clinically healthy men who were followed for 15 years and found that the PON1-activity was lower by 20% in those who had a cardiovascular event (p=0.039). In the study group, lower values in subjects with past heart attack are confirmed. Perhaps significantly reduced activity in patients with past CVE may be a prognostic marker for recurrent ischemic attacks during follow-up of PON1. The interesting point was that we did not receive the expected proportional relationship between two enzyme activities of PON1. This observation can be explained by the existence of different genotypes of the two functional polymorphisms in the PON1 gene [Gln(Q)192Arg(R) and Leu(L)55Met(M)] and of the proven differences in genetically determined variants of the enzyme for various substrates, including paraoxon and phenylacetate. The amino acid substitution Q/R at position 192 has a significant effect on catalytic activity of the enzyme: wild-type (PON1Q192) enzyme is more active in the hydrolysis of sarin and zoman, hydrolyzes more slowly paraoxon and phenytroxon and has the same activity towards phenylacetate and

diazoxon compared with variant aloenzyme PON1 R192 (11, 20).

CONCLUSION

Studies conducted with clinically healthy individuals with and without risk factors and those with overt coronary artery disease, and tracking over time the values of the enzyme would have give us information about the prognostic role of PON.

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