

Paraoxonase 1 Gene Polymorphisms and Enzyme Activities in Diabetes Mellitus

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Summary

Paraoxonase 1 (PON1), an antioxidant enzyme closely associated with HDL (high-density lipoproteins), preserves LDL (low-density lipoproteins) against oxidation. Less protection may be therefore supposed by decreased PON1 activity. This study was undertaken to investigate the association of PON1 gene polymorphisms with diabetic angiopathy and to evaluate the relationship of these polymorphisms with PON1 activity. Total of 86 Type 1 (T1DM) and 246 Type 2 (T2DM) diabetic patients together with 110 healthy subjects were examined. DNA isolated from leukocytes was amplified with polymerase chain reaction (PCR) followed by restriction enzyme digestion. The products were analyzed for L55M and Q192R polymorphisms in coding region and for -107 C/T and -907 G/C in promotor sequence of PON1. Serum enzyme activity was measured spectrophotometrically. Significant differences were found between T1DM or T2DM and control persons in L55M polymorphism (allele M more frequent in T1DM and T2DM vs. controls, $p < 0.05$) and Q192R polymorphism (R allele less frequent in T1DM and T2DM vs. controls, $p < 0.01$) of the PON1 gene. Serum PON1 activity was significantly decreased in T1DM (110 ± 68 nmol/ml/min) and T2DM patients (118 ± 69 nmol/ml/min) compared to the control persons (203 ± 58 nmol/ml/min), both $p < 0.01$. The presence of MM and QQ genotypes was accompanied by lower PON1 activity than of LL and RR genotypes ($p < 0.05$), respectively. Better diabetes control was found in patients with LL than with MM genotypes and similarly in RR genotype than QQ genotype with $p < 0.05$. Significantly different allele frequencies were found in diabetic patients with macroangiopathy than in those without it (M: 0.59 vs. 0.44. R: 0.12 vs. 0.19, $p < 0.01$). The association of PON1 polymorphisms, lower PON1 activity and poorer diabetes control found in patients with macroangiopathy further support the idea of genetic factors contributing to the development of vascular disorders in diabetes.

Key words

Paraoxonase • Genetic polymorphisms • Serum activity • Diabetic macroangiopathy

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Introduction

Human serum paraoxonase (PON1, EC 3.1.8.1.), a 43-kDa protein, catalyses the hydrolysis of organophosphate esters, aromatic carboxylic acid esters, and carbamates (Li *et al.* 2005). PON1 is synthesized in the liver and is mainly associated with high-density lipoprotein (HDL) (Tomas *et al.* 2004). The enzyme decreases accumulation of the lipid peroxides in low-density lipoprotein (LDL) due to its ability to reduce hydroperoxides (Kinumi *et al.* 2005) and attenuates biological effects of mildly oxidized LDL. Both HDL and LDL isolated from PON1-knockout mice were equally susceptible to oxidation in co-cultured cells (Tward *et al.* 2002). This mice developed significantly larger aortic lesions than the wild type animals (Bradshaw *et al.* 2005).

PON1 activity was found to be decreased in cardiovascular disease (Mackness *et al.* 2004) and in diabetes mellitus (Karabina *et al.* 2005). Several factors may take part in these changes. Firstly, oxidative stress is accelerated and thus lipid peroxidation may contribute to vascular wall impairment (Gross *et al.* 2003). Secondly, glycation of proteins including enzymes may decrease their activities in diabetes (Kalousová *et al.* 2005,

Maritim *et al.* 2003). When HDL were incubated at very high concentrations of glucose (1 mol/l), the esterolytic PON1 activity was preserved. In contrast, HDL incubated in normal (5 mmol/l) or elevated (up to 100 mmol/l) glucose concentrations caused a loss of the esterolytic PON1 activity (Furlong *et al.* 1989).

In the present study we evaluate the association of four single nucleotide polymorphisms (SNPs) in the PON 1 gene with serum paraoxonase activity in type 1 (T1DM) and type 2 diabetes mellitus (T2DM) with or without macro- and microangiopathy. The molecular basis of the paraoxonase activity polymorphisms is a missense mutation in the coding region of PON1, resulting in a glutamine (Q)/arginine (R) substitution at codon 192 (Humbert *et al.* 1993). The PON1Q192 alloform hydrolyzes paraoxon much less efficiently than does PON1R192, while the opposite is true in case of soman or sarin. PON1Q192 is also more efficient at metabolizing oxidized HDL or LDL than PON1R192 (Aviram *et al.* 2000). Another coding region polymorphism, resulting in aminoacid substitution at position 55 Leu(L)/Met(M), has been associated with plasma PON1 protein levels, with PON1M55 being associated with low plasma PON1 level. From the polymorphisms characterized in the promotor region, the C-108T substitution has the most significant effect on plasma PON1 levels, with the -107C allele providing levels of PON1 about twice as high as those seen with the -107T allele (Brophy *et al.* 2001). The other polymorphisms in promotor region recently identified have for the most part not been yet characterized, but may affect splicing activity, message stability or efficiency of polyadenylation. We have focused on four SNP in our study: L55M, Q192R, -107 C/T, -907 G/C.

Methods

Subjects

Total of 86 Type 1 (mean age 43±18 years, 42 males, 44 females), 246 Type 2 diabetic patients (mean age 58±18, 114 males, 132 females) and control group of 110 healthy subjects without family history of diabetes (mean age 41±9 years, 55 males, 45 females) were examined in this study. Microangiopathy was confirmed by ophthalmoscopy or by the presence of peripheral neuropathy (diagnose was based on clinical features and by physical examination by 10 g monofilament, tuning fork and biothesiometry) in 167 patients who did not have any evidence of macrovascular

disease from the clinical picture (no history of angina pectoris, normal ECG records or normal coronarogram). In case of suspicion on autonomic neuropathy made from physical examination (tachycardia recorded by ECG in resting state, systolic blood pressure reaction on orthostatism) patients were excluded from this group. Forty-five subjects had macrovascular complications manifested by ischemic heart disease (diagnosis was based on ECG or coronarography), ischemic disease of the lower limbs (diagnosis was based on angiography of lower limbs arteries) or had history of stroke (diagnosis based on clinical features and CT). The remaining 120 diabetic patients were free of any complications. Clinical and laboratory characteristics are shown in Table 1.

Laboratory measurements.

Venous blood samples were drawn after an overnight fast. Plasma (Li-heparine) glucose and creatinine were measured in central biochemistry laboratory. Serum total cholesterol, HDL-cholesterol and triglycerides (TG) were measured by automated enzymatic methods on Hitachi analyzer, LDL cholesterol was calculated according to Friedwald's formula. HbA1c was measured by high-performance liquid chromatography.

Paraoxonase (PON1) activity was determined spectrophotometrically (Furlong *et al.* 1989). Serum was preincubated with 5×10^{-6} mol/l eserine (Sigma-Aldrich) for 10 min at room temperature to inhibit serum butyrylcholinesterase activity, which is markedly elevated in diabetes and interferes with determination of paraoxonase activity. Preliminary experiments showed that these conditions completely inhibited butyrylcholinesterase without any effects on paraoxonase activity. Paraoxonase activity was measured by adding 6.6 µl of serum to 1 ml Tris/HCl buffer (100 mmol/l, pH 8.0. Sigma-Aldrich) containing 2 mmol/l CaCl₂ and 5.5 mmol/l paraoxon (O,O-diethyl-O-*p*-nitrophenyl-phosphate; Sigma-Aldrich). The rate of *p*-nitrophenol generation was determined on spectrophotometer Spectronic (USA) at 405 nm and 25 °C and the PON1 activity was expressed in nmol/min/ml.

DNA analysis

Blood was drawn from the peripheral veins (5-10 ml) and genomic DNA was prepared from leucocytes (minimal amount of leucocytes 3.5×10^9 /l) by sodium dodecylsulphate (SDS) lysis by ammonium acetate extraction and ethanol precipitation (Miller *et al.* 1988).

Table 1. Clinical and laboratory characteristics.

	T1DM	P values (a)	T2DM	Controls	P values (b)
Gender (males/females)	42/44	0.532	114/132	55/45	0.185
Mean age (years)	43±18	0.016	58±18	41±9	0.038
Duration of DM (years)	15±9	0.811	13±8	0	-
BMI (kg/m ²)	23±4	0.021	30±8	22±4	0.039
Systolic BP (mmHg)	120±10	0.656	125±25	115±20	0.586
Diastolic BP (mmHg)	60±20	0.493	70±30	70±15	0.601
Microvascular complications (n)	29	0.041	138	0	-
Macrovascular complications (n)	8	0.012	37	0	-
FPG (mmol/l)	6.50±1.85	0.052	7.71±2.29	4.85±0.56	0.008
HbA1c (%)	6.8±1.2	0.230	6.9±1.6	0	
GFR (MDRD) (ml/s/1.73 m ²)	1.19±0.29	0.219	1.13±0.32	1.42±0.18	0.037
Total cholesterol (mmol/l)	4.90±0.16	0.042	5.12±0.96	4.86±0.21	0.102
HDL-cholesterol (mmol/l)	1.72±0.31	0.216	1.60±0.28	1.75±0.51	0.458
LDL-cholesterol (mmol/l)	3.15±0.55	0.116	3.61±0.78	3.10±0.72	0.257
Triglycerides (mmol/l)	1.29±0.30	0.031	2.01±0.72	1.23±0.42	0.051

Data are shown as account or mean ± SD. FPG means fasting plasma glucose, HbA1c – glycated hemoglobin. GFR – glomerular filtration rate, MDRD – modification of diet in renal disease (used for calculation of GFR). BP – blood pressure. P (a) values are referred to T1DM vs T2DM group. P (b) values are referred to both types of diabetes vs. control group. Statistically significant differences among two groups are marked in bold. Comparison of continuous variables were performed with t-test, the others with χ^2 test.

Table 2. Oligonucleotides used as primers for polymerase chain reaction and restriction endonucleases for restriction analysis.

SNP	sequence of used primer	PCR product	restriction endonuclease
L55M	5'-TTGAGGAAAAGCTCTAGTCCA-3' TTG/ATG 5'-GAAAGACTTAAACTGCCAGTCC-3' 5'-TTGTTGCTGTGGGACCTGAG-3'	384 bp	Hsp92II (CATG)
Q192R	CAA/CGA 5'-AATCCTTCTGCCACCACTCG-3' 5'-AGTTTAATTATGTATTTTCGCGGACCCGCGGGGAGGAG-3'	150 bp	AlwI (GGATC(N)4)
-107 C/T	GCG/GTG 5'-GGGGCTCGTGGAGCTGGCAG-3' 5'-CAATGTGAGGCCAAAGAAGC-3'	289 bp	BsrBI (CCGCTC)
-907G/C	AGA/ACA 5'-CCTTTACCCCTCATTCCCTGAGGTGCCTCTGTACACCCATAT-3'	244 bp	NdeI (CATATG)

Determination of the PON1 polymorphism was achieved by polymerase chain reaction followed by restriction digestion. The nucleotide substitution corresponding to position 55(Met/Leu) and 192(Gln/Arg) creates an Hsp 92II (Biogen-Fermentas) and AlwI (Biogen-Fermentas) restriction site. Polymorphisms in promoter region of PON 1 gene -107C/T and -907 G/C were studied using restriction endonucleases Bsr BI (Biogen-Fermentas) and NdeI (Biogen-Fermentas). Characteristics of polymorphisms studied by restriction analysis are described in Table 2.

The PCR products were digested with

restrictases described above, separated by non-denaturing acrylamide gel (10 %) electrophoresis and visualized by using of ethidium bromide. Individuals homozygous for the L allele showed only the presence of a 384 bp product and those homozygous for the M allele showed 282 and 102 bp products. In Q192R polymorphism individuals homozygous for Q allele had 150 bp and homozygous for the R allele 89 and 61 bp products.

Statistical analysis

Age, BMI and duration of diabetes were compared between studied groups using Student's *t*-test.

Table 3. The occurrence of genotypes in studied polymorphisms among T1, T2 diabetic patients and healthy subjects, serum paraoxonase activities separated according to the genotypes in compared groups.

		LL	LM	MM	QQ	QR	RR	CC	CT	TT	GG	GC	CC
T1DM	n (%)	30 (27)	55 (50)	25 (23)*	56 (65)	27 (31)	3 (4) *	26 (30)	42 (49)	18 (21)	42 (49)	36 (42)	8 (9)
	PON1 act	122±44 [#]	119±40	103±48 [#]	113±32 [#]	115±48	121±33 [#]	115±34	119±40	118±36	113±38	120±30	118±34
T2DM	n (%)	94 (34)	118 (48)	44 (18)*	177 (72)	64 (26)	5 (2)*	71 (29)	123 (50)	52 (21)	117 (48)	104 (42)	25 (9)
	PON1 act	119±34 [#]	114±48	108±39 [#]	106±48 [#]	114±34	118±31 [#]	112±30	114±32	113±39	114±31	118±34	115±38
Controls	n (%)	42 (48)	35 (42)	9 (10)*	32 (29)	54 (49)	24 (22)*	37 (33)	55 (50)	18 (17)	57 (51)	44 (40)	9 (9)
	PON1 act	206±48	205±52	202±48	202±43	204±31	204±29	199±42	205±38	202±46	206±40	200±32	203±38

Data are expressed in mean±SD, n means number of cases, brackets mean genotype frequencies (allele frequencies and differences among them are mentioned in text), PON1 means enzyme activity in nmol/min/ml. * means differences between genotype frequencies MM (L55M) or RR (Q192R) in T1 or T2 DM vs. control subjects with $p \leq 0.05$. # means differences between PON1 activity in LL vs. MM or QQ vs. RR genotype in T1 and T2 DM with $p \leq 0.05$.

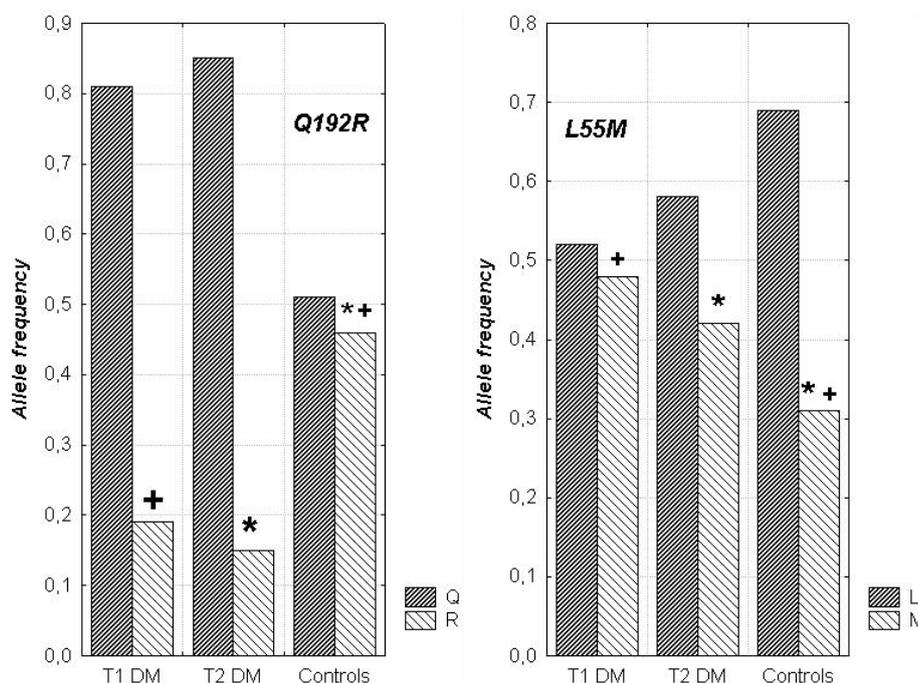


Fig. 1. The frequencies of alleles in L55M and Q192R polymorphisms in diabetic patients and healthy subjects. Statistically significant differences ($p < 0.05$): + between T1DM vs. control subjects; * between T2DM vs. control subjects.

Statistical analyses of frequency counts were performed using the Chi-square (χ^2) test. Comparison of continuous variables (HbA_{1c}) among the PON genotypes was performed by analysis of variance. A logistic regression analysis was performed to evaluate the interaction between the PON1 genotypes and other variables in relation to the prevalence of macro- or microangiopathy. In this analysis, the dependent variable was the presence or absence of vascular complication. Independent variables included in this analysis were BMI, age, present HbA_{1c} level, type of diabetes, duration of diabetes, PON1 activity and PON1 genotype. $P < 0.05$ values were considered as significant. The laboratory data are expressed as means \pm S.D. The analysis was performed

using program Statistica 6.0 (StatSoft).

Results

Serum PON1 activity

Serum PON1 activity was significantly decreased in T1DM (110±68 nmol/min/ml; 95 % CI: 96-120 nmol/min/l) and in T2DM patients (118±69 nmol/min/l; 95 % CI: 111-127 nmol/min/l) compared to the control subjects (203±58 nmol/min/ml; 95 % CI: 190-226 nmol/l/min), both $p < 0.01$. No gender or age influence on its activity was found in diabetic or healthy subjects. The lower serum PON1 activity was found in patients (T1DM and T2DM) with macrovascular (109±71

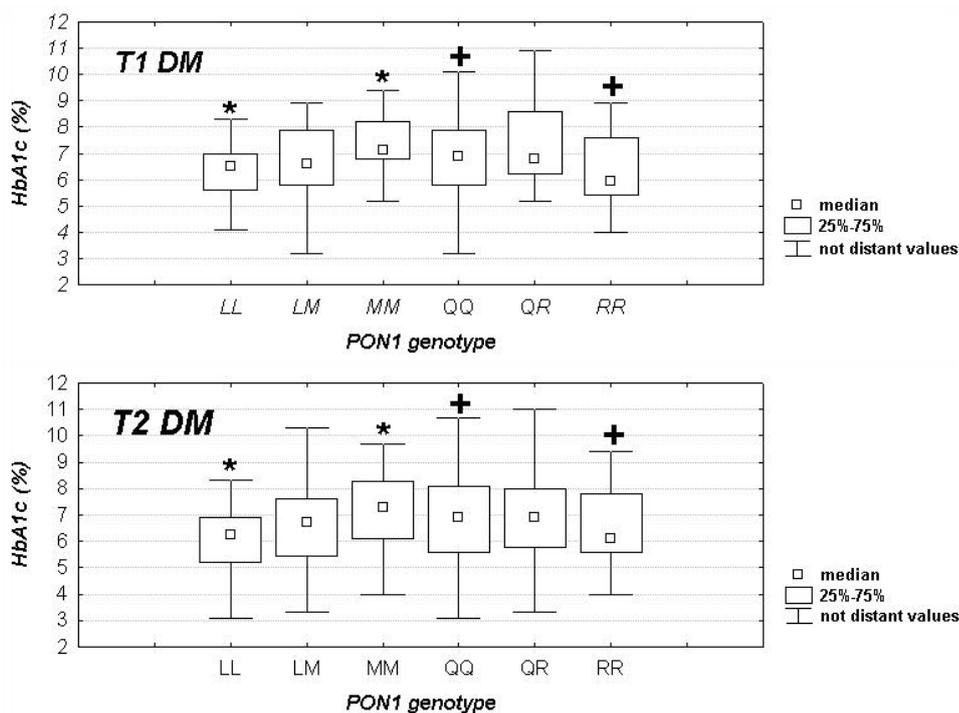


Fig. 2. Box diagram demonstrates glycated hemoglobin values (HbA1c %) in subgroups of Type 1 and Type 2 diabetic patients distinguished according to L55M and Q192R genotypes. Statistically significant differences ($p < 0.05$): * between LL vs. MM genotype in L55M polymorphism; + between QQ vs. RR genotype in Q192R polymorphism.

nmol/min/ml; 95 % CI: 91-113 nmol/min/l) than in those with microvascular complications (119 ± 69 nmol/min/ml; 95 % CI: 108-128 nmol/min/ml, $p < 0.05$).

Table 4. Genotype frequencies of promotor polymorphisms of PON1 gene according to the presence of vascular complications in patients with diabetes mellitus.

Genotype	MA+	MI+	MA-MI-	HbA1c
CC	0.27	0.26	0.24	6.62 ± 1.35
CT	0.49	0.49	0.49	6.74 ± 1.30
TT	0.23	0.24	0.26	6.58 ± 1.12
GG	0.39	0.36	0.38	6.77 ± 1.39
GC	0.46	0.48	0.48	6.58 ± 1.46
CC	0.15	0.16	0.14	6.61 ± 1.32

MA+ indicates the presence of macroangiopathy, MI+ indicates the presence of microangiopathy, MA-MI- group involves patients with no vascular complications. HbA1c is glycated hemoglobin (%) marked as mean \pm SD.

The effect of the PON1 L55M polymorphism on PON1 activity in healthy subjects and diabetic patients

The LL (Leu/Leu) genotype was the most common in healthy subjects followed by the LM (Leu/Met) genotype, whereas the LM was more common than the LL genotype in T1DM and T2DM patients (Table 3). Significant differences between the allele frequencies for the PON1 55 polymorphism were observed in T1DM

as compared to controls (L: 0.52 vs. 0.69, $p < 0.01$; M: 0.48 vs. 0.31, $p < 0.05$) and similarly in T2DM (L: 0.58 vs. 0.69; M: 0.42 vs. 0.31, $p < 0.05$). The 55 gene polymorphism was related to PON1 serum activity. Higher activities were found in LL than in MM genotypes of diabetic patients but not in control subjects (Table 3).

Relationship between the PON1 Q192R polymorphism and PON1 activity in the controls and DM population

The QQ genotype (Gln/Gln) was the most common in both T1DM and T2DM patients, whereas the RR genotype was the rarest one as well as in healthy subjects (Table 3). The allele frequency of the PON1 192 polymorphisms was significantly different in healthy persons compared to T1DM and T2DM patients (Q: 0.54 (controls) vs. 0.81 (T1DM) or 0.85 (T2DM), $p < 0.05$; R: 0.46 (controls) vs. 0.19 (T1DM) or 0.15 (T2DM), $p < 0.05$) (Fig. 1).

In both groups of diabetic patients PON1 activity was the highest in the RR genotype and the lowest in the QQ genotype whereas no differences were found in healthy persons (Table 3).

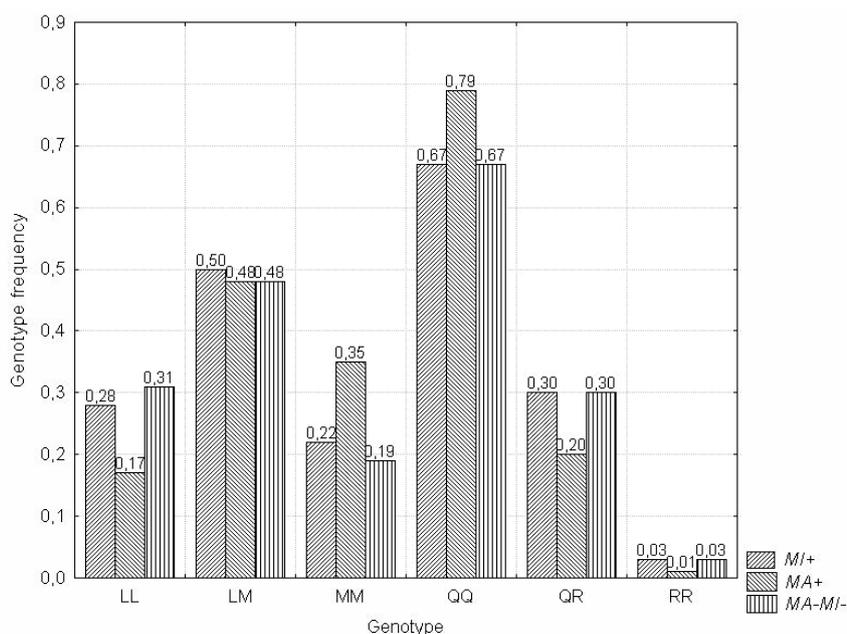
Relationship between the PON1 promotor polymorphisms and PON1 activity in the controls and DM population

We found no statistically significant differences between frequencies in alleles of both promotor SNPs between DM patients and healthy subjects (Table 3). PON1 activity, as well as diabetes control, were not

Table 5. Logistic regression analysis for risk factors of vascular complications in diabetes mellitus.

Variable	p (MA)	OR; 95%CI (MA)	p (MI)	OR; 95%CI (MI)
<i>L55M genotype</i>	0.005	3.11; 1.33-8,86	0.813	0.92; 0.49-1.76
<i>Q192R genotype</i>	0.028	0.51; 0.27-0.98	0.662	0.94; 0.45-1.65
<i>-107 C/T</i>	0.345	0.94; 0.86-1.08	0.565	0.78; 0.56-1.08
<i>-907 G/C</i>	0.357	0.92; 0.76-1.14	0.61	0.90; 0.78-1.12
<i>PON1 activity</i>	0.035	0.48; 0.25-0.84	0.042	0.52; 0.34-0.82
<i>Present HbA1c</i>	0.032	1.35; 1.22-1.57	0.025	1.56; 1.22-1.91
<i>BMI</i>	0.397	0.96; 0.91-1.08	0.452	0.88; 0.77-1.04
<i>Duration of diabetes</i>	0.025	1.91; 1.37-4.12	0.032	2.01; 1.80-5.31
<i>Sex</i>	0.66	0.99; 0.89-1.23	0.83	0.98; 0.88-1.22
<i>Age</i>	0.324	0.93; 0.59-1.34	0.452	0.96; 0.72-1.28
<i>Type of diabetes</i>	0.034	1.96; 1.79-2.65	0.049	1.98; 1.63-2.13

MA in brackets indicates the presence of macroangiopathy, MI in brackets indicates the presence of microangiopathy, OR means odds ratio, 95%CI means confidence interval ($\alpha=0.05$). MA and MI are dependent variables. Genotype, PON activity, HbA1c, duration of diabetes, BMI, sex, age and type of diabetes act as independent variables. Variables significantly associated with macro- or microangiopathy are marked in bold.

**Fig. 3.** Distribution of genotypes in PON1 gene in both types of diabetes mellitus according to presence macro- (MA+) or microangiopathy (MI-) or no complications (MA-MI-). Results are explained in the text.

influenced by polymorphisms in the promoter region (Table 4). Glycated hemoglobin (%) was 6.69 ± 1.34 ; 95 % CI: 6.19-7.02 in C allele carriers vs. 6.61 ± 1.45 ; 95 % CI: 6.29-7.10 in T allele carriers with $p=0.261$ in $-107C/T$ polymorphism and 6.70 ± 1.69 ; 95 % CI: 6.53-7.12 in G allele carriers vs. 6.58 ± 1.55 ; 95 % CI: 6.28-7.07 in C allele carriers with $p=0.326$ in $-907 G/C$ polymorphism.

The association of PON1 polymorphisms and PON1 activity with diabetes control and vascular complications

In T1DM and T2DM patients control of diabetes

expressed by glycated hemoglobin values was poorer in MM genotype (7.10 ± 1.51 ; 95 % CI: 6.42-7.91 in T1DM and 7.29 ± 1.49 ; 95 % CI: 6.70-8.46 in T2DM) than in LL genotype (6.39 ± 1.1 ; 95 % CI: 5.7-7.0 in T1DM and 6.71 ± 1.21 ; 95 % CI: 5.73-6.99) with $p<0.05$ and similarly in QQ genotype (6.9 ± 1.4 ; 95 % CI: 5.53-7.35 in T1DM and 6.9 ± 1.4 ; 95 % CI: 5.58-7.09 in T2DM) than in RR genotype (5.95 ± 1.51 ; 95 % CI: 5.39-7.14 in T1DM and 6.1 ± 1.51 ; 95 % CI: 5.70-7.35 in T2DM) with $p<0.05$. (Fig. 2). The patients with LM and QR genotypes had intermediate diabetes control.

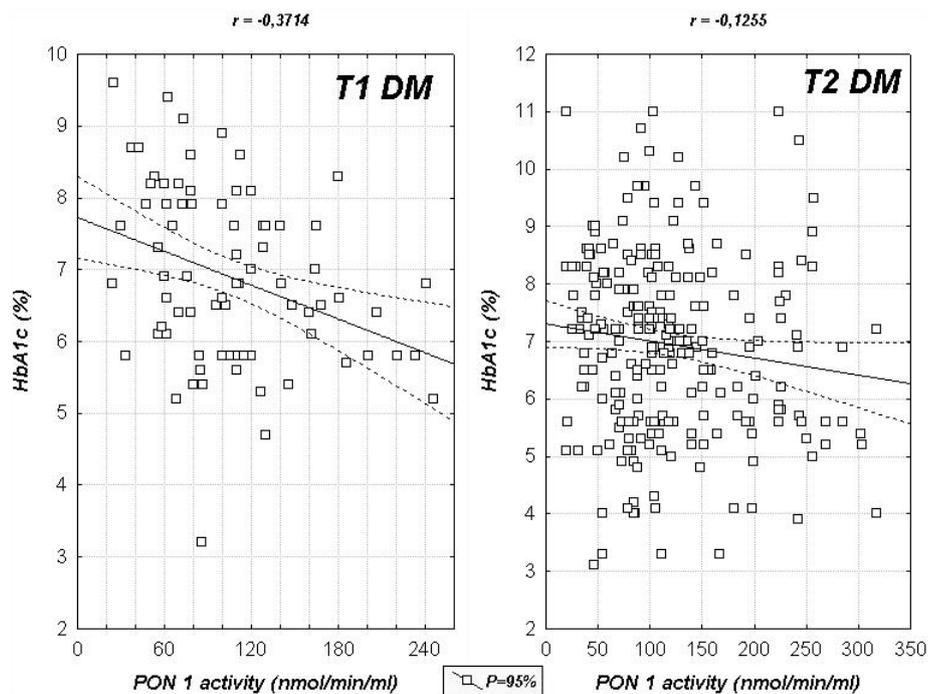


Fig. 4. The correlations between glycated hemoglobin (HbA1c %) and serum paraoxonase activity (PON1) in both types of diabetes mellitus. The correlation coefficients are $r_1 = -0.37$ (T1DM), $r_2 = -0.13$ (T2DM) with $p \leq 0.05$. Dotted lines represent 95 % confidence intervals.

Significantly different genotype frequencies of both SNPs in coding region of gene were found in diabetic patients (T1DM and T2DM) with macroangiopathy (ma+). When compared these with LL vs. (LM and LM) genotypes: OR (odds ratio) 3.07; 95 % CI: 1.55-7.44 with $p < 0.01$ and QQ vs. (QR and RR) genotypes: OR 0.62; 95 % CI: 0.38-0.88 with $p < 0.01$. No differences in genotype frequencies were associated with microangiopathy (mi+). When compared these with LL vs. (LM and MM) genotypes (odds ratio): OR genotypes (odds ratio) 0.92; 95 % CI: 0.58-1.62 with $p = 0.843$ and QQ genotype vs. (QR and RR) genotypes: OR 0.96; 95 % CI: 0.62-1.78 with $p = 0.752$.

Macroangiopathy was associated with significantly higher frequency of M allele (0.59 in ma+ group vs. 0.44 in group without complications, $p \leq 0.01$) and lower frequency of R allele (0.12 in ma+ group vs. 0.19 in group without vascular complications, $p \leq 0.05$) whereas no such distribution was found in microangiopathy (M allele was 0.47 in mi+ group vs. 0.44 in group without complications with $p = 0.218$ and R allele was 0.18 in mi+ group vs. 0.19 in group without complications with $p = 0.542$). Frequencies of genotypes ranged according to presence of vascular complications in both types of diabetes mellitus are showed in Figure 3. We found no significant association of promotor polymorphisms with macro- or microangiopathy (Table 4).

We found also negative correlation between serum paraoxonase activity in both types of diabetes

mellitus and the values of glycated hemoglobin (Fig. 4), as well as the presence of vascular complications in both types of diabetes (Fig. 5).

Association of the PON1 polymorphism in coding and promotor region, BMI, age, duration of diabetes, sex, type of diabetes and PON1 activity as independent variables with the presence of micro- or macroangiopathy as dependent variable was performed using a logistic regression model. This analysis indicated that PON1 L55M and Q192R genotypes are significantly associated with macroangiopathy. Another variables significantly associated ($p \leq 0.05$) with angiopathy were HbA1c, PON1 activity, type and duration of diabetes. No independent contribution has been demonstrated for age, sex and BMI (Table 5).

Discussion

In present study we found significantly different proportion of allele distribution for two coding region but not for promotor sequence of PON1 gene in Type 1 and Type 2 diabetic patients as compared with healthy subjects. Our findings of L55M and Q192R polymorphisms in diabetes are in agreement with previous observation of other authors (Agachan *et al.* 2004). We confirmed that serum PON1 activity is significantly reduced in diabetic patients (Karabina *et al.* 2005). The presence of PON1-55 MM and PON1-192 QQ genotypes was associated with poorer diabetes control than LL and RR genotypes. Finally, macro-

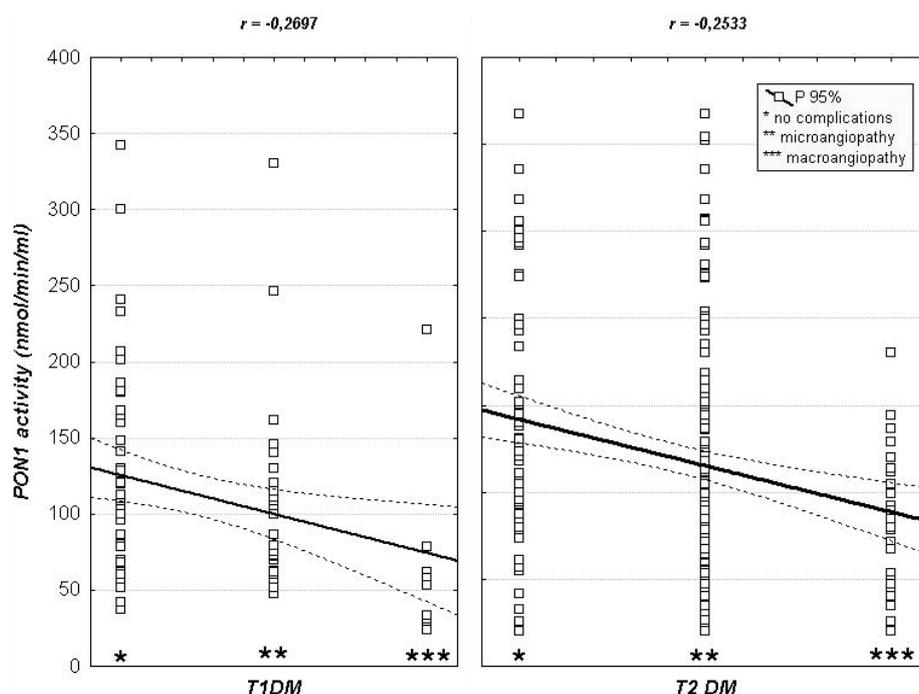


Fig. 5. Cross-correlation between the presence of vascular complications in diabetic patients and the level of serum paraoxonase activity. The correlation coefficients are $r_1 = -0.27$ (T1DM), $r_2 = -0.25$ (T2DM) with $p \leq 0.05$. Dotted lines represent 95 % confidence intervals.

angiopathy was associated with significantly higher frequency of M allele and lower of R allele, whereas no such distribution was found in microangiopathy.

Peroxidation of low density lipoproteins (LDL) plays a central role in atherogenesis (Maritim *et al.* 2003). Enzymes associated with HDL particles, including paraoxonase 1, platelet-activating factor acyltransferase and lecithin-cholesterol acyltransferase (LCAT), can cleave oxidized lipids from LDL. High-density lipoproteins (HDL) diminish the accumulation of lipid peroxides in LDL mainly due to paraoxonase activity. *In vitro* PON 1 protects LDL from the copper-induced generation of oxidation products, particularly LDL-conjugated dienes. Associations between PON-1 gene polymorphisms and cardiovascular disease could be therefore influenced by these enzyme-protective effects *in vivo*.

PON1 gene polymorphisms may influence the variability of enzyme activity and some cross-sectional and case-control studies have described the association between cardiovascular disease or cardiovascular events and PON1 gene polymorphisms in diabetes mellitus and non-diabetic subjects (Fortunato *et al.* 2003). Low PON1 activity decreases the ability to prevent lipid-peroxide formation with consequent acceleration of the oxidative stress. Overproduction of the reactive oxygen species in diabetic patients may be due to chronic hyperglycemia, hyperinsulinemia, elevated free fatty acids (FFA) and dyslipidemia (Maritim *et al.* 2003). Plasma lipids also modifies composition, function and concentration of the

HDL. Elevated plasma triglyceride-rich lipoproteins may substitute cholesterol esters in HDL by driving cholesterol ester transfer protein (CETP) with subsequent HDL depletion of cholesterol esters. As a result, both the conformation and function of HDL may be altered. Glycation of HDL or directly of PON1 in HDL as occurs in diabetes may result in detachment of PON1 itself from the HDL and PON1 inactivation (Ferretti *et al.* 2001, Karabina *et al.* 2005). The low enzyme activity is caused rather by glycation of the PON1 protein than by its reduced synthesis (Hedrick *et al.* 2000). PON1 is bound by HDL in lesser extent in diabetic patients as compared to healthy persons and its activity is then poorly stabilized (Baum *et al.* 2006).

Our results support an idea that lipid protection against oxidation by PON1 may be reduced in diabetic patients because of lower enzyme activity. The association of MM and QQ genotypes in two tested regions with poorer diabetes control and more decreased enzyme activity in macroangiopathy relates to the assumption that L and R carriers might be better protected against atherosclerosis. On the contrary, in some studies the RR genotype was more prevalent in subjects with history of cardiovascular disease than in those without it (Ranade *et al.* 2005, Jarvik *et al.* 2000). Other authors did not find any relationship of the PON1 Q192R polymorphism to cardiovascular disease (Mackness *et al.* 2001, Durrington *et al.* 2001).

The strongest candidate for the natural substrate of PON1 today seems to be one of the modified LDL

phospholipids, an oxidized arachidonic acid derivative (Dantoine *et al.* 2003). Our observation indicates that the activity of PON1, measured by non-physiological substrate paraoxon, may be adequate to predict its antioxidative properties, which may take part in the development of macrovascular complications in diabetes mellitus.

Large differences between ethnic populations are known in the PON1 genotype distribution which may be the reason for differences among studies (Koda *et al.* 2004). The study inconsistency in the association between PON genotypes and diabetes mellitus or cardiovascular disease is partly due to limits of conventional cross-sectional and retrospective case-control studies because selection bias has to be considered.

We conclude that the PON1 192 RR and 55 LL genotypes are associated with higher PON1 activity than

QQ and MM genotypes. This may be more protective to lipid peroxidation. Moreover, higher prevalence of QQ and MM genotypes in diabetes is associated with poorer glucose control and therefore advanced non-enzymatic glycation as well as greater oxidative stress. Genetic background may be at least partly associated with diabetes control and consequently enzyme activities protecting against oxidative stress. Vascular disorders like atherosclerosis are then the results of combined genetic and metabolic changes.

Conflict of Interest

There is no conflict of interest.

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