

# Paraoksonaza/arilesteraza u serumu ispitanika s dijabetesom tipa II

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## Paraoxonase/arylesterase in serum of patients with type II diabetes mellitus

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The aim of this study was to determine whether the paraoxonase (PON1) status, *i.e.* PON1 activities and phenotypes (AA, AB and BB), and its relationship with lipid status are different in patients with type II diabetes as compared to healthy population. Diabetic group comprised 175 patients with type II diabetes mellitus (94 men and 81 women) who came to their regular control examination and took the oral glucose tolerance test. Patients with type II diabetes mellitus diagnosis for 12 years on average were on peroral antidiabetics, or insulin or diet, and 3 patients had no therapy prescribed yet. Control group comprised 114 apparently healthy individuals (28 men and 86 women) who were not on any medication. The paraoxonase activity was measured with 2.0 mmol L<sup>-1</sup> paraoxon in the absence and in the presence of 1.0 mol L<sup>-1</sup> NaCl, and with 2.0 mmol L<sup>-1</sup> phenylacetate. Both activities were measured spectrophotometrically at 37 °C in 0.1 mol L<sup>-1</sup> Tris-HCl buffer, pH = 8.0, containing 2.0 mmol L<sup>-1</sup> CaCl<sub>2</sub>. Sera of diabetic and control subjects were assigned to the paraoxonase phenotypes on the basis of the basal paraoxonase activity distribution. We assigned 45% sera of male and 49% sera of female diabetic patients, and 64% sera of both genders of the control group to the AA low activity phenotype. There were no differences in paraoxonase activities between the gender- and phenotype-matched diabetic and control groups. Enzyme activity against the phenylacetate was higher, and phenotype-dependent, only in diabetic patients. In contrast to AA phenotype individuals, total cholesterol and LDL-cholesterol in the female diabetic group and triglyceride concentration in the male diabetic group assigned to pooled AB and BB phenotypes were higher than in the corresponding controls. It follows from PON1 phenotype distribution that less antiatherogenic paraoxonase B allele is more frequent in type II diabetes mellitus than in the healthy population. Their lipid status is more atherogenic, which could indicate a risk of premature atherosclerosis.

**Keywords:** paraoxonase, arylesterase, type II diabetes mellitus, lipid status

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Paraoxonase (aryldialkylphosphatase, EC 3.1.8.1; PON) was originally found to catalyze the hydrolysis of paraoxon (*O,O*-diethyl-*O-p*-nitrophenylphosphate), a catabolite of the insecticide parathion. It has been shown later on that this enzyme hydrolyzes arylesters and has a wide range of important hydrolytic activities and functions in lipid metabolism. In mammals, three genes PON1, PON2 and PON3 were identified in adjacent positions on chromosome 7 in humans and on chromosome 6 in mice. Only PON1 has been extensively clinically studied to date because PON3 levels are very low and PON2 is not present at all in human serum (1–4).

Paraoxonase 1 (PON1) is a protein of 354 amino acids with a molecular mass of 43 kDa. The amino acid arginine at position 192 of the protein specifies high activity whereas glutamine at that position specifies low-activity against paraoxon, the former has been also termed B and the latter A (the current recommended nomenclature is R and Q, respectively). The B enzyme is stimulated by 1 mol L<sup>-1</sup> NaCl to a greater extent than the A enzyme. Arylesterase activity of PON1 does not exhibit polymorphism (5).

PON1 is tightly bound with the hydrophobic N-terminal domain to apoprotein A-I of high density lipoprotein (HDL) and circulates as a HDL component in the blood of humans and other vertebrates (6, 7).

Several studies have shown that PON1 protects low density lipoprotein (LDL) and HDL against oxidative modification. It can destroy active lipids in mildly oxidized LDL and thereby protect against the induction of inflammatory responses in arterial wall cells. Oxidation of LDL is recognized as an early stage in the development of atherosclerosis, leading to LDL uptake by the macrophage scavenger receptor and hence to formation of foam cells (8, 9).

Serum PON1 activity greatly varies among individuals and populations due to the PON1 codon 192 genetic polymorphism, but also to the gene-environment and/or gene-gene interactions (10). Factors influencing serum levels of PON1, either genetic or environmental, will in turn affect the capacity of HDL to protect LDL from oxidation and, consequently, may be linked to atherosclerosis (11). This is of particular relevance to diabetic patients where higher risk of oxidative stress is suggested to contribute to the greatly increased incidence of vascular disease and other complications (12). Some studies have shown that PON1 levels and activities were independent of PON1 polymorphism in patients with type I diabetes (13, 14). The enzyme has also been identified as an independent, genetic risk factor for vascular disease, particularly in type II diabetic patients. High frequency of PON1 R allele has also been reported in type II diabetic patients with accompanying vascular diseases (15–17). Mackness *et al.* (11) and Aviram *et al.* (18) demonstrated that purified PON1 Q (low activity phenotype) was more effective than PON1 R in protection against LDL peroxidation, while Cao *et al.* (19) showed that PON1 from QQ and RR diabetic patients decreased LDL peroxidation to a similar extent, suggesting that the PON1 codon 192 genetic polymorphism has no effect on the enzyme's antioxidative capacity. As suggested by Jarvik *et al.* (20), the PON1 phenotype is a better predictor of vascular and carotid artery disease than the PON1 codon 192 genetic polymorphism itself.

The aim of this study was to determine whether PON1 activities against paraoxon and phenylacetate as well as phenotypes, *i.e.* PON1 status and lipid profile, are different in patients with type II diabetes as compared to the healthy population.

## EXPERIMENTAL

*Subjects*

Control group comprised 114 apparently healthy individuals who were not on any medication and who attended a systemic medical examination. The inclusion criterion for the control group was living in a similar socioeconomic environment as diabetic patients. There were 28 men and 86 women of an average age of 59 years (range 17–74 years) and 52 years (range 21–80), respectively.

Diabetic group comprised 175 patients with type II diabetes mellitus who came to their regular control examination and took the oral glucose tolerance test. There were 94 men and 81 women of an average age of 64 years (range 31–90) and 65 years (range 46–86), respectively. Patients were with type II diabetes mellitus diagnosis for 12 years on average, one man had this diagnosis for 41 years, and one woman for 36 years. The patients were on peroral antidiabetics, or insulin, or diet and 3 patients with recently confirmed diabetes had no therapy prescribed yet (Table I).

*Table I. Clinical characteristics of the patient group*

	Male	Female
Number of individuals (N)	94	81
Age (years)	63 (30–89)	64 (45–84)
Duration of diabetes type II (years)	11 (1–41)	12 (1–36)
Glucose (mmol L <sup>-1</sup> )	9.43 ± 2.98	10.06 ± 2.95
Therapy		
diet	6	11
insulin	42	44
oral hypoglycemic drugs	46	35
not prescribed yet	2	1
HbA <sub>1c</sub>		
< 6.5%	23	9
6.5–7.5%	17	19
> 7.5%	51	52

*Serum samples*

Blood samples were collected by venipuncture in the fasting state at the Health Center Zagreb Centar (control group) and at the Vuk Vrhovac Institute, University Clinic for Diabetes, Endocrinology and Metabolic Diseases, Zagreb (diabetic group). Blood was centrifuged for 15 min at 1500–2000 g. Serum was analyzed immediately on an Olympus AU 600 analyzer (Olympus Mishima Co., Japan) for glucose, cholesterol, triglycerides and HDL-cholesterol. Sera were then stored at 4 °C until the next day when the PON1 activity was measured. Serum aliquots of 2 mL were frozen at –20 °C and arylesterase activity was measured within 5 weeks. Before analysis, samples were thawed and homogenized.

This study was approved by the Research Ethics Committee of the Vuk Vrhovac Institute and Health Center Zagreb Centar.

### *Enzyme activity assays*

PON1 activity assays were performed with 2 mmol L<sup>-1</sup> paraoxon in the absence of NaCl (basal activity) and in the presence of 1 mol L<sup>-1</sup> NaCl (NaCl-stimulated activity) according to the method described by Mackness *et al.* (21, 22). Initial hydrolysis rates of paraoxon (Sigma Chemical Co, UK) were determined by measuring the released *p*-nitrophenol at 405 nm at 37 °C on a Technicon RA-1000 autoanalyzer (Bayer, Italy).

The basal assay mixture included 2.0 mmol L<sup>-1</sup> paraoxon and 2.0 mmol L<sup>-1</sup> CaCl<sub>2</sub> in 0.1 mol L<sup>-1</sup> Tris-HCl buffer, pH = 8.0. To 350 µL of reagent mixture, 10 µL of serum was added. For the NaCl-stimulated assay, 1.0 mol L<sup>-1</sup> NaCl was added into the reaction mixture described above.

Arylesterase activity was measured using phenylacetate as a substrate. Stock solutions of phenylacetate were prepared in 40% methanol in water (V/V). Final concentration of methanol in the reaction mixture was 0.8%. Serum was diluted 200 times in 0.1 mol L<sup>-1</sup> Tris-HCl buffer, pH = 8.0. To 2.7 mL buffer, 0.3 mL diluted serum was added, mixed vigorously, and 20 mmol L<sup>-1</sup> phenylacetate was added. Initial rates of hydrolysis were determined by following the increase of phenol concentration at 270 nm at 37 °C on a CE 7250 spectrophotometer (Cecil Instruments Limited, UK).

The reaction mixture contained 2.0 mmol L<sup>-1</sup> phenylacetate (Sigma Chemical Co) and 2.0 mmol L<sup>-1</sup> CaCl<sub>2</sub> in 0.1 mol L<sup>-1</sup> Tris-HCl buffer, pH = 8.0.

Enzyme activities were expressed in international units (U) or kilounits (kU) per 1 litre of sera. An international unit is the amount of hydrolyzed substrate in µmol per minute.

### *Paraoxonase phenotype distribution*

Sera of the control and diabetic patients were assigned to the phenotypes on the basis of the basal paraoxonase activity distribution (23).

### *Lipid and lipoprotein assays*

Serum triglycerides were measured by the GPO-PAP method (Olympus System Reagent Cat. No.: OSR 6133). Total serum cholesterol was measured by the CHOD-PAP method (Trace Scientific Ltd., Australia, PIN: TR 13303). HDL-cholesterol was measured using a direct method based on selective inhibition of the non-HDL fractions using polyanions (Olympus Mishima Co., Ltd., Japan, Olympus System Reagent Cat. No.: OSR 6187). LDL-cholesterol was calculated using the Friedewald formula (24).

### *Statistical analysis*

According to the test of the normality of data distribution, the Mann-Whitney rank sum-test or Student *t*-test was used. Differences between the values were considered sig-

nificant at the significance level  $p < 0.05$ . Statistical analysis was performed using the SigmaStat program, version 2.0 (Jandel Corporation, USA).

## RESULTS AND DISCUSSION

Diabetic patients and control individuals were analyzed by assigning their sera to the paraoxonase phenotypes using the method of Eckerson *et al.* (23), based on the basal paraoxonase activity distribution profile (Fig. 1). Only the low activity AA phenotype was well defined at the nadir of  $400 \text{ U L}^{-1}$ , while the other two phenotypes, AB and BB, were not distinctive. Therefore, sera were classified to the paraoxonase phenotype AA and to a group comprising both the subjects with AB (heterozygous intermediate activities) and BB phenotypes (homozygous high activities).

We found that 64% of male and 64% of female control individuals belong to the AA phenotype (Table II). This is in agreement with another study in a population from Zagreb, where 60% individuals were attributed to group AA, also based on visual estimation of the basal paraoxonase activity distribution profile (25). In the diabetic group, a significantly lower percentage of male sera (45%,  $p < 0.05$ ) and female sera (49%,  $p < 0.05$ ) were assigned to AA phenotype. This is in agreement with the report of Ruiz *et al.* (26), the first in a series of studies on higher frequency of B allozyme and coronary heart disease in type II diabetic patients. There were no differences in paraoxonase activities between the gender- and phenotype-matched diabetic and control groups. Enzyme activities against phenylacetate were significantly higher in the sera of diabetic patients compared to the gender- and phenotype-matched control groups. Additionally, statistically signifi-

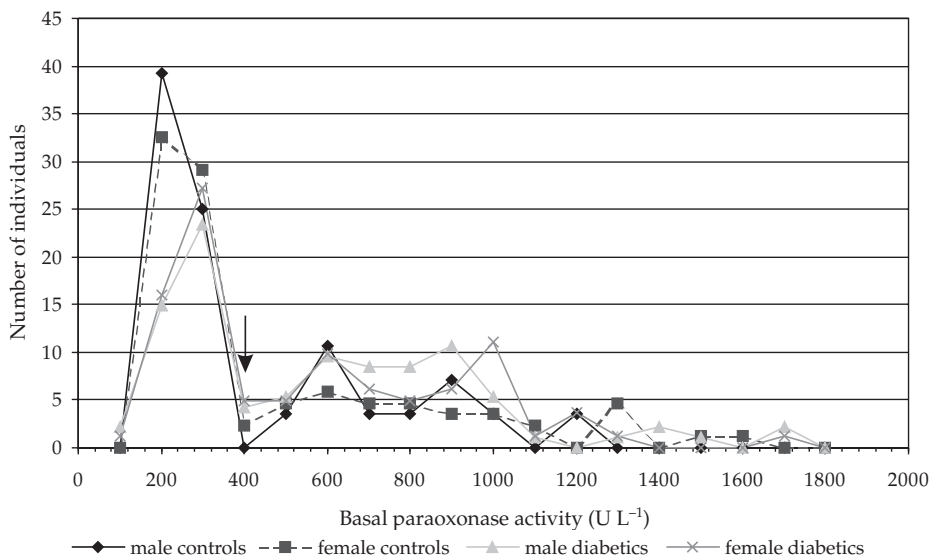


Fig. 1. Paraoxonase activity distribution in control and diabetic individuals according to gender.

Table II. Activities against paraoxon and phenylacetate in sera of control and diabetic groups classified according to phenotypes

Phenotype	Control group		Diabetic group		
	Male	Female	Male	Female	
	N (%)	N (%)	N (%)	N (%)	
AA	18 (64)	55 (64)	42 (45)	40 (49)	
AB + BB	10 (36)	31 (36)	52 (55)	41 (51)	
Substrate	Activity		Activity		
Paraoxon (U L <sup>-1</sup> )	AA	192 (102–300)	198 (102–387)	216 (78–339)	241 (81–345)
	AB + BB	702 (429–1116) <sup>b</sup>	759 (420–1527) <sup>b</sup>	717 (426–1623) <sup>b</sup>	759 (429–1623) <sup>b</sup>
Paraoxon + NaCl (U L <sup>-1</sup> )	AA	408 (231–630)	417 (207–723)	444 (141–702)	483 (153–714)
	AB + BB	1337 (831–2106) <sup>b</sup>	1419 (786–1419) <sup>b</sup>	1482 (854–3386) <sup>b</sup>	1569 (540–2787) <sup>b</sup>
Phenylacetate (kU L <sup>-1</sup> )	AA	41 ± 27	50 ± 26	66 ± 26 <sup>a</sup>	65 ± 22 <sup>a</sup>
	AB + BB	40 ± 24	46 ± 30	81 ± 27 <sup>a,b</sup>	80 ± 26 <sup>a,b</sup>

N – number of individuals

Activities against paraoxon and phenylacetate are presented as median (range) and mean ± standard deviation, respectively. Statistically significant difference ( $p < 0.05$ ) between: <sup>a</sup> gender- and phenotype-matched diabetic groups and control groups; <sup>b</sup> diabetic groups and control groups with respect to the phenotype.

cant higher arylesterase activities were obtained in the diabetic groups with pooled AB and BB phenotypes compared to the diabetic AA phenotype group. Therefore, the ratio of paraoxon to phenylacetate activities was significantly lower in diabetic patients than in the control groups. Nevin *et al.* (27) suggested that arylesterase activity reflects the enzyme mass, which might explain our results of lower specific paraoxonase activity in diabetic groups. The ratio of paraoxon to phenylacetate activities could be a more sensitive marker of the enzyme activity changes than estimation of the activity against paraoxon alone.

Jarvik *et al.* (20) found that subjects having paraoxonase with arginine at position 192 were at higher risk of cardiovascular disease than homozygous subjects carrying glutamine at that position because of their more atherogenic lipid profile. Therefore, the lipid status of the control and diabetic patients was analyzed for each phenotype group. Triglycerides were higher in all diabetic groups compared to the gender- or phenotype-matched control group, while total cholesterol and LDL-cholesterol were higher only in the female diabetic group classified as a group comprising subjects with both AB and BB phenotypes. Significantly higher total cholesterol and LDL-cholesterol concentrations were found in the female diabetic sera with AB and BB paraoxonase phenotypes compared to the AA phenotype. HDL-cholesterol concentrations were significantly higher in the female diabetic sera of both paraoxonase phenotypes compared to the same male diabetic group. LDL-cholesterol concentration was higher only in the female diabetic group comprising subjects with both AB and BB phenotypes compared to the same male diabetic group (Table III).

Table III. Serum triglyceride, total cholesterol, HDL-cholesterol and LDL-cholesterol concentrations in control and diabetic groups classified according to phenotypes

Parameter	Phenotype	Control group		Diabetic group	
		Male	Female	Male	Female
		N (%)	N (%)	N (%)	N (%)
	AA	18 (64)	55 (64)	42 (45)	40 (49)
	AB + BB	10 (36)	31 (36)	52 (55)	41 (51)
Triglyceride (mmol L <sup>-1</sup> )	AA	1.32 ± 0.49	1.17 ± 0.80	2.27 ± 1.43 <sup>a</sup>	2.29 ± 1.74 <sup>a</sup>
	AB + BB	1.08 ± 0.28	1.04 ± 0.34	3.09 ± 1.23 <sup>a,b</sup>	2.34 ± 1.41 <sup>a</sup>
Total cholesterol (mmol L <sup>-1</sup> )	AA	5.34 ± 0.74	5.57 ± 0.77	6.00 ± 1.27	5.84 ± 1.07
	AB + BB	5.38 ± 0.65	5.51 ± 0.65	5.98 ± 1.66	6.51 ± 1.39 <sup>a,b,c</sup>
HDL-cholesterol (mmol L <sup>-1</sup> )	AA	1.38 ± 0.33	1.50 ± 0.28	1.25 ± 0.28	1.44 ± 0.30 <sup>c</sup>
	AB + BB	1.34 ± 0.30	1.47 ± 0.41	1.22 ± 0.25	1.39 ± 0.30 <sup>c</sup>
LDL-cholesterol (mmol L <sup>-1</sup> )	AA	3.35 ± 0.70	3.53 ± 0.66	3.81 ± 1.10	3.38 ± 0.90
	AB + BB	3.56 ± 0.67	3.56 ± 0.58	3.60 ± 0.96	4.17 ± 1.22 <sup>a,b,c</sup>

N – number of individuals.

Data are presented as mean ± standard deviation. Statistically significant difference ( $p < 0.05$ ) between:

<sup>a</sup> gender- and phenotype-matched diabetic groups and control groups; <sup>b</sup> diabetic or control groups with respect to the phenotype, <sup>c</sup> genders within phenotype groups.

## CONCLUSIONS

This study shows that the frequency of paraoxonase AA low activity phenotype is lower in diabetic patients than in controls and that enzyme activity against the phenylacetate was higher and phenotype-dependent only in diabetic patients. The lipid status of the diabetic and control groups showed that total cholesterol and LDL-cholesterol were higher only in the female diabetic group and triglyceride concentration was higher in the male diabetic group with AB and BB phenotypes pooled together compared to AA phenotype.

This fact might constitute supportive evidence to other reports that PON1 status does play a role in the protection against lipid oxidation and that AB and BB phenotypes are mainly associated with the more atherogenic lipid profile in type II diabetic patients.

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## S A Ž E T A K

### Paraoksonaza/arylesteraza u serumu ispitanika s dijabetesom tipa II

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Cilj rada je usporediti katalitičku aktivnost paraoksonaze (PON1) te učestalost fenotipova AA, AB i BB paraoksonaze i njihovu povezanost s lipidnim statusom u serumu ispitanika s dijabetesom tipa II i u kontrolnoj skupini. U skupini ispitanika s dijabetesom tipa II bilo je 175 osoba (81 žena i 94 muškarca), s prosječnim trajanjem bolesti od 12 godina, koji su bili na peroralnoj terapiji antidijabeticima ili inzulinom ili na dijeti, dok trojici ispitanika još nije predložena terapija. Aktivnost paraoksonaze mjerena je paraoksonom (*O,O*-dietil-*O-p*-nitrofenilfosfat). Koncentracije reagensa u reakcijskoj smjesi za određivanje bazalne aktivnosti paraoksonaze iznosile su: 2.0 mmol L<sup>-1</sup> paraoksona i 2.0 mmol L<sup>-1</sup> CaCl<sub>2</sub> u 0.1 mol L<sup>-1</sup> Tris-HCl puferu, pH = 8.0. Reakcijska smjesa za određivanje NaCl-stimulirane aktivnosti paraoksonaze sadržavala je još 1.0 mol L<sup>-1</sup> NaCl. Arylesterazna aktivnost enzima mjerena je fenil-acetatom. Reakcijska smjesa je sadržavala 2.0 mmol L<sup>-1</sup> fenil-acetata i 2.0 mmol L<sup>-1</sup> CaCl<sub>2</sub> u 0.1 mol L<sup>-1</sup> Tris-HCl puferu, pH = 8.0. Broj ispitanika s AA fenotipom odnosno skupno AB i BB fenotipom paraoksonaze određen je iz raspodjelne krivulje bazalnih aktivnosti (bez prisutnosti 1.0 mol L<sup>-1</sup> NaCl) paraoksonaze u serumu. U serumima 45% žena i 49% muškaraca skupine ispitanika s dijabetesom tipa II te u 64% seruma oba spola u skupini zdravih ispitanika potvrđen je AA homozigotni fenotip paraoksonaze. Katalitičke aktivnosti enzima prema paraoksonu nisu se značajno razlikovale ovisno o spolu i fenotipu između dijabetične i kontrolne skupine, dok su aktivnosti enzima prema fenil-acetatu bile veće i ovisne o fenotipu samo u

dijabetičnoj skupini ispitanika. Značajno veće koncentracije ukupnog kolesterola i LDL-kolesterola izmjerene su u serumima žena, te veće koncentracije triglicerida u serumima muškaraca s dijabetesom tipa II koji su razvrstani u zajedničku skupinu AB+BB fenotipova, što bi ukazivalo na to da su AB i BB fenotipovi uglavnom povezani s lipidnim statusom većeg rizika za razvoj ateroskleroze u ispitanika s dijabetesom tipa II.

Ključne riječi: paraoksonaza, arilesteraza, dijabetes tipa II, lipidni status

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