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**Gene polymorphisms of antioxidant
enzymes and diabetes mellitus**

Summary of PhD thesis

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List of abbreviations

ADA	American Diabetes Association
AGE	advanced glycation endproduct
ANOVA	analysis of variance
ATP	adenosine triphosphate
CAT	catalase
CE	cholesterolester
CETP	cholesterolester transfer protein
CI	confidence interval
CT	computer tomography
CuZnSOD	copper-zinc SOD
DM	diabetes mellitus
ECG	electrocardiogram
FFA	free fatty acid
HbA1c	glycated haemoglobin
HDL	high density lipoprotein
HWE	Hardy Weinberg equilibrium
LCAT	lecitin-cholesterol acyltransferase
LDL	low density lipoprotein
MnSOD	manganese SOD
NCBI	National Center for Biotechnology Information
oxLDL	oxidized LDL
p38 MAPK	p38 mitogen activated protein kinase
PCR-RFLP	polymerase chain reaction-restriction fragment length polymorphism
PKC	protein kinase C
PON1	paraoxonase 1
RAGE	receptor for advanced glycation endproduct
ROS	reactive oxygen species
SNP	single nucleotide polymorphism
SOD	superoxide dismutase
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TBE	tris borate EDTA buffer
WHO	World Health Organisation

Introduction

It is a well-established fact that diabetes mellitus is a risk factor for cardiovascular disease [1] which is leading cause of death in diabetic population [2]. It is also well-known fact that tight control of diabetes is effective in reducing vascular complications [3]. One of the principal pathways to develop vascular complications is the production of ROS [4]. There are multiple sources of oxidative stress in diabetes including nonenzymatic and enzymatic pathways [5]. In the process of mitochondrial respiration, molecular oxygen is essential for the complete metabolism of glucose and other substrates during the production of ATP. During the course of normal oxidative phosphorylation between 0.4 and 4% of all oxygen consumed is converted into superoxide. While ROS are generated under physiological conditions [6], excess generation of ROS has pathological consequences [7]. They can stimulate oxidation of LDL, forming ox-LDL, which is not recognized by the LDL receptor leading to foam cell formation [8]. ROS can activate formation of AGE [9], polyol pathway [10], hexosamine pathway and PKC [11], involved in pathogenesis of microvascular and macrovascular complications [12].

There is considerable evidence that hyperglycemia causes many of the major complications of diabetes including macro- and microvascular damage [13,14,15,16,17]. Oxidative stress resulting from increased production of ROS (or their inadequate removal) plays a key role in the pathogenesis of diabetic complications.

In vivo studies reveal that oxidative stress due to hyperglycaemia occurs before vascular complications become clinically evident [18,19,20,21,22,23,24,25,26, 27,28,30,31,32,33]. Hyperglycaemia regulates stress-activated signaling pathways including nuclear

factor- κ B, p38 MAPK, NH₂-terminal Jun kinases/stress-activated protein kinases (JNK/SAPK), AGE/ RAGE, and protein kinase C.

It still have to be elucidated the fact that some patients with DM develop vascular complications but this cannot be seen in the other patients with the same duration of diabetes mellitus or level of disease control [34]. We have focused on genes coding leading antioxidative enzymes superoxide dismutase (SOD), catalase (CAT) and paraoxonase 1 (PON1).

Specifically, we have targeted SNPs for their likely functional role: SOD1 +35A/C (*rs2234694*) which is located adjacent to a splice site (exon3/intron3 boundary), SOD2 Ala16Val (*rs4880*) which has been suggested to alter protein structure [35] and function (C/T substitution in exon 2, codon position 2, aminoacid position 16) and catalase - 21A/T (*rs7943316*) which is located in the promotor region just proximal to the start site. The molecular basis of the PON1 polymorphisms is a missence mutation in the coding region of PON1, resulting in a glutamine (Q)/arginine (R) substitution at codon 192 (*rs662*) [36]. PON1Q192 is more efficient at metabolizing oxidized HDL or LDL than PON1R192 [37]. Polymorphism at position 55 Leu(L)/Met(M) (*rs854560*) has been associated with plasma PON1 protein levels, with PON1M55 being associated with low plasma PON1 level. Polymorphisms in promotor region of PON1 have for the most part not been yet characterized, but may affect splicing activity, message stability or efficiency of polyadenylation.

Superoxide dismutase 1 (CuZnSOD, SOD1, EC 1.15.1.1) is one of the cellular defense systems for oxidative insults [38]. The increase of CuZnSOD expression in human smooth muscle cells protects against oxidant injury. OxLDL cause an increase in the DNA binding

activity of activator protein-1 and nuclear factor κ B, which is inhibited by CuZn-SOD overexpression.

Superoxide dismutase 2 (MnSOD, SOD2, EC 1.15.1.1) is present in mitochondria. C/T substitution (GCT/GTT) has been shown to change the structural conformation of the mitochondrial targeting sequence (MTS) of the enzyme. Associations have been found between the Ala16Val SNP and human neurodegenerative disorders [39].

Catalase (CAT, EC 1.11.1.6) is present in the peroxisomes and exists as a dumbbell-shaped tetramer of four identical subunits. Several SNPs in the CAT gene have been reported, most of which are associated with acatalasaemia [40].

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 [41]. PON1 is synthesized in the liver and is mainly associated with high-density lipoprotein (HDL) [42]. The enzyme decreases accumulation of the lipid peroxides in low-density lipoprotein (LDL) due to its ability to reduce hydroperoxides [43] and it attenuates biological effects of mildly oxidized LDL.

Aims

We have focused on genes encoding leading antioxidative enzymes superoxide dismutase (SOD), catalase (CAT) and paraoxonase 1 (PON1). These enzymes play key role in the pathogenesis of chronic complications of diabetes mellitus. One of the principal pathways to develop vascular complications is the production of ROS. Etiology of the vascular damage in patients with diabetes mellitus is multifactorial with considerable interindividual variability and the influence of the genetic background.

Hypothesis:

- we have considered that escalated oxidative stress relate to the formation and development of chronic vascular changes in patients with diabetes mellitus.
- we have also presumed that activity of ROS is involved in the development of both types of vascular complications in diabetes mellitus.
- we have expected that displacement of the equilibrium among ROS production and removing is caused not only by hyperglycaemia but also by insufficiency of antioxidative defense system. It has been still elucidated which of them is primary event.
- dysfunction of antioxidative enzymes may be caused not only secondarily by the hyperglycaemia (e.g. process of nonenzymatic glycation) but also in primary association with the presence of gene polymorphisms of antioxidative enzymes.

- it has been evidenced that some of studied polymorphisms may influence activity or concentration of the enzymes. We expected that minor SNP or mutations preferable cause alteration of the enzyme than play a protective role.
- we suppose that studied candidate genes for macroangiopathy do not play a major role in the process of microangiopathy.

Aims of research:

- to analyse polymorphisms of candidate genes for chronic vascular complications in patients with diabetes mellitus – genes for PON1, SOD1, SOD2, CAT.
- to study an association between gene polymorphisms of antioxidant enzymes and the presence of macroangiopathy or microangiopathy in patients with diabetes.
- to determine enzyme activity of paraoxonase 1 and superoxide dismutase.
- to investigate gene polymorphism influence on enzyme activity.
- to study the correlation of polymorphisms with some laboratory parameters monitored in patients with diabetes.

Methods

Subjects

Total of 120 T1DM, 306 T2DM and control group of 140 healthy subjects without family history of diabetes were examined in this study. Diagnosis of T1 and T2DM was based on WHO/ADA definition of diabetes (1999), healthy subject didn't fulfill the criteria for this diagnosis. They were in good health and namely free of any co-morbidities often associated with diabetes, especially with T2DM (hypertension, obesity, hyperlipoproteinemia) and other endocrine disorders. 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. 66 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 161 diabetic patients were free of any complications. Clinical and laboratory characteristics are shown in Table 1. The research has been carried out within an ethical framework, informed consents of all participants are documented.

Laboratory measurements

Venous blood samples were drawn after an overnight fast. Plasma 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 cholesterol was calculated according to Friedwalds formula. HbA1c was measured by high-performance liquid chromatography.

Paraoxonase activity was determined spectrophotometrically [44]. Serum was preincubated with 5×10^{-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 μ l 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 at 405 nm and 25°C. PON1 activity was expressed in nmol/min/ml.

Superoxid dismutase activity was determined also spectrophotometrically by xanthine/xanthine oxidase system. The method is based on the reaction described by McCord and Fridovich [45]. SOD activity was expressed in international units (U).

DNA analysis

Blood was extracted from the peripheral blood (5-10 ml) and genomic DNA was prepared from leucocytes (minimal amount of leucocytes was $3,5 \cdot 10^9 / l$) by sodium dodecylsulphate (SDS) lysis by ammonium acetate extraction and ethanol precipitation. Determination of the PON1, SOD1, SOD2 and CAT polymorphisms was achieved by PCR-RFLP analysis. Details are shown in Tab.2. Digested PCR products were visualised by UV transillumination following ethidium bromide staining and migration compared against DNA ladder and a positive RFLP control sample. 3% agarose gel including 0,5 µg/ml ethidium bromide, 10 µl of molecular markers (two different types used simultaneously) and 20 µl of amplicon for the other wells were applied for electrophoresis. 0,5xTBE buffer (pH 8) including 0,5 µl/ml ethidium bromide was used. Running conditions were 100 V, 40 mA and 140 min. Information about all SNPs and SNP ID were obtained from the NCBI homepage and all SNPs have been validated by multiple, independent submissions to the refSNP cluster. The genotyping success rate was 95.0% (range 91.1 to 98.4%). Water control, internal controls and previously genotyped samples were included in each plate to ensure accuracy of genotyping. Positive and negative controls were used in each genotyping assay. To ensure quality control, the genotyping analysis was performed "blind" with respect to case/control status. About 10% of the samples were randomly selected to be genotyped again by a different investigator, who was also unaware of the status of studied subjects. The results were concordant. The polymorphisms were also examined by PCR and RFLP analysis described previously [46,47,48].

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 SOD genotypes was performed with the use of ANOVA. A logistic regression analysis was performed to evaluate the interaction between the 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, SOD activity and genotype. P values <0.05 were considered as significant. The laboratory data are expressed as means \pm SD. The analysis was performed using programme Statistica 6.0 (StatSoft). Testing for deviation from Hardy-Weinberg equilibrium (HWE) was performed and all the observed genotype frequencies were in agreement with HWE.

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 patients

The LL (Leu/Leu) genotype was the most common in healthy subjects followed by the LM (Leu/Met) genotype, whereas the MM 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 $6,69 \pm 1,34$; 95% CI: 6,19-7,02 in C allele carriers vs. $6,61 \pm 1,45$; 95% CI: 6,29-7,10 in T allele carriers with $p = 0,261$ in -107C/T polymorphism and $6,70 \pm 1,69$; 95%CI: 6,53-7,12 in G allele carriers vs. $6,58 \pm 1,55$; 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 ($7,10 \pm 1,51$; 95%CI: 6,42-7,91 in T1DM and $7,29 \pm 1,49$; 95%CI: 6,70-8,46 in T2DM) than in LL genotype ($6,39 \pm 1,1$; 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 patients (T1DM and T2DM) with macroangiopathy (ma+). When compared these with LL vs. (LM and MM) 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, $p \leq 0,01$) 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 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).

SOD activity

Serum SOD activity was significantly decreased in T1DM ($0,75\pm 0,18$ U; 95% CI: 0,72, 0,79) and in T2DM patients ($0,71\pm 0,33$; 95% CI: 0,67, 0,74) compared to the control subjects ($1,67\pm 0,33$, 95% CI: 1,61, 1,72), both $p < 0,01$. Differences between T1DM and T2DM in SOD activity were not found statistically significant ($p=0,14$). No gender or age influence on its activity was found in diabetic patients or healthy subjects. Difference in SOD activity between diabetic patients and healthy subjects is probably accountable not only by genotype background but also by various effects in terms of diabetes, e.g. enzyme glycation. The lower serum SOD activity was found in patients (T1DM and T2DM) with macrovascular complications ($0,51\pm 0,31$ U; 95%CI: 0,43, 0,58) than in those with microvascular complications ($0,74\pm 0,16$ U; 95%CI: 0,71, 0,76), $p < 0,01$ or without any vascular complications ($0,76\pm 0,34$ U; 95%CI: 0,71, 0,81), $p < 0,05$.

The effect of the SOD1 +35A/C polymorphism on SOD activity in healthy subjects and diabetic patients with DM

The AA genotype was the most common observed in the healthy subjects followed by the AC genotype, whereas the AC was more common than the AA genotype in T1DM and T2DM patients (Tab. 3). Significant differences between the allele and genotype frequencies for the SOD1 +35A/C polymorphism was observed in T1DM as

compared to controls (A: 0.69 vs 0.52, $p < 0.01$; C: 0.31 vs. 0.48, $p < 0.05$) and similarly in T2DM (A: 0.58 vs. 0.52; C: 0.42 vs 0.48, $p < 0.05$). This SNP was related to SOD serum activity. Higher activities were found in AA than in CC genotypes of diabetic patients (Tab. 3). Statistical analysis (analysis of variance) showed significant trend towards possible association of AA genotype with higher activity (P (trend) = 0.029). Diabetic and healthy subjects have been pooled together as one group in the study of association between SOD activity and genotypes to improve statistical power of analysis. Differences among these subjects in age, duration of diabetes, presence of other co-morbidities were included.

Relationship between the SOD2 Ala16Val (C/T) polymorphism and SOD activity in healthy subjects and patients with DM

The TT genotype (Val/Val) was the most common in both T1DM and T2DM patients, CT genotype was the most common in healthy subjects whereas the CC genotype (Ala/Ala) was the rarest one in all groups (Tab. 3). The allele frequency of the SOD2 polymorphisms was significantly different in healthy persons compared to T1DM and T2DM patients (T allele (Val): 0.54 (controls) vs. 0.81 (T1) or 0.85 (T2), $p < 0.05$ and C allele (Ala): 0.46 (controls) vs. 0.19 (T1) or 0.15 (T2), $p < 0.05$ (Fig. 6). In all groups of diabetic patients SOD activity was the highest in the CC genotype (Ala/Ala) and the lowest in the TT genotype (Val/Val) (Tab. 3.).

CAT polymorphism in diabetes mellitus

We found no statistically significant differences between frequencies in alleles of CAT SNP between diabetic patients and

healthy subjects ($p=0,294$). Control of diabetes was not influenced by polymorphisms in the CAT gene (Tab. 5).

The association of enzyme activity and polymorphisms in SOD1 and SOD2 with diabetes control and vascular complications of diabetes mellitus

Diabetes control expressed by glycated haemoglobin values was poorer in TT genotype (Val/Val) of SOD2 ($7,10\pm 1,51$; 95%CI: 6,42-7,91 in T1DM and $7,29\pm 1,49$; 95%CI: 6,70-8,46 in T2DM) than in CC genotype (Ala/Ala) of SOD2 ($6,39\pm 1,1$; 95%CI: 5,7-7,0 in T1DM and $6,71\pm 1,21$; 95%CI: 5,73-6,99 in T2DM), $p<0,05$.

No effect of SNP in SOD1 gene on diabetes control was found. Glycated haemoglobin was $6,7\pm 1,4$; 95%CI: 5,41-6,23 in T1DM and $6,9\pm 1,4$; 95%CI: 5,48-6,09 in T2DM, both in AA genotype of SOD1 gene and $6,59\pm 1,22$, 95%CI: 5,08-6,24 in T1DM and $6,61\pm 1,51$; 95%CI: 5,02-6,05 in T2DM, both in CC genotype of SOD1 gene, with $p=0,124$.

Similar findings were made in CAT gene. Glycated haemoglobin was $6,14\pm 1,11$; 95% CI: 6,06-6,57 in T1DM and $6,50\pm 0,85$; 95%CI: 6,32-7,25 in T2DM, both in AA genotype of CAT and $6,19\pm 1,32$, 95% CI: 6,09-6,60 in T1DM and $6,61\pm 0,54$; 95%CI: 6,45-7,05 in T2DM, both in TT genotype of CAT gene, with $p=0,249$.

Significantly different genotype frequencies of SNPs were found in diabetic patients (T1DM and T2DM) with macroangiopathy (MA+) in SOD1 and SOD2 genes. When compared these with CC genotype vs. AC and AA genotypes of SOD1: OR (odds ratio) was 1.73; 95% CI 1.45-5.37 with $p<0,05$, CC genotype (Ala/Ala) vs. CT (Ala/Val) and TT (Val/Val) genotypes of SOD2: OR was 0,62; 95%CI 0,58-

0,90 with $p < 0,01$. When compared AA genotype vs. AT and TT genotypes of CAT: OR was 1.05; 95%CI 0,78-1,13, $p = 0,851$.

Macroangiopathy was associated with significantly higher frequency of C allele in SOD1 gene (0,58 in MA group vs. 0,42 in DM group without complications, $p < 0,01$), lower frequency of C allele (Ala) in SOD2 gene (0,28 in MA group vs. 0,39 in DM group without vascular complications, $p < 0,05$) whereas no such distribution was found in CAT gene, $p = 0,594$.

No differences in genotype frequencies were associated with microangiopathy (MI+). When compared these with CC genotype vs. AC and AA genotypes in SOD1: OR was 0,91; 95%CI 0.74-1.32 with $p = 0.783$, CC genotype (Ala/Ala) vs. CT (Ala/Val) and TT (Val/Val) genotypes in SOD2: OR was 0,96; 95% CI 0.52-1.38 with $p = 0.852$ and AA genotype vs. AT and TT genotypes in CAT: OR 1,04 95%; CI 0,37-1,26 with $p = 0,814$. No statistically significant differences in allele frequencies were found in all SNPs of the studied genes in the patients with microangiopathy when compared with patients without vascular complications (C allele in SOD1 was 0,47 in MI group vs. 0,42 in DM group without complications, $p = 0,118$, T allele in SOD2 was 0,35 in MI group vs. 0,39 in DM group without complications, $p = 0,242$). Frequencies of genotypes ranged according to presence of vascular complications in both types of diabetes mellitus are showed in Figure 6.

We found negative correlation between serum superoxide dismutase activity (SOD) in both types of diabetes mellitus and the values of glycated haemoglobin (HbA1c %) (Fig. 7), as well as the presence of vascular complications in both types of diabetes (Fig.8).

Association of the PON1 polymorphism in coding and promotor region, SOD1, SOD2 and CAT polymorphisms, BMI, age, duration of

diabetes, sex, type of diabetes, PON1 and SOD 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, Q192R, SOD1 and SOD2 genotypes are significantly associated with macroangiopathy. Another variables significantly associated ($p < 0,05$) with angiopathy were HbA1c, PON1 activity, SOD activity and duration of diabetes. No independent contribution has been demonstrated for age, sex, BMI and the type of diabetes (Tab. 6).

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 observations of other authors [49]. We confirmed that serum PON1 activity is significantly reduced in diabetic patients [50]. 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 allele whereas no such distribution was found in microangiopathy.

Peroxidation of low density lipoproteins (LDL) plays a central role in atherogenesis [51]. Enzymes associated with HDL particles, including paraoxonase 1, platelet-activating factor acyltransferase and lecithin-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 [52]. 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 [51]. Plasma lipids also modifies composition, function and concentration of the HDL. Elevated plasma triglyceride-rich lipoproteins may substitute cholesteroesters (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 [50]. The low enzyme activity is caused rather by glycation of the PON1 protein than by reduced synthesis of its molecules [53]. PON1 is bound by HDL in lesser extent in diabetic patients as compared to healthy persons and its activity is then poorly stabilized [54]

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 [55,56]. Other authors did not find any relationship of PON1 Q192R polymorphism to cardiovascular disease [57,58].

The strongest candidate for the natural substrate of PON seems to be one of the modified LDL phospholipids, an oxidised arachidonic acid derivative [59]. Previous observations indicate 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 [60].

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.

Our findings in SOD2 gene are in agreement with previous observations of other authors [61]. We also confirmed known fact that serum SOD activity is significantly reduced in patients with DM [62]. The presence of TT (Val/Val) genotype in SOD2 gene was associated with poorer diabetes control in comparison with CT (Ala/Val) and CC (Ala/Ala) genotypes. Macroangiopathy was associated with significantly lower frequency of C (Ala) allele of Ala16Val SNP of SOD2 gene. This has not been confirmed by another study focused on the role of antioxidative enzymes (including SOD2) in determining genetic susceptibility to the coronary artery disease in patients with T2DM [63]. Other studies suggest that Ala16Val SNP of SOD2 gene is not related to pathogenesis of diabetes but is associated with microangiopathy expressed as

microalbuminuria [64] or macular edema in patients with T2DM [65]. No such distribution was found in microangiopathy in our study. Finally, we found negative correlation between SOD activity in both types of diabetes and level of control (expressed by glycated haemoglobin) and presence of microangiopathy or macroangiopathy.

SNP in the signal sequence of SOD2 (Ala16Val) appears to be a minor determinant of carotid atherosclerosis [40]. The Ala type of SOD2 might have a common alpha-helical structure while the Val type might change its conformation to beta-sheet [66]. The Val variant of the SOD2 might be present at a lower concentration in the mitochondria. The processing study of these 2 leader signals has suggested that the basal level of the SOD2 activity might be the highest for Ala/Ala genotype (C/C) [66]. Observed positive association of macroangiopathy and high levels of glycated haemoglobin with the SOD2-Val/Val genotype could be explained, at least in part, by the Val isoform of the SOD2. It may lead to decrease resistance against ROS produced in the mitochondria and to oxidative damage of proteins [67]. The Ala allele of the SOD2 gene is more widespread than the Val allele in Caucasian population in contrast to Asian populations.

Our results are indicative of potential effect of A/C SNP in SOD1 gene on enzyme activity. It is known that deficiency in SOD1 results in increased levels of vascular superoxide and peroxynitrite and impaired endothelium-dependent relaxation in both large arteries and microvessels [68] and caused hypertrophy of arteries [69]. Macroangiopathy was associated with significantly higher frequency of C allele of +35 A/C SNP of SOD1 gene whereas no such distribution was found in microangiopathy.

We found no statistically significant differences in distribution of CAT alleles in studied SNP and no impact of this SNP on the presence of vascular complications and the level of glycated haemoglobin. Hypocatalasaemic patients were found to have higher plasma levels of homocysteine and lower levels of folate [40], suggesting these patients are at greater risk for cardiovascular diseases. SNPs in the catalase promoter have been identified in a Swedish population [70] but their relationship to the vascular disease risk has not been determined. A variant within the catalase promoter region has been associated with essential arterial hypertension in an isolated Chinese population [71]. T1DM susceptibility locus on the chromosome 11p13 near to the catalase gene supports the idea of CAT gene may play a role in DM [72]. On the other side another authors found no evidence for a major effect of CAT SNPs on T1DM susceptibility in two large sample collections [73].

The study inconsistency in the association between genotypes and DM or cardiovascular disease is partly due to the limits of cross-sectional case-control studies because selection bias have to be considered and the statistical analysis might have failed to demonstrate any significant differences.

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.

Conclusions

- enzyme activity of PON1 was significantly decreased in patients with both types of diabetes mellitus in comparison with control subjects.
- allele and genotype frequency of L55M a Q192R in the coding region of PON1 gene differed significantly between patients with diabetes mellitus and healthy subjects.
- we found no such distribution of allele and genotype frequency of polymorphisms in the promotor region of PON1 gene in both compared groups (diabetic and nondiabetic).
- patients with both types of diabetes had better glycaemic control of the disease (expressed by glycated haemoglobin) in the presence of LL genotype in L55M SNP and RR genotype in Q192R SNP of PON1 gene.
- the presence of macroangiopathy was significantly associated with higher frequency of M allele and lower frequency of R allele.
- no such distribution has been found in the promoter region of PON1 gene according to the presence of macroangiopathy nor level of glycaemic control of the disease (expressed by glycated haemoglobin).
- we found no association between microangiopathy and studied polymorphisms both in coding and promoter region of PON1 gene.
- enzyme activity of PON1 correlated negatively with the values of glycated haemoglobin in patients with diabetes mellitus.
- enzyme activity of PON1 was significantly decreased in patients with vascular complications of diabetes mellitus.

- enzyme activity of superoxide dismutase has been found significantly lower in patients with diabetes mellitus in comparison with healthy subjects. Moreover, patients with macroangiopathy had SOD activity lower than those with microangiopathy.
- allele and genotype frequency differed statistically significantly when compared patients with both types of diabetes mellitus with control subjects in +35A/C SNP in SOD1 gene and Ala16Val in SOD2 gene. No such difference has been found in -21A/T in CAT gene.
- patients with both types of diabetes mellitus have better long-term glycaemic control of the disease (expressed by glycated haemoglobin) in the presence of CC (Ala/Ala) genotype in SOD2 gene.
- no such distribution has been found in the studied SNPs of SOD1 and CAT genes according to the level of glycaemic control of the disease (expressed by glycated haemoglobin).
- the presence of macroangiopathy was significantly associated with higher frequency of C allele of +35A/C polymorphism in SOD1 gene and lower frequency of C(Ala) allele of Val16Ala polymorphism in SOD2 gene. No such distribution has been found in the CAT gene according to the presence of macroangiopathy.
- we found no association between microangiopathy and studied polymorphisms in SOD1, SOD2 and CAT.
- enzyme activity of SOD correlated negatively with the values of glycated haemoglobin in patients with diabetes mellitus. SOD activity was significantly decreased in patients with vascular complications of diabetes mellitus.

Tables

Table 1 Clinical and laboratory characteristics.

	T1DM	P values (a)	T2DM	Controls	P values (b)
Gender (males/females)	58/62	0,602	156/150	93/87	0,198
Mean age (years)	40±12	0,022	57±15	39±9	0,043
Duration of DM (years)	18±9	0,853	17±8	0	-
BMI (kg/m²)	22±4	0,03	30±5	21±5	0,027
Systolic BP (mmHg)	120±10	0,748	125±25	120±20	0,676
Diastolic BP (mmHg)	60±20	0,521	70±30	70±15	0,621
Microvascular complications(n)	40	0,048	159	0	-
Macrovascular complications(n)	14	0,016	52	0	-
FPG (mmol/l)	6,60±1,35	0,058	7,82±2,29	4,95±0,76	0,009
HbA1c (%)	6,1±1,9	0,321	6,7±1,8	0	-
GFR (MDRD) (ml/s/1,73m²)	1,23±0,35	0,179	1,09±0,28	1,35±0,22	0,041
Total cholesterol (mmol/l)	4,8±0,5	0,042	5,3±0,7	4,8±0,3	0,205
HDL-C (mmol/l)	1,55±0,35	0,216	1,31±0,32	1,75±0,66	0,325
LDL-C (mmol/l)	3,22±0,53	0,116	3,57±0,81	3,10±0,74	0,234
Triglycerides (mmol/l)	1,31±0,30	0,031	1,99±0,79	1,23±0,48	0,038

Table 2 Sequences of primers and used restrictases.

SNP	sequence of used primers	restrictase	restriction fragments
SOD1 35 A/C	5'CTATCCAGAAA ACACGGTGGGC C 3'	HhaI	C allele 71bp and 207 bp A allele 278 bp
	5'TCTATATTCAA TCAAATGCTACA AAAC3'		
SOD2 A16V (C/T)	5'GCTGTGCTTTC TCGTCTTCAG 3'	BsaWI	C allele 267 bp T allele 183bp and 84bp
	5'TGGTACTTCTC CTCGGTGACG3'		
CAT -21 A/T	5'- AATCAGAAGGCA GTCCTCCC-3'	HinfI	A allele 203 bp and 47bp T allele 250bp
	5'- TCGGGGAGCAC AGAGTGAC-3'		
PON1 L55M	5'- TTGAGGAAAAGC TCTAGTCCA-3'	Hsp92II	L allele 384 bp M allele 282 bp and 102 bp

	5'- GAAAGACTTAAA CTGCCAGTCC-3'		
PON1 Q192R	5'- TTGTTGCTGTGG GACCTGAG-3'	AlwI	Q allele 150 bp R allele 89 bp and 61 bp
	5'- AATCCTTCTGCC ACCACTCG-3'		
PON1 -107 C/T	5'- AGTTTAATTATGT ATTTTCGCGGAC CCGGCAGGGGAG GAG-3'	BsrBI	C allele 210 bp T allele 95 bp and 115 bp
	5'- GGGGCTCGTGG AGCTGGCAG-3'		
PON1 -907 G/C	5'- CAATGTGAGGCC AAAGAAGC-3'	NdeI	G allele 314 bp C allele 84 bp and 230 bp
	5'- CCTTACCCTC ATTCCCTGAGGT GCCTCTGTACAC CCATAT-3'		

Table 3 The occurrence of genotypes in studied polymorphisms.

		LL	LM	MM	QQ	QR	RR
T1DM	n (%)	23 (27)	43 (50)	19 (22)*	49 (57)	22 (26)	14 (17)*
	PON1 act	122±44¶	119±40	103±48¶	113±32¶	115±48	121±33¶
T2DM	n (%)	69 (28)	124 (51)	53 (21)*	156 (63)	59 (24)	31 (13)*
	PON1 act	119±34¶	114±48	108±39¶	106±48¶	114±34	118±31¶
Controls	n (%)	76 (70)	29 (26)	5 (4)*	39 (36)	36 (32)	35 (31)*
	PON1 act	206±48	205±52	202±48	202±43	204±31	204±29
		CC	CT	TT	GG	GC	CC
T1DM	n (%)	26 (30)	42 (49)	18 (21)	42 (49)	36 (42)	8 (9)
	PON1 act	115±34	119±40	118±36	113±38	120±30	118±34
T2DM	n (%)	71 (29)	123 (50)	52 (21)	117 (48)	104 (42)	25 (9)
	PON1 act	112±30	114±32	113±39	114±31	118±34	115±38
Controls	n (%)	37 (33)	55 (50)	18 (17)	57 (51)	44 (40)	9 (9)
	PON1 act	199±42	205±38	202±46	206±40	200±32	203±38

		AA	AC	CC	TT (Val/Val)	CT (Ala/Val)	CC (Ala/Ala)
T1DM	n (%)	58 (48)	50 (42)	12 (10)	79 (66)	36 (30)	5 (4)
	SOD act (U)	0,76±0,1 5	0,74±0,1 3	0,73±0,1 9	0,73±0,1 4	0,74±0,2 3	0,77±0,1 8
T2DM	n (%)	104 (34)	147(48)	55(18)	220(72)	80(26)	6 (2)
	SOD act (U)	0,73±0,2 9	0,72±0,3 6	0,70±0,3 1	0,72±0,2 8	0,72±0,1 9	0,75±0,1 5
Controls	n (%)	49 (27)	90 (50)	41(23)	52(29)	90 (50)	38(21)
	SOD act (U)	1,66±0,3 1	1,67±0,3 5	1,66±0,2 8	1,58±0,2 8	1,60±0,3 5	1,63±0,0 8
		AA	AT	TT			
T1DM	n (%)	48(40)	53 (44)	19(16)			
T2DM	n (%)	110(36)	147(48)	49(16)			
Controls	n (%)	67(37)	86(48)	27 (15)			

The occurrence of genotypes in studied polymorphisms among T1, T2 diabetic patients and healthy subjects (controls), serum superoxide dismutase activity separated according to the genotypes in compared groups (AA/AC/CC in SOD1 gene, TT/CT/CC in SOD2 gene, AA/AT/TT in CAT gene). 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 act, resp. *SOD act* means enzyme activity in nmol/min/ml, resp. U.* 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 $p \leq 0.05$.

Table 4 Genotype frequencies of promotor polymorphisms of PON1 gene.

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

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.

Table 5 Genotype frequencies in CAT gene according to presence of vascular complications and values of glycated haemoglobin according to the genotype.

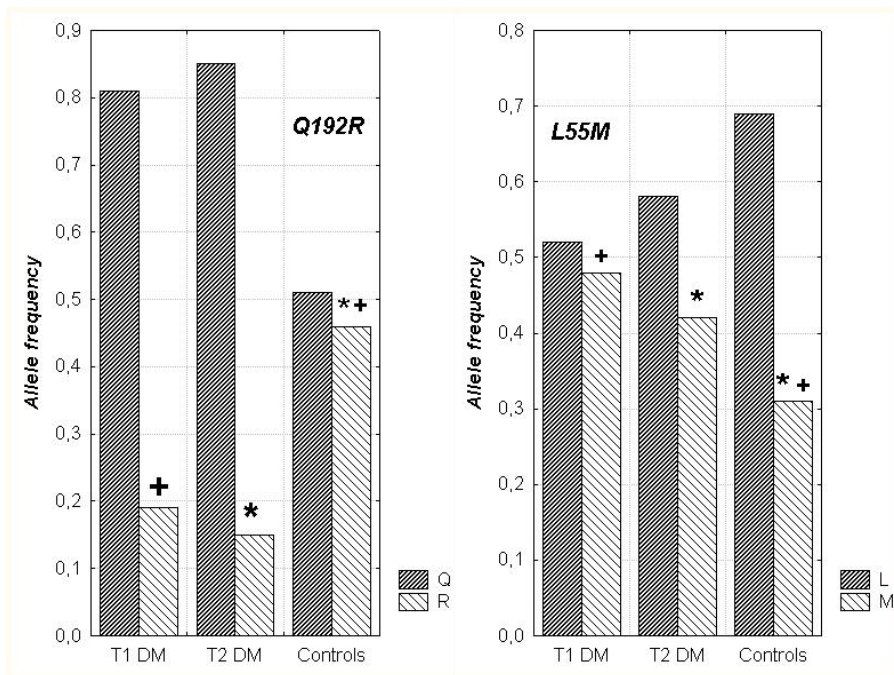
Genotype	MA+	MI+	MA-MI-	HbA1c
AA	0,28	0,32	0,34	6,28±1,25
AT	0,50	0,49	0,49	6,35±1,10
TT	0,22	0,19	0,17	6,32±1,22

Table 6

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
SOD1 35 A/C	0,048	1,73; 1,45-5,37	0,783	0,91; 0,74-1,32
SOD2 A16V	0,009	0,62; 0,58-0,90	0,852	0,96; 0,52-1,38
CAT -21 A/T	0,851	1,05; 0,78-1,13	0,814	1,04; 0,37-1,26
SOD activity	0,040	0,48; 0,25-0,9	0,048	0,62; 0,44-0,91
Type of diabetes	0,084	0,96; 0,89-1,65	0,079	0,88; 0,73-1,17
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

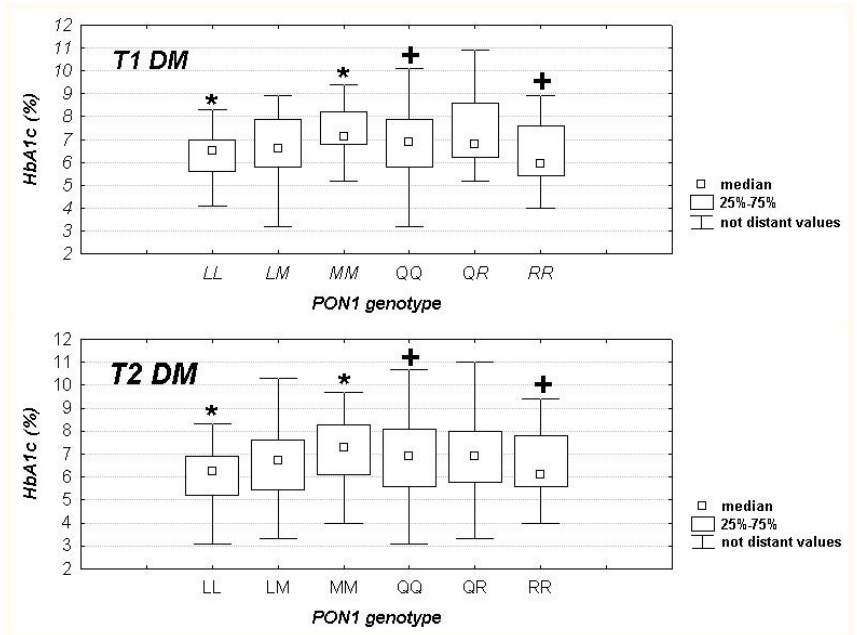
Figures

Figure 1



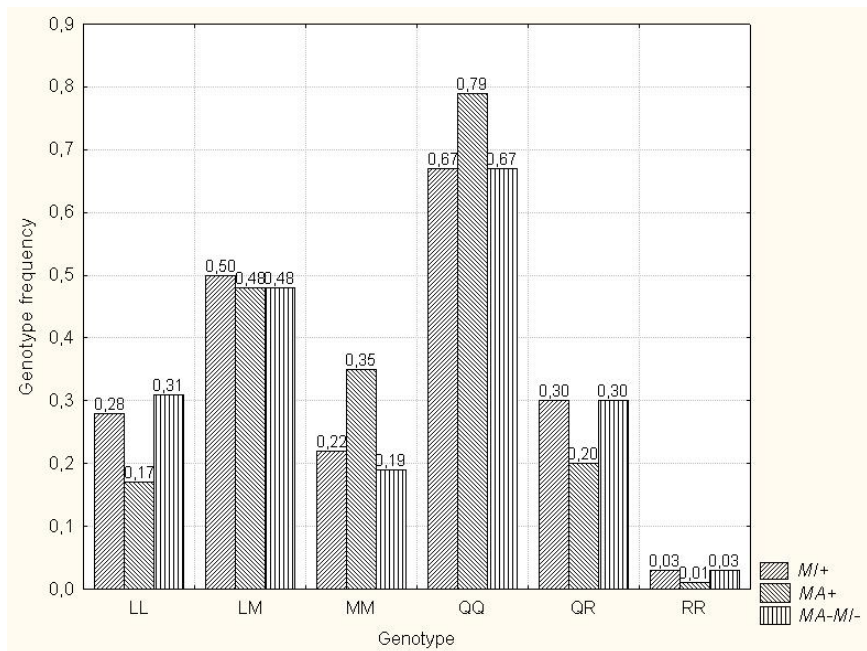
The frequencies of alleles in L55M and Q192R polymorphisms in diabetic patients and healthy subjects. Statistically significant differences between T1DM vs. control subjects are expressed by + with $p < 0,05$, between T2DM vs. control subjects are expressed by * with $p < 0.05$.

Figure 2



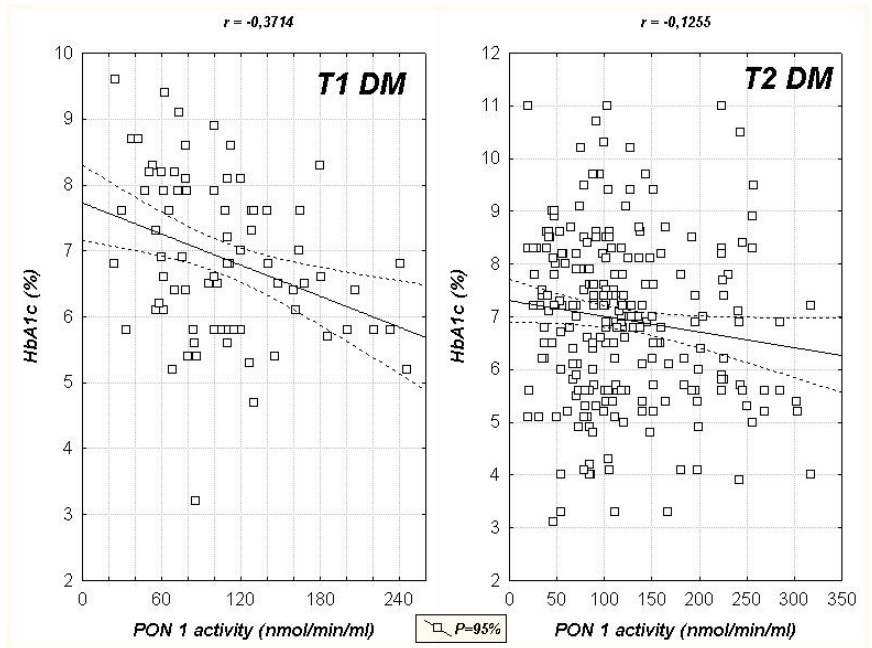
Box diagram demonstrates glyated hemoglobin values (HbA1c %) in subgroups of Type 1 and Type 2 diabetic patients distinguished according to L55M and Q192R genotypes. Statistically significant differences between LL vs. MM genotype in L55M polymorphism are expressed by * with $p < 0.05$ and between QQ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

Figure 3



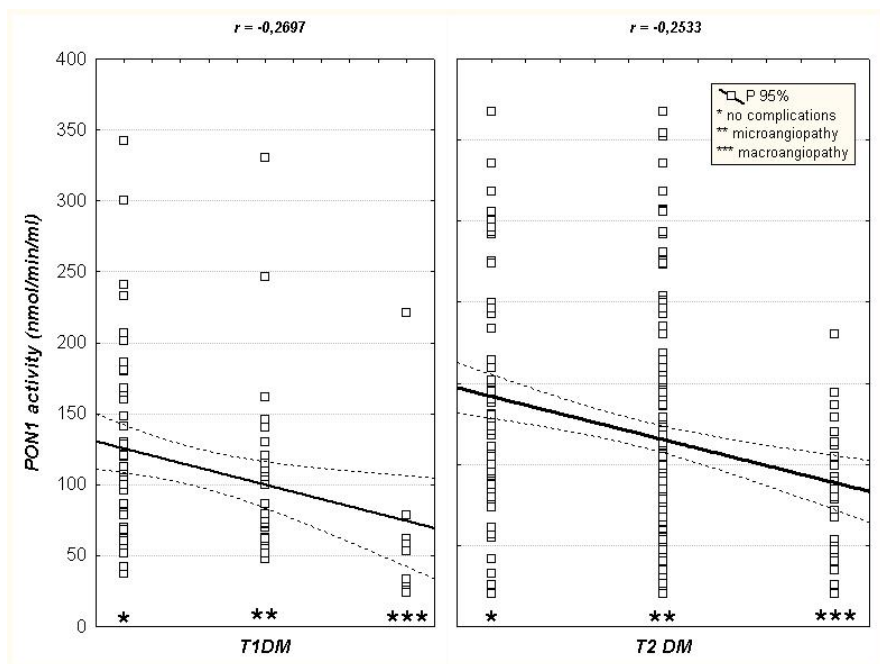
Distribution of genotypes in PON1 gene in both types of diabetes mellitus according to presence macro-(MA+) or microangiopathy (MI-) or no complications (MA-MI-). Explanation of results is mentioned in the text.

Figure 4



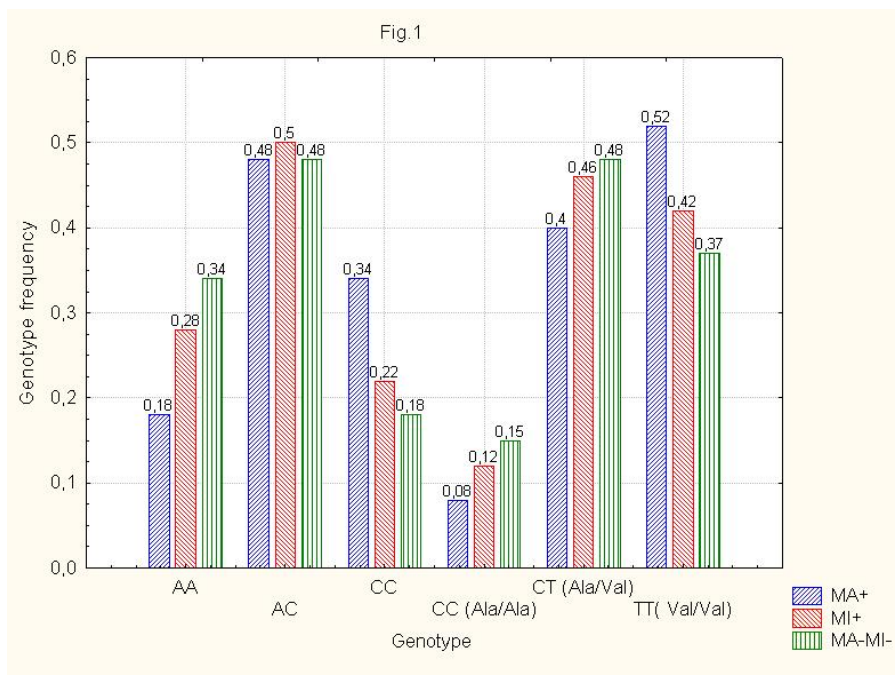
Data correlation between the values of glycated haemoglobin (HbA1c %) and serum paraoxonase activity (PON1) in both types of diabetes mellitus. The correlation coefficients (Spearman) are $r_1 = -0,37$ (T1DM), $r_2 = -0,13$ (T2DM) with $p \leq 0,05$. Dotted lines mean 95% confidence intervals.

Figure 5



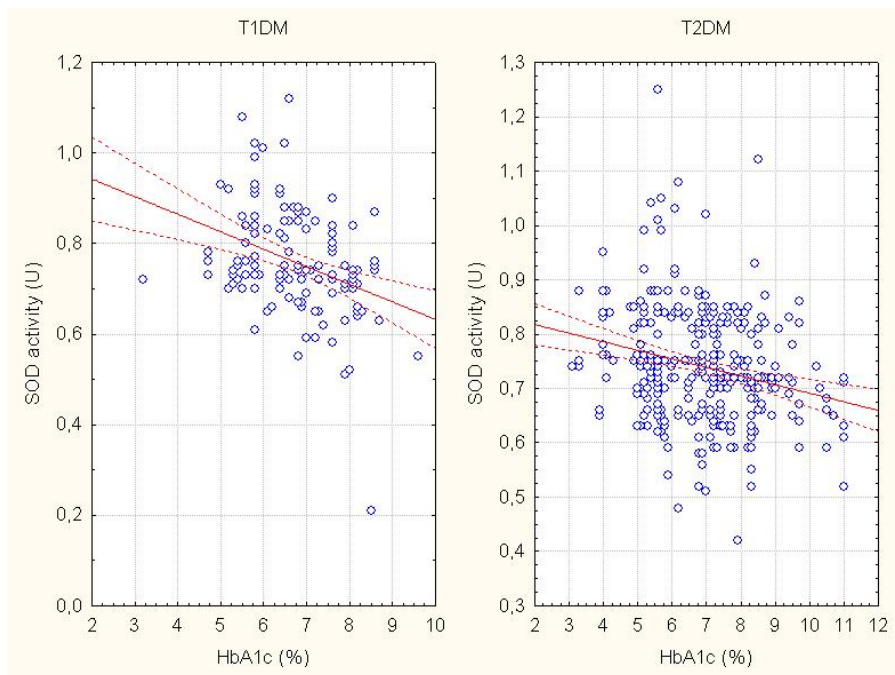
Cross-correlation between the presence of vascular complications in diabetic patients and the level of serum paraoxonase activity. The correlation coefficients are $r_1 = -0,27$ (T1DM), $r_2 = -0,25$ (T2DM) with $p < 0,05$. Dotted lines mean 95% confidence intervals.

Figure 6



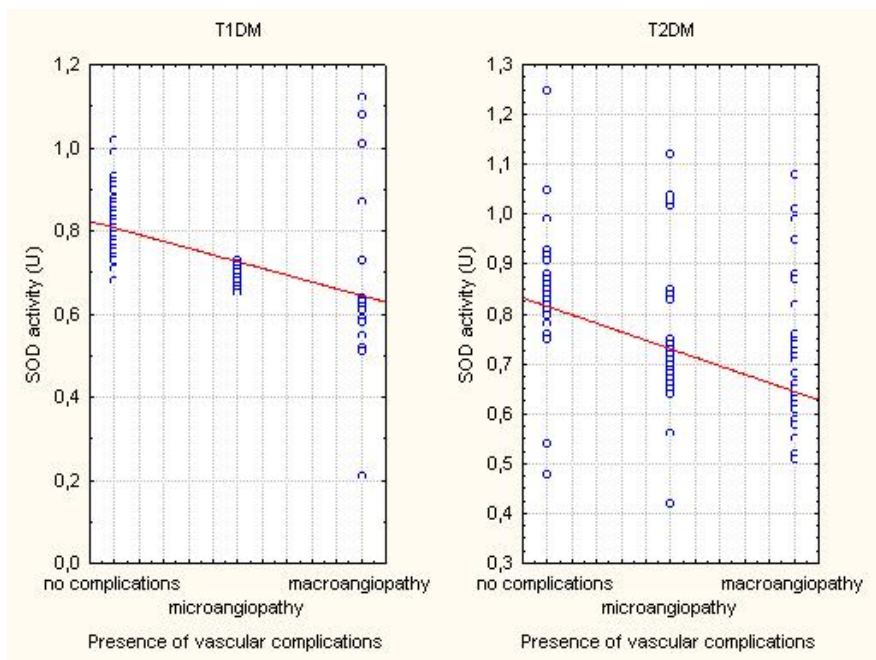
Distribution of genotypes in SOD1 (A/C allele) and SOD2 (C/T allele, Ala/Val) in both types of diabetes mellitus according to presence of macroangiopathy (MA+) or microangiopathy (MI+) or no complications (MA-MI-). Explanation of results is mentioned in the text.

Figure 7



Data correlation between the values of glycated haemoglobin (HbA1c %) and serum superoxide dismutase activity (SOD) in both types of diabetes mellitus. The correlation coefficients (Spearman) are $r_1 = -0,41$ (T1DM), $r_2 = -0,23$ (T2DM) with $p < 0,05$. Dotted lines mean 95% confidence intervals.

Figure 8



Cross-correlation between the presence of vascular complications in diabetic patients and the level of serum superoxide dismutase activity. The correlation coefficients (Spearman) are $r_1 = -0,29$ (T1DM), $r_2 = -0,28$ (T2DM) with $p < 0,05$. Dotted lines mean 95% confidence intervals.

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