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Paraoxonase 1 gene polymorphisms and enzyme activities in diabetes mellitus

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Short title: Paraoxonase and diabetes mellitus

Summary

Background:

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.

Methods:

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.

Results:

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, $p<0,01$).

Conclusions:

The association of PON1 polymorphisms, lower PON1 activity and poorer diabetes control found in patients with macroangiopathy further support an idea of genetic factors contributing to development of vascular disorders in diabetes.

Key words: Paraoxonase, genetic polymorphisms, serum activity, diabetic macroangiopathy.

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

M. 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 it 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 littermates (Bradshaw et al. 2005).

PON1 activity was found to be decreased in cardiovascular disease (CVD) (Mackness et al. 2004) and in diabetes mellitus (DM) (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 (Kalousova et al. 2005, Maritim et al. 2003). When HDL was incubated in 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 missence 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 (T1DM) (mean age 43±18 years, 42 males, 44 females), 246 Type 2 (T2DM) 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 suspection 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. 45 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 Tab.1.

Laboratory measurements.

Venous blood samples were drawn after an overnight fast. Plasma (Li-heparine) glucose, 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 cholestrol was calculated according to Friedwalds formula. HbA1c was measured by high-performance liquid chromatography.

Paraoxonase (PON1) activity was determined spectrophotometrically (Furlong et al. 1989). Serum was preincubated with $5x10^{-6}$ mol/l eserine (Sigma- Aldrich) for 10 minutes 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 $CaCl_2$ and 5.5 mmol/l paraoxon (O,O-diethyl-O-*p*-nitrophenylphosphate; 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 was $3,5 . 10^9$ /l) by sodium dodecylsulphate (SDS) lysis by ammonium acetate extraction and ethanol precipitation (Miller et al. 1988).
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 promotor 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 Tab 2.
The PCR products were digested with restrictases described above, separated by nondenaturing 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 150bp 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. 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 with the use of analysis of variance (ANOVA). 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 values <0.05 were considered as significant. The laboratory data are expressed as means ± S.D. The analysis was performed using programme 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 (T1 and T2) with macrovascular (109±71 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).

The effect of the L55M PON 1 polymorphism on PON 1 activity in healthy subjects and diabetic patiens

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 (Tab. 3). Significant differences between the allele and genotype frequencies (Tab.3) for the PON1 55 polymorphism was observed in T1DM as compared to controls (L: 0.69 vs 0.52, p<0.01; M: 0.31 vs. 0.48, p<0.05) and similarly in T2DM (L: 0.58 vs. 0.52; M: 0.42 vs 0.48, 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 (Tab. 3).

Relationship between the PON 1 Q192R polymorphism and PON 1 activity in the controls and DM population.

The QQ genotype (Gln/Gln) was the most common in both T1DM and T2DM patients as well as in healthy subjects whereas the RR genotype was the rarest one (Tab. 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 (T1) or 0.85 (T2), $p<0.05$, R: 0.46 (controls) vs. 0.19 (T1) or 0.15 (T2), $p<0.05$) (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 (Tab. 3.).

Relationship between the PON 1 promotor polymorphisms and PON 1 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 (Tab.3). PON1 activity, as well as diabetes control, were not influenced by polymorphisms in the promotor region (Tab. 4). Glycated haemoglobin (%) 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 PON 1 polymorphisms and PON1 activity with diabetes control and vascular complications.

In T1DM and T2DM patients diabetes control expressed by glycated haemoglobin 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.

Significantly different genotype frequencies of both SNPs in coding region of gene were found in diabetic patiens (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 genotype vs. (LM and MM): OR 95%, OR 0,92; 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≤0,01) and lower frequency of R alelle (0,12 in ma+ group vs. 0,19 in group without vascular complications, p≤0,05) whereas no such distrubution 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 Fig. 3.

We found no statistically significant association of promotor polymorphisms with macro- or microangiopathy (Tab. 4).

We found also negative correlation between serum paraoxonase activity (PON1) in both types of diabetes mellitus and the values of glycated haemoglobin (HbA1c %) (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. (Tab.5)

Discussion

In present study we found significantly different proportion of alelle 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 is 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, macroangiopathy was associated with significantly higher frequency of M allele and lower of R alelle whereas no such distrubution 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 lecitin-cholesterol acyltransferase (LCAT), can cleave oxidised 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 variability of the enzyme activity and some cross-sectional and case-control studies have described an association between cardiovascular disease or cardiovascular events and PON1 gene polymorphisms in diabetes mellitus and non-diabetic subjects (Fortunato et al. 2003). The low PON1 activity decreases 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, hyperinsulinaemia, elevated free fatty acids (FFA) and dyslipidemia (Maritim et al. 2003, Le 2001). Plasma lipids also modifies composition, function and concentration of the HDL. Elevated plasma triglyceride-rich lipoproteins may substitute cholesterolesters (CE) in HDL by driving cholesterol ester transfer protein (CETP) with subsequent HDL depletion of CE. 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 (Karabina et al. 2005). The low enzyme activity is caused rather by glycation of the PON1 protein than by reduced synthesis of its molecules (Hadrick et al. 2000). PON1 is bound by HDL in lesser extent in diabetic patients as compered to healthy persons and its activity is then poorly stabilized (Baum et al. 2005).

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 PON today seems to be one of the modified LDL phospholipids, an oxidised arachidonic acid derivative (Dantoine et al. 2003). Our observation indicates that the activity of PON, 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 DM or cardiovascular disease is partly due to limits of conventional cross-sectional and retrospective case-control studies because selection bias have 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 and 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.

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References

AGACHAN, B., YILMAZ, H., KARAALI, Z. AND ISBIR, T. (2004) Paraoxonase 55 and 192 polymorphism and its relationship to serum paraoxonase activity and serum lipids in Turkish patients with non-insulin dependent diabetes mellitus. *Cell. Biochem. Funct*. **22**, 163–168.

AVIRAM M, HARDAK E, VAYA J, MAHMOOD S, MILO S, HOFFMAN A, et al.Human serum paraoxonase (PON1) Q and R selectively decrease lipid peroxides in human coronary and carotid arteriosclerotic lesions. *Circulation* 2000;**101**:2510–7.

BAUM L, NG HK, WOO KS, TOMLINSON B, RAINER TH, CHEN X, CHEUNG WS, CHAN DK, THOMAS GN, TONG CS, WONG KS. Paraoxonase 1 gene Q192R polymorphism affects stroke and myocardial infarction risk. *Clin Biochem*. 2006 Mar;**39**(3):191-5.

BRADSHAW G, GUTIERREZ A, MIYAKE JH, DAVIS KR, LI AC, GLASS CK, CURTISS LK, DAVIS RA. Facilitated replacement of Kupffer cells expressing a paraoxonase-1 transgene is essential for ameliorating atherosclerosis in mice. *Proc Natl Acad Sci U S A.* 2005 Aug 2;**102**(31):11029-34.

BROPHY VH, JAMPSA RL, CLENDENNING JB, MCKINSTRY LA, JARVIK GP, FURLONG CE. Effects of 50 regulatory-region polymorphisms on paraoxonase- gene (PON1) expression.*AmJHumGenet* 2001;**68**:1428–36.

DANTOINE T, DEBORD J, MERLE L, CHARMES JP.From organophosphate compound toxicity to atherosclerosis: role of paraoxonase 1. Rev Med Interne. 2003 Jul;**24**(7):436-42

DURRINGTON N., MACKNESS B. AND MACKNESS MI. Paraoxonase and atherosclerosis, *Arterioscler. Thromb. Vasc. Biol.* **21** (2001), pp. 473–480.

FERRETTI G, BACCHETTI T, MARCHIONNI C, CALDARELLI L, CURATOLA G. Effect of glycation of high density lipoproteins on their physicochemical properties and on paraoxonase activity.*Acta Diabetol*. 2001 Dec;**38**(4):163-9.

FORTUNATO G, RUBBA P, PANICO S, TRONO D, TINTO N, MAZZACCARA C, DE MICHELEM, IANNUZZI A, VITALE DF, SALVATORE F, SACCHETTI L.:A paraoxonase gene polymorphism, PON 1 (55), as an independent risk factor for increased carotid intima-media thickness in middle-aged women. *Atherosclerosis.* 2003 Mar;**167**(1):141-8.

FURLONG C,RICHTER RJ,SEIDEL SL,COSTA LG,MOTULSKY AG. Spectrophotometric assays for the enzymatic hydrolysis of the active metabolites of chlorpyrifos and parathion by plasma paraoxonase/arylesterase. *Anal Biochem* 1989; **180**: 242-7.

GROSS ER, LADISA JF JR, WEIHRAUCH D, OLSON LE, KRESS TT, HETTRICK DA, PAGELPS, WARLTIER DC, KERSTEN JR. Reactive oxygen species modulate coronary wall shear stress and endothelial function during hyperglycemia. *Am J Physiol Heart Circ Physiol*. 2003 May;**284**(5):H1552-9.

HEDRICK CC, THROPE SR, FU MX, HARPER CM, YOO J, KIM SM.Glycation impairs high-density lipoprotein function.*Diabetologia* 2000;**43**:312–20.

HUMBERT R, ADLER DA, DISTECHE CM, HASSETT C, OMIECINSKI CJ, FURLONG CE. The molecular basis of the human serum paraoxonase polymorphisms. *Nat Genet* 1993;**3**:73–6.

JARVIK G.P., L.S. ROZEK, V.H. BROPHY, T.S. HATSUKAMI, R.J. RICHTER, G.D. SCHELLENBERG AND C.E. FURLONG. Paraoxonase (PON1) phenotype is a better predictor of vascular disease than is $PON1_{192}$ or $PON1_{55}$ genotype, *Arterioscler. Thromb. Vasc. Biol.* **20** (2000), pp. 2441–2447.

KALOUSOVA M, ZIMA T, TESAR V, DUSILOVA-SULKOVA S, SKRHA J. Advanced glycoxidation end products in chronic diseases-clinical chemistry and genetic background. *Mutat Res*. 2005 Nov 11;**579**(1-2):37-46.
KARABINA SA, LEHNER AN, FRANK E, PARTHASARATHY S, SANTANAM N. Oxidative inactivation of paraoxonase--implications in diabetes mellitus and atherosclerosis.Biochim Biophys Acta. 2005 Sep 15;1725(2):213-21.
KINUMI T, OGAWA Y, KIMATA J, SAITO Y, YOSHIDA Y, NIKI E. Proteomic characterization of oxidative dysfunction in human umbilical vein endothelial cells (HUVEC) induced by exposure to oxidized LDL. *Free Radic Res.* 2005 Dec;**39**(12):1335-44.
KODA Y, TACHIDA H, SOEJIMA M, TAKENAKA O, KIMURA H. Population differences in DNA sequence variation and linkage disequilibrium at the PON1 gene. Ann Hum Genet. 2004 Mar;**68**(Pt 2):110-9.
LI B, SEDLACEK M, MANOHARAN I, BOOPATHY R, DUYSEN EG, MASSON P, LOCKRIDGE O. Butyrylcholinesterase, paraoxonase, and albumin esterase, but not carboxylesterase, are present in human plasma. *Biochem Pharmacol*. 2005 Nov 25;**70**(11):1673-84.
MACKNESS B., G.K. DAVIES, W. TURKIE, E. LEE, D.H. ROBERTS, E. HILL, C. ROBERTS, P.N. DURRINGTON AND M.I. MACKNESS. Paraoxonase status in coronary heart disease: are activity and concentration more important than genotype?, *Arterioscler. Thromb. Vasc. Biol.* **21** (2001), pp. 1451–1457.
MACKNESS MI, DURRINGTON PN, MACKNESS B. The role of paraoxonase 1 activity in cardiovascular disease: potential for therapeutic intervention. *Am J Cardiovasc Drugs*. 2004;**4**(4):211-7.
MARITIM AC, SANDERS RA, WATKINS JB 3rd. Diabetes, oxidative stress, and antioxidants: a review. *J Biochem Mol Toxicol*. 2003;**17**(1):24-38.
MILLER SA, DYKES DD, POLESKY HS: Simples salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acid Res.* 1988; **16**: 1215.
RANADE K, KIRCHGESSNER TG, IAKOUBOVA OA, DEVLIN JJ, DELMONTE T, VISHNUPAD P,HUI L, TSUCHIHASHI Z, SACKS FM, SABATINE MS, BRAUNWALD E, WHITE TJ, SHAW PM,D RACOPOLI NC. Evaluation of the paraoxonases as candidate genes for stroke: Gln192Argpolymorphism in the paraoxonase 1 gene is associated with increased risk of stroke. *Stroke*. 2005 Nov; **36**(11):2346-50.
TOMAS M, LATORRE G, SENTI M, MARRUGAT J. The antioxidant function of high density lipoproteins: a new paradigm in atherosclerosis. *Rev Esp Cardiol*. 2004 Jun;**57**(6):557-69.
TWARD A, XIA YR, WANG XP, SHI YS, PARK C, CASTELLANI LW, LUSIS AJ, SHIH DM. Decreased atherosclerotic lesion formation in human serum paraoxonase transgenic mice.*Circulation.* 2002 Jul 23;**106**(4):484-90.

Table legends

Tab.1
Clinical and laboratory characteristics. Data are shown as account or mean ±SD. FPG means

fasting plasma glucose, HbA1c means glycated haemoglobin. GFR means glomerular filtration

rate, MDRD means modification of diet in renal disease (used for calculation of GFR).BP means blood pressure. P (a) values are refered to T1DM vs T2DM group. P (b) values are refered 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.

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

Tab.3

The occurrence of genotypes in studied polymorphisms among T1, T2 diabetic patiens and healthy subjects, serum paraoxonase activities separated according to the genotypes in compared groups. 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 $p \leq 0.05$.

Tab.4

Genotype frequencies of promotor polymorphisms of PON1 gene according to the presence of vascular complications in patients with diabetes mellitus. MA+ means presence of macroangiopathy, MI- means presence of microangiopathy, MA-MI- group involves patients with no vascular complications. HbA1c is glycated haemoglobin (%) marked as mean±SD.

Tab. 5

Logistic regression analysis for risk factors of vascular complications in diabetes mellitus. MA in brackets means presence of macroangiopathy, MI in brackets means presence of microangiopathy, OR means odds ratio, 95%CI means confidence interval (α=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 asssociated with macro- or microangiopathy are marked in table as bold.

Figure legends

Fig. 1

The frequencies of alleles in L55M and Q192R polymorphisms in diabetic patiens and healthy subjects. Statistically significant differences between T1DM vs. control subjects are expressed by + with $p<0,05$, between T2DM vs. control subjects are expressed by * with $p<0.05$.

Fig. 2

Box diagram demonstrates glycated hemoglobin values (HbA1c %) in subgroups of Type 1 and Type 2 diabetic patiens distinquished according to L55M and Q192R genotypes. Statistically significant differences between LL vs. MM genotype in L55M polymorphism are expressed by * with $p<0.05$ and between QQ vs. RR genotype in Q192R polymorhism are expressed by + with $p<0.05$.

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-). Explanation of results is mentioned in the text.

Fig.4

Data correlation between the values of glycated haemoglobin (HbA1c %) and serum paraoxonase activity (PON1) in both types of diabetes mellitus. The correlation coeficients (Spearman) are r1= -0,37 (T1DM), r2= -0,13 (T2DM) with $p \leq 0,05$. Dotted lines mean 95% confidence intervals.

Fig. 5

Cross-correlation between the presence of vascular complications in diabetic patients and the level of serum paraoxonase activity. The correlation coeficients are r1=-0,27 (T1DM), r2= -0,25 (T2DM) with $p \leq 0,05$. Dotted lines mean 95% confidence intervals.

Tables

Tab.1

	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/m2)	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	0,23	6,9±1,6	0	
GFR (MDRD) (ml/s/1,73m ^{2})	1,19±0,29	0,219	1,13±0,32	1,42±0,18	**0,037**
Total cholesterol (mmol/l)	4,9±0,16	**0,042**	5,12±0,96	4,86±0,21	0,102
HDL-C (mmol/l)	1,72±0,31	0,216	1,60±0,28	1,75±0,51	0,458
LDL-C (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

Tab.2

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

Tab.3

		LL	LM	MM	QQ	QR	RR	CC	CT	TT	GG	GC	CC
T1DM	n (%)	23 (27)	43 (50)	19 (22)*	49 (57)	22 (26)	14 (17)*	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 (%)	69 (28)	124 (51)	53 (21)*	156 (63)	59 (24)	31 (13)*	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 (%)	76 (70)	29 (26)	5 (4)*	39 (36)	36 (32)	35 (31)*	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

Tab.4

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

Tab.5

Variable	p (MA)	OR; 95%CI (MA)	p (MI)	OR; 95%CI (MI)
L55M genotype	**0,005**	3.11; 1,33-8,86	0,813	0,92; 0,49-1,76
Q192R genotype	**0,028**	0,51; 0,27-0,98	0,662	0,94; 0.45-1.65
-107 C/T	0,345	0,94; 0,86-1,08	0,565	0,78; 0,56-1,08
- 907 G/C	0,357	0,92; 0,76-1,14	0,61	0,90; 0,78-1,12
PON1 activity	**0,035**	0,48; 0,25-0,84	**0,042**	0,52; 0,34-0,82
Present HbA1c	**0,032**	1,35; 1,22-1,57	**0,025**	1,56; 1,22-1,91
BMI	0,397	0,96; 0,91-1,08	0,452	0,88; 0,77-1,04
Duration of diabetes	**0,025**	1,91; 1,37-4,12	**0,032**	2,01; 1,80-5,31
Sex	0,66	0,99; 0,89-1,23	0,83	0,98; 0,88-1,22
Age	0,324	0,93; 0,59-1,34	0,452	0,96; 0,72-1,28
Type of diabetes	**0,034**	1,96; 1,79-2,65	**0,049**	1,98; 1,63-2,13

Fig. 1

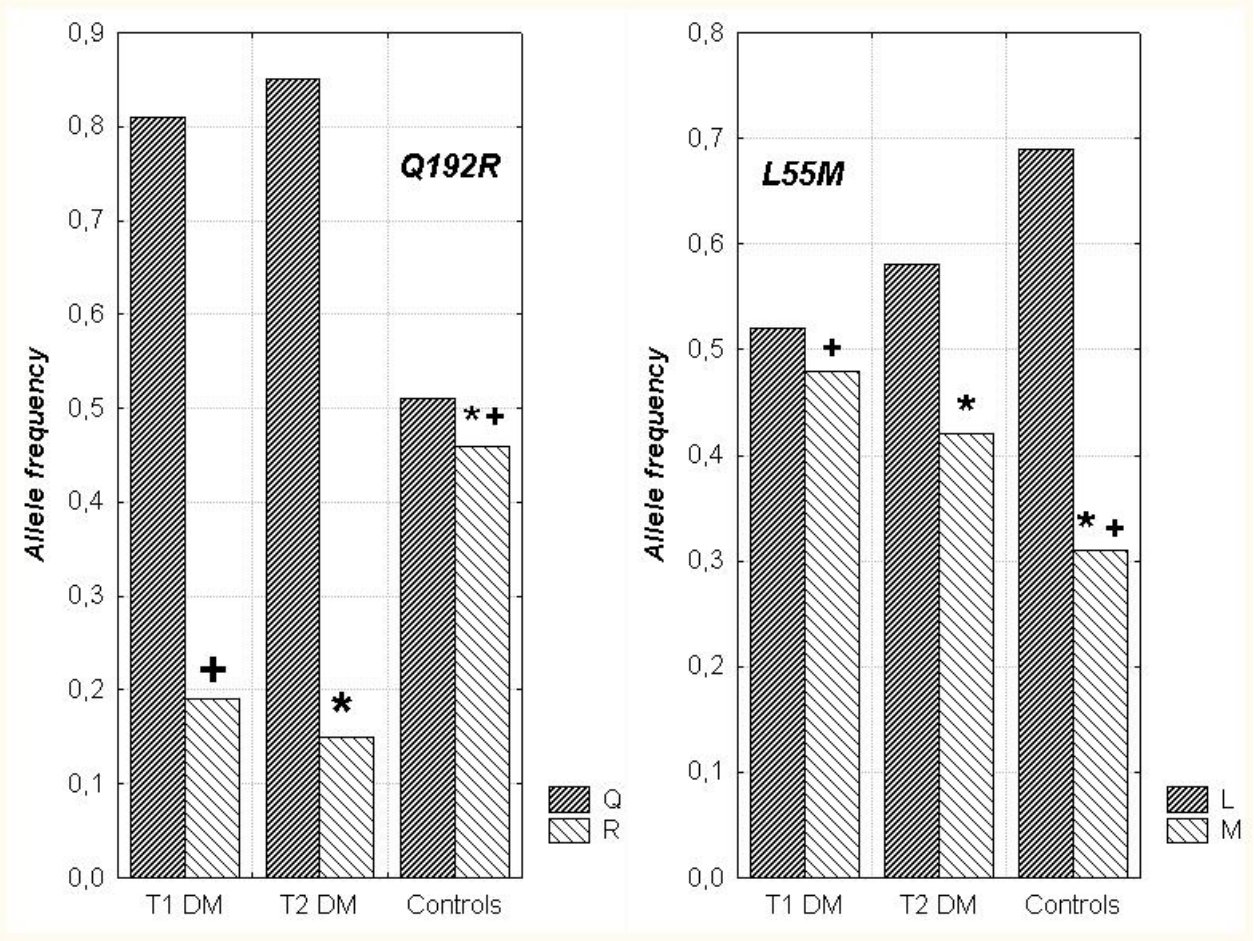

Q192R
Allele frequency
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
T1 DM
T2 DM
Controls
+
*
*+
Q
R
L55M
Allele frequency
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
T1 DM
T2 DM
Controls
+
*
*+
L
M

Fig. 2

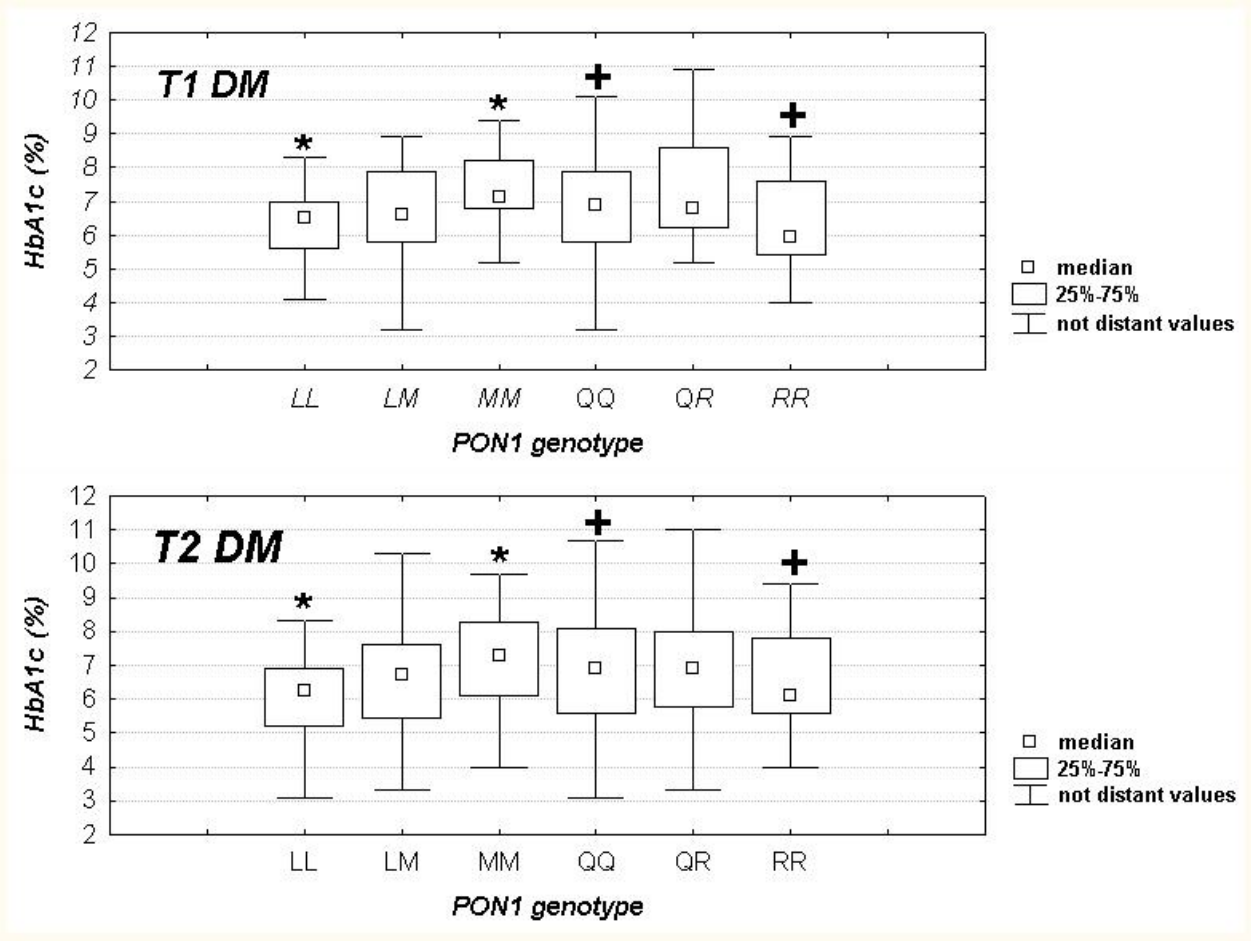

T1 DM
HbA1c (%)
12
11
10
9
8
7
6
5
4
3
2
LL
LM
MM
QQ
QR
RR
PON1 genotype
median
25%-75%
not distant values
T2 DM
HbA1c (%)
12
11
10
9
8
7
6
5
4
3
2
LL
LM
MM
QQ
QR
RR
PON1 genotype
median
25%-75%
not distant values

Fig. 3

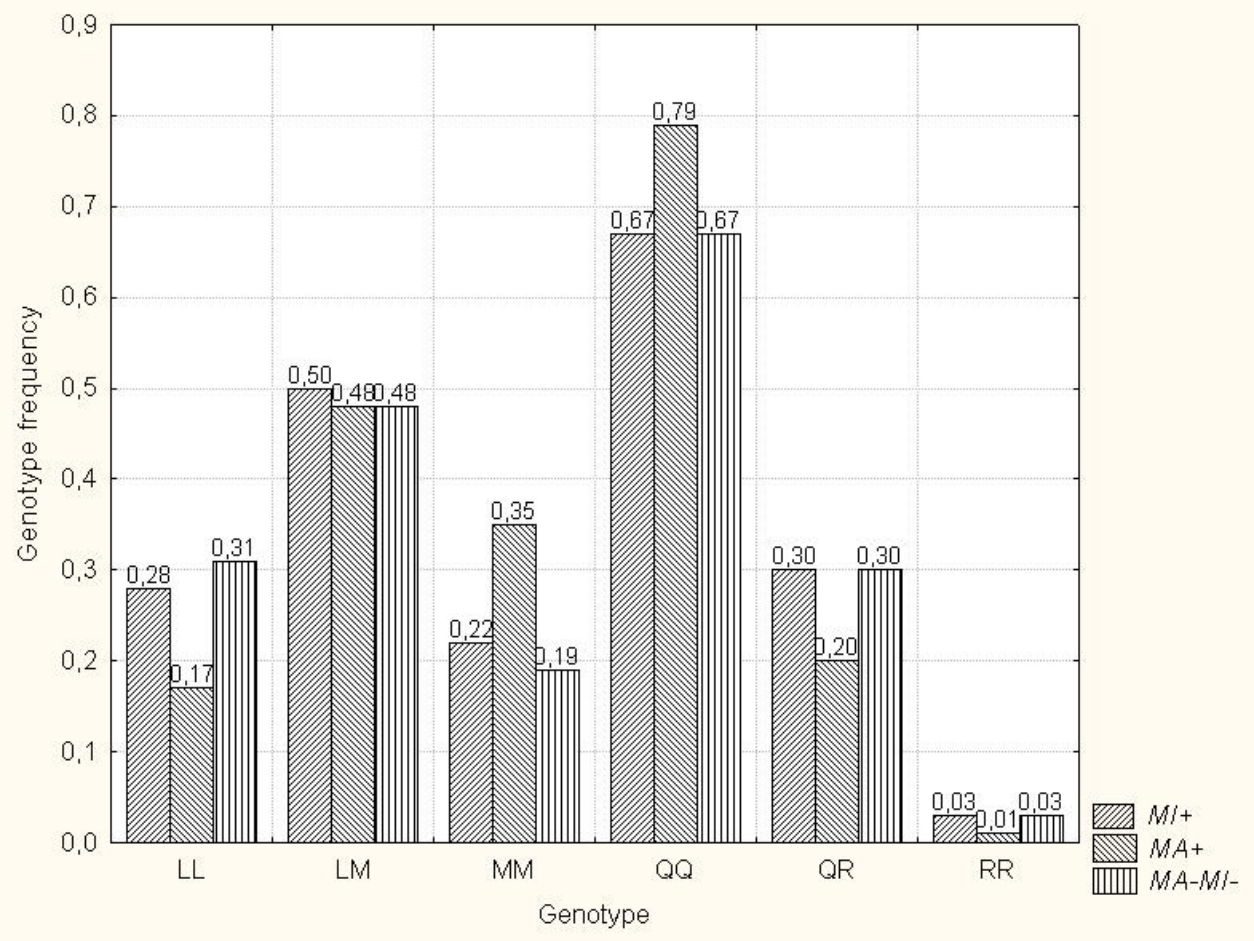

0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
Genotype frequency
0,28
0,17
0,31
0,50
0,48
0,48
0,22
0,35
0,19
0,67
0,79
0,67
0,30
0,20
0,30
0,03
0,01
0,03
LL
LM
MM
QQ
QR
RR
Genotype
MI+
MA+
MA-MI-

Fig. 4

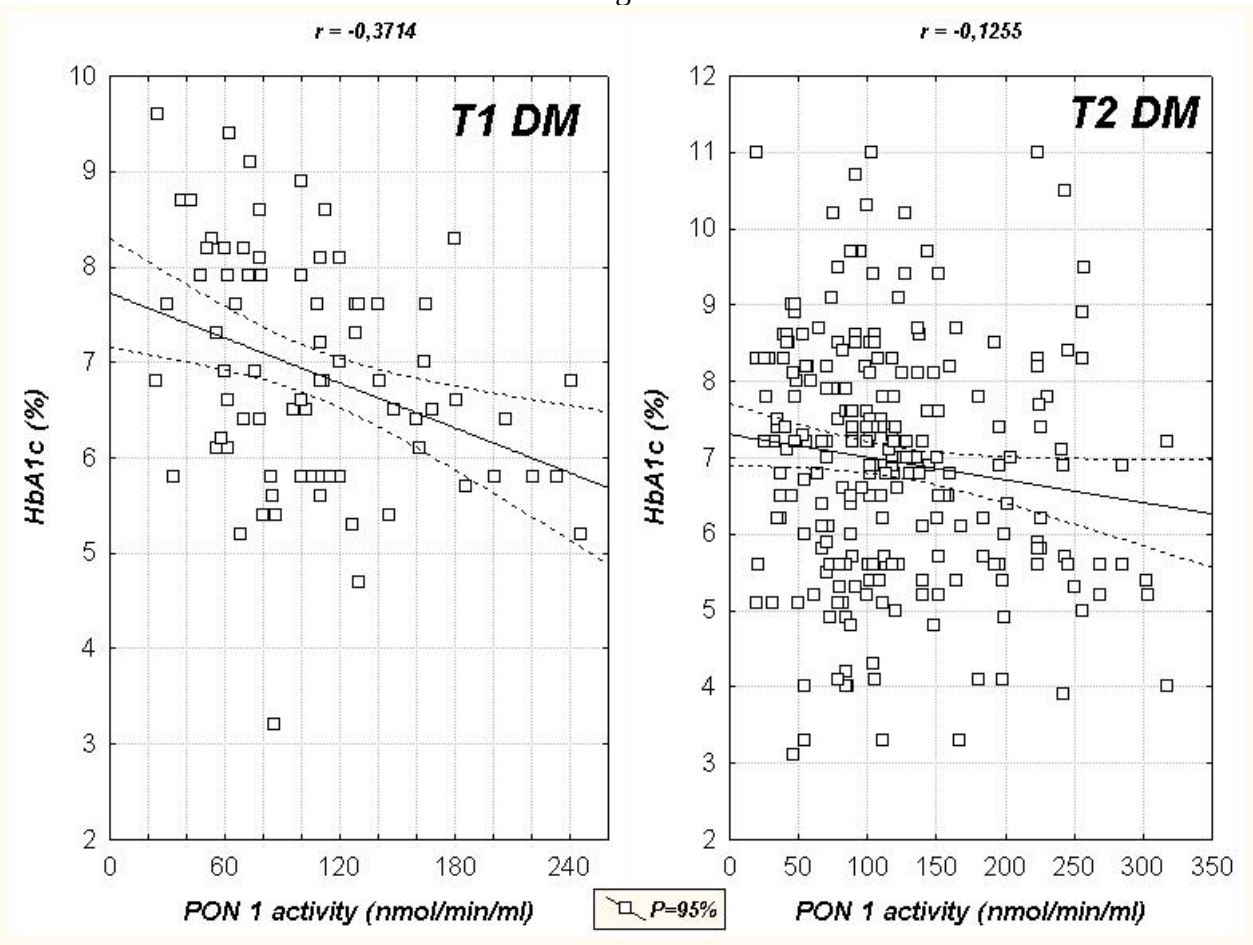
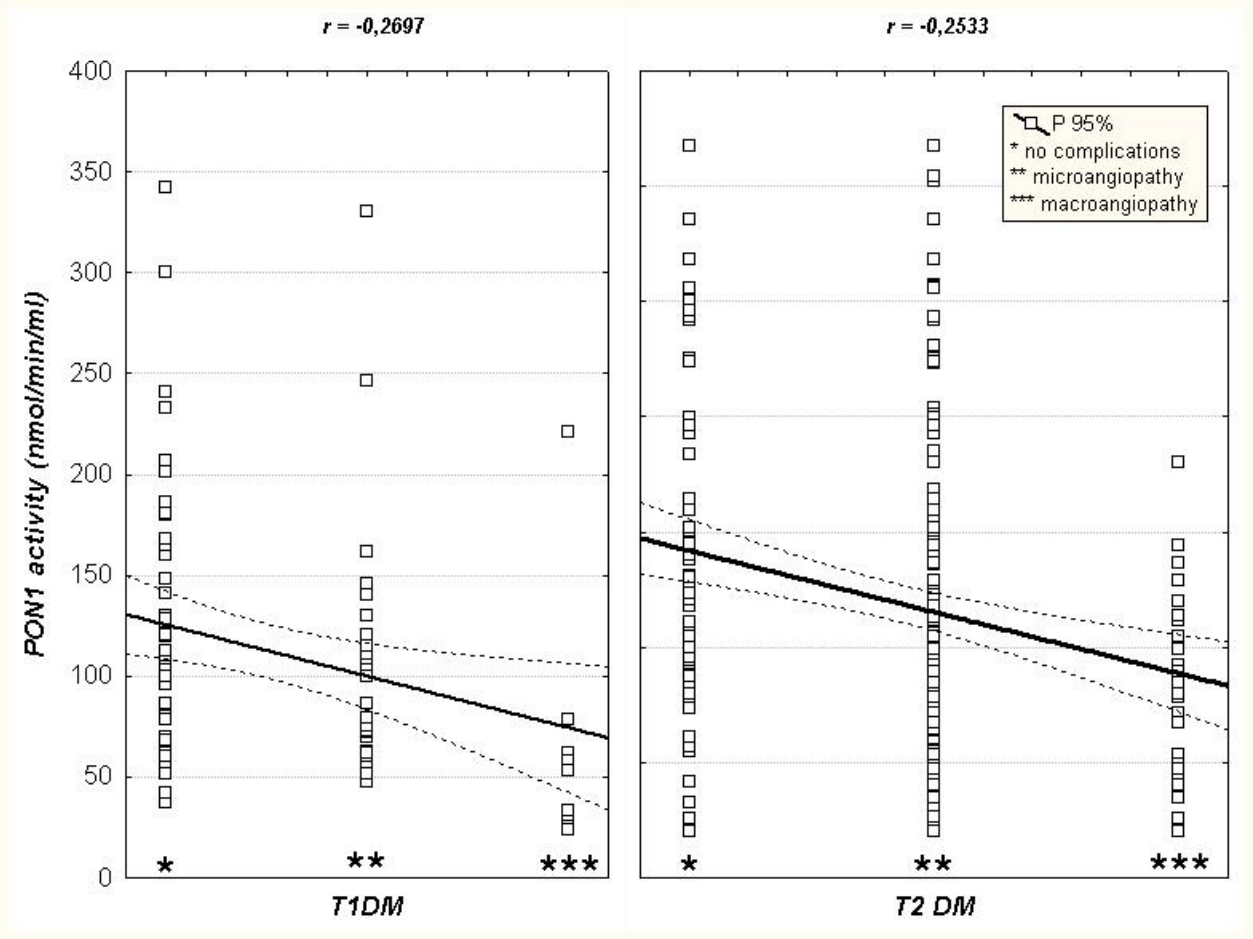

r = -0,3714
T1 DM
HbA1c (%)
PON 1 activity (nmol/min/ml)
r = -0,1255
T2 DM
HbA1c (%)
PON 1 activity (nmol/min/ml)
P=95%

Fig.5


r = -0,2697
r = -0,2533
PON1 activity (nmol/min/ml)
400
350
300
250
200
150
100
50
0
P 95%
* no complications
** microangiopathy
*** macroangiopathy
*
**
***
T1DM
T2 DM