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Diabetic nephropathy in type 2 diabetes: MPO *T-764C* genotype is associated with oxidative stress

Research Article

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Abstract: Background: Oxidative stress is a single mechanism relating all major pathways responsible for diabetic damage and plays an important role in diabetes development, progression and related vascular complications. To investigate the impact of oxidative stress related gene polymorphisms on development of diabetic nephropathy (DN), we tested 7 polymorphic variants that could hypothetically affect the ability of the antioxidant defense system and thus accelerate oxidative stress. Methodology: 197 Slovenian (Caucasian) type 2 diabetic (T2D) patients, age 34-83, classified into two groups according to the presence of DN, were tested for SOD2 Val16Ala (rs4880), p22 phox C242T (rs4673), CAT C-262T (rs1001179), MPO T-764C (rs2243828), GSTP1 Ile105Val (rs1695), GSTT1 and GSTM1 deletion polymorphisms using PCR, RFLP and qPCR. Oxidative stress was assessed through serum 8-hydroxy-2-deoxyguanosine (8-OHdG) level. Results were analyzed using ANOVA, Chi-square test and multivariate logistic regression. Results and Conclusions: Despite the commonly recognized link between oxidative stress and diabetes and its complications we found no association between the selected polymorphisms and DN. However, we confirmed an association between oxidative stress level and MPO T-764C genotype, which was tested in relation to DN for the first time.

Keywords: *SOD2 Val16Ala • p22 phox C242T • CAT C-262T • MPO T-764C • GSTP1 Ile105Val • GSTT1 deletion • GSTM1 deletion • Oxidative stress • Diabetic nephropathy*

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Abbreviations:

8-OHdG - 8-hydroxy-2-deoxyguanosine;
BMI - body mass index;
CVD - cardiovascular disease;
DF - diabetic foot;
DBP - diastolic blood pressure;
DN - diabetic nephropathy;
DNeur - diabetic neuropathy;
DR - diabetic retinopathy;
eGFR - estimated glomerular filtration rate;
Hb - haemoglobin;
HbA1c - haemoglobin A1c, glycated haemoglobin;
HDL - high-density lipoprotein;
LDL - low-density lipoprotein;
MDRD - modification of diet in renal disease;
NO - nitric oxide;
SPB - systolic blood pressure;
TG - triglycerides;
SNP - single-nucleotide polymorphism.

1. Introduction

Diabetic nephropathy (DN) occurs in up to one third of patients after 20 years of diabetes. It is defined as a rise in urinary albumin excretion rate in the absence of other causes of renal disease. Usually, it is accompanied by retinopathy and an increase in blood pressure [1]. DN is associated with high morbidity and mortality, mainly due to cardiovascular disease (CVD) and before end-stage renal disease develops. All-cause mortality of patients with DN is 20-40 times higher in comparison to diabetic patients without DN and 2-5 times higher than with other forms of chronic kidney disease. Susceptibility to DN has a familial

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basis, diabetic siblings of probands with DN have a 3-fold increase in the risk of DN. Presumably, DN is a complex, polygenic disease - genetic susceptibility is most likely determined by a large number of relatively common allelic variants, possibly interlinked and interacting with environmental influences, each individually conferring a modest increase in relative risk [1-4].

Oxidative stress is a single mechanism relating all major pathways responsible for diabetic damage. It plays an important role in diabetes development, progression and related vascular complications, including DN [5-13]. It occurs when the production of oxidants exceeds local antioxidant capacity and is derived from the two main chemical pathways: reactive oxygen species (ROS) and reactive nitrogen oxide species (RNS) [14]. Direct measurement of oxidative stress is difficult because of low oxidant serum levels and short half-life. However, it can be assessed indirectly by their oxidative byproducts, e.g. 8-OHdG, an abundant oxidative DNA product and therefore a reliable DNA damage marker [15]. There is no consensus on what it's true levels are in human DNA, but a significant increase was noticed from healthy to prediabetic and finally diabetic individuals [16,17]. A marked increase was confirmed in diabetic nephropathy in comparison to diabetes without vascular complications, especially in patients with proteinuria greater than 3 g/day [18,19]. Oxidative stress can be accelerated due to an increased production of oxidants caused by hyperglycemia, and due to the a reduced ability of the antioxidant defense system.

Superoxide dismutase (SOD), probably the most important free radical scavenger, converts superoxide (O_2^-) into hydrogen peroxide (H_2O_2). Three SOD isoforms exist in mammals: cytosolic SOD 1 (also termed CuZnSOD), mitochondrial SOD 2 (MnSOD) and extracellular SOD 3 (EC-SOD), each derived from distinct genes but catalyzing the same reaction [20]. Catalase (CAT) has a predominant role in controlling the concentration of hydrogen peroxide [21]. Glutathione S-transferases (GSTs) inactivate secondary metabolites of ROS by catalyzing their conjugation with glutathione. GST isoforms can be classified into 7 groups: GSTA, GSTM, GSTK, GSTO, GSTP, GSTT and GSTM [22]. Some enzymes produce and utilize oxidants as a part of body's defense system, e.g. mieloperoxidase (MPO) and NADPH oxidases. MPO catalyses the conversion of hydrogen peroxide to hypoclorous acid - a cytotoxic antimicrobial agent in neutrophils and monocytes [23,24]. NADPH oxidases, a family of multi-subunit enzymes, are an important source of ROS in phagocytes and non-phagocytic cells [25].

Numerous oxidative stress-related genes are positional candidates (determined by GWAS) and candidate genes studies have confirmed the association of their polymorphisms with DN [26]. Considering these facts we analyzed the association of 7 commonly reported polymorphic variants with DN in T2D patients: *SOD2* Val16Ala (rs4880), C242T polymorphism of the *p22 phox* gene (rs4673), encoding a subunit of NADPH oxidase; *CAT* C-262T (rs1001179), *MPO* T-764C (rs2243828), *GSTT1* and *GSTM1* deletion polymorphisms and *GSTP1* Ile105Val (rs1695). Genotype status was compared to serum 8-OHdG, oxidative stress marker.

2. Experimental Procedures

2.1 Patients and study design

In our cross sectional study, reflecting a real diabetic population, 197 unrelated Slovenian (Caucasian) T2D patients, age 34-83, were classified into two groups according to the presence of DN: a study group of 88 patients with DN (DN+) and a control group of 109 patients without DN but T2D lasting over 10 years (DN-). Diagnosis of diabetes was made according to WHO 1999 diagnostic criteria [27]. Diabetic nephropathy was defined by increased albumin/creatinin ratio (>3 g/mol) in two out of three successive urine samples, separated by 3-month intervals; or decreased eGFR in combination with characteristic morphological changes and presence of diabetic retinopathy; both criteria in the absence of other renal disease. To avoid the confounding effect of impaired kidney function, patients with overt nephropathy were not enrolled in the study. Patients with poor glycaemic control, significant heart failure (NYHA II-IV), alcoholism, infection and other causes of renal disease were also excluded. The study was approved by the national medical ethics committee. All patients signed an informed consent for participation in the study and were interviewed in person [28].

2.2 DNA isolation and genotyping

Information on smoking, presence of CVD, family history of CVD, duration of arterial hypertension and diabetes, diabetes management and complications (retinopathy - DR, neuropathy, diabetic foot - DF), therapy and routine laboratory measurements were obtained from their medical records. DNA was extracted from peripheral blood samples using a commercial isolation kit according to manufacturer's protocol (DNeasy Blood & Tissue Kit, Qiagen). Selected polymorphisms were tested using PCR, RFLP or real-time PCR (qPCR) with protocols in Tables 1 and 2. Genotyping was performed by two researchers (JM, DP), blinded for case or control status of the patients; duplicate samples were used. *P22 phox* and *GST* polymorphisms were tested using

Gene	Marker details	Primer sequence	PCR protocol	PCR reaction mix	Enzyme used	Product size
p22 phox	C242T rs4673	5´-AAC ACT GAG GTA AGT GGG GGT GGC TCC TGT-3´ 5´-CGC TGC GTT TAT TGC AGG T-3´	Primary denaturation: 95°C 5 min; Denaturation: 95°C 1 min Annealing 58°C 1 min Extension 72°C 1 min; 30x Final elongation 72°C 5 min.	0,3 μl 10 mM dNTP (0,2 mM) 3 μl 5-times conc. buffer (Promega) 0,6 μl 25 mM $MgCl_2$ (1 mM) 0,5 μl 10 μM primers (0,3 μM) 1 μl (500 ng) DNA 8,9 μl H_2O 0,2 μl (1U) GoTaq DNA polymerase	*RsaI*	348 (188+160)
GSTT1	+/-	5´-TTC CTT ACT GGT CCT CAC ATC TC-3´ 5´-TCA CCG GAT CAT GGC CAG CA-3´	Primary denaturation: 95°C 5 min; Denaturation: 95°C 1 min Annealing 64°C 1 min Extension 72°C 1 min; 30x Final elongation 72°C 5 min.	0,1 μl 10 mM dNTP (0,2 mM) 0,5 μl 10-krat conc. buffer (Fermentas); 0,3 μl 25 mM $MgCl_2$ (1,5 mM) 0,1 μl 10 μM primer pair + internal control primers (0,2 μM) 1 μl (500 ng) DNA 2,3 μl H_2O 0,2 μl (1U) Taq DNA polymerase	/	215
GSTM1	+/-	5´-GAA CTC CCT GAA AAG CTA AAG C-3´ 5´-GTT GGG CTC AAA TAT ACG GTG G-3´			/	480
Albumin (internal replication control)		5´-GCC CTC TGC TAA CAA GTC CTA C-3´ 5´-GCC CTA AAA AGA AAA TCG CCA ATC-3'			/	350
GSTP1	Ile105Val rs1695	5´-ACC CCA GGG CTC TAT GGG AA-3´ 5´-TGA GGG CAC AAG AAG CCC CT-3´	Primary denaturation: 95°C 5 min; Denaturation: 95°C 30 sec Annealing 56°C 30 sec Extension 72°C 1 min; 30x Final elongation 72°C 5 min.	0,1 μl 10 mM dNTP (0,2 mM) 1 μl 5-times conc. buffer (Promega) 0,3 μl 25 mM $MgCl_2$ (1,5 mM) 0,15 μl 10 μM primers (0,3 μM) 0,5 μl (500 ng) DNA 2,35 μl H_2O 0,2 μl (1U) GoTaq DNA polymerase	*AlwI261*	176 (91+85)

Table 1. PCR and RFLP information.

Gene	Marker details	qPCR protocol	qPCR reaction mix
SOD2	rs4880	Pre – PCR Read: 60°C 30 sec Predenaturation: 95°C 10 min Cycling: Denaturation: 95°C 15 sec Annealing and extension: 60°C 60 sec; 35x Post – PCR Read: 60°C 30 sec	2x TaqMan Genotyping Master Mix 40x specific TaqMan® SNP Genotyping Assay for rs4880 (C_8709053) 0,5 μl (500 ng) DNA.
CAT	rs1001179	Pre – PCR Read: 60°C 30 sec Predenaturation: 95°C 10 min Cycling: Denaturation: 95°C 15 sec Annealing and extension: 60°C 60 sec; 35x Post – PCR Read: 60°C 30 sec	2x TaqMan Genotyping Master Mix 40x specific TaqMan® SNP Genotyping Assay for rs1001179 (C_11468118) 0,5 μl (500 ng) DNA.
MPO	T-764C rs2243828	Initial denaturation: 94° C 15 min Cycling (Touchdown qPCR): Denaturation 94°C 20 sec Annealing and extension; 61°C 60 sec; 10x (0,6°C per cycle until 55°C) Adittional cycling: Denaturation 94°C 10 sec Annealing and extension 55° C 60 sec; 26x	2x KASPar Reaction Mix v3 2,5 μl 40x specific Assay Mix for rs2243828 (KBioScience) 0,07 μl 1,0 μl (3-10 ng) DNA 1,38 μl H_20 MgCl2 0,05 μl (50 mM)

Table 2. Real time PCR (gPCR) protocol.

"Applied Biosystem 2720 Thermal Cycler" instrument and the results were visualized after electrophoresis in a SYBR Green (Invitrogene) stained 2% agarose gel. *SOD2*, *CAT* and *MPO* qPCR genotyping was performed in a 48-well StepOne™ Real-Time PCR instrument.

2.3 Serum 8-OHdG

Level of serum 8-OHdG, a reliable DNA damage marker, was used to assess oxidative stress and was measured by enzyme-linked immunosorbent assay (ELISA).

2.4 Statistical analysis

Statistical analysis was performed using SPSS version 20.0 software. Numeric variables are reported as mean ± standard deviation (SD); variance between both groups was assessed using ANOVA. Chi-square test was used to compare categorical variables. Genotypes were tested for Hardy-Weinberg equilibrium among DN+ and DN- groups. Finally, a multivariate logistic regression model was performed for the risk factors and genotypes with respect to DN. A P value of <0.05 was considered statistically significant.

3. Results and Discussion

Susceptibility to DN is most likely determined by a large number of relatively common allelic variants, possibly interlinked and interacting with environmental influences, each individually conferring a modest increase in relative risk [2]. Our previously published results in this population (Table 3) [28] confirmed duration of diabetes (P<0.001), male gender (P=0.008) and poor glycaemic control (HbA1c; P=0.012) as DN risk factors by multivariate logistic regression model. Using this model, testing all unmatched variables and important risk factors (gender, duration of T2D and hypertension, SBP, DBP, BMI, smoking, fasting glucose, HbA1c, total cholesterol, HDL, serum creatinine, cystatin C, eGFR, urine albumine/creatinine ratio) with respect to DN, we could also predict a significant increase in cardiovascular morbidity in DN+ group (P=0.037). Importantly, there was no significant difference between groups regarding blood pressure, which is also a substantial contributor to renal dysfunction.

As for the genetic component, we selected 7 polymorphic variants that could hypothetically affect the ability of the antioxidant defense system and thus accelerate oxidative stress (Table 4). Of the three SOD isoforms, SOD2 is an essential defender against mitochondrial superoxide radicals. *SOD2* C47T polymorphism (also called Ala16Val; rs4880) alters the SOD 2 protein structure and function. This C/T substitution results in a missense mutation (Ala/Val) that disrupts the enzyme's α-helix structure, changing the structural conformation of the mitochondrial targeting sequence (MTS). Ala-SOD 2/MTS allows efficient SOD 2 import into the mitochondrial matrix, while the Val-variant causes partial arrest of the precursor within the inner membrane and the decreased formation of the

	Study group (DN)	Control group (-DN)	Significance (P)
No.	88	109	
Sex (M/F)	66/22	64/46	0.011
Age (years)	61.6 ± 9.9	62.1 ± 8.9	0.709
Duration of T2D (years)	12.2 ± 8.1	15.0 ± 4.9	0.003
Duration of hypertension (years)	8.3 ± 9.6	7.1 ± 7.6	0.363
SBP [mm Hg]	153.1 ± 19.4	147.9 ± 20.1	0.067
DBP [mm Hg]	87.9 ± 13.6	85.6 ± 10.7	0.184
BMI	30.9 ± 3.9	30.0 ± 4.1	0.108
Active smokers	10.2%	8.3%	0.634
CVD	22.7%	6.4%	0.001
Family history of CVD	30.7%	29.4%	0.520
DR	37.5%	23.9%	0.115
Duration of DR (years)	3.7 ± 2.9	3.8 ± 3.9	0.955
DNeur	37.5%	33.0%	0.513
DF	8.0%	2.75%	0.097
S-HbA1c [%]	8.12 ± 1.59	7.47 ± 1.15	0.001
S-fasting glucose [mmol/l]	9.16 ± 2.67	8.41 ± 2.06	0.028
S-Hb [g/l]	142.06 ± 15.17	138.44 ± 12.41	0.067
S-urea [mmol/l]	6.39 ± 2.62	5.88 ± 1.82	0.115
S-creatinine [μmol/l]	76.31 ± 28.91	66.73 ± 15.2	0.003
S-cystatin C [mg/l]	0.78 ± 0.28	0.68 ± 0.18	0.002
eGFR [MDRD equation, ml/min]	79.70 ± 15.79	84.51 ± 9.88	0.010
S-Total cholesterol [mmol/l]	4.51 ± 1.36	4.48 ± 1.10	0.858
S-HDL [mmol/l]	1.17 ± 0.33	1.28 ± 0.37	0.038
S-LDL [mmol/l]	2.63 ± 1.16	2.64 ± 0.85	0.921
S-TG [mmol/l]	2.19 ± 2.06	1.83 ± 1.31	0.141
U-albumin/creatinine ratio [g/mol] - sample No. 1	32.78 ± 61.70	1.46 ± 2.59	0.000
U-albumin/creatinine ratio [g/mol] - sample No. 2	39.49 ± 96.07	1.99 ± 6.08	0.000
U-albumin/creatinine ratio [g/mol] – sample No. 3	42.26 ± 102.01	1.39 ± 2.04	0.000

Table 3. Population characteristics.

BMI = body mass index, CVD = cardiovascular disease, DR = diabetic retinopathy, DNeur = diabetic neuropathy, DF = diabetic foot, eGFR - estimated glomerular filtration rate, HDL = high-density lipoprotein, LDL = low-density lipoprotein, S- = serum, TG = triglycerides, U- = urine

active SOD 2 tetramer in the mitochondrial matrix [29]. In previous studies, in contrast to healthy controls with predominant CT genotype, genotype TT (Val/Val) was most common in T1D and T2D patients and was related to lower enzyme activity [30]. The T allele has been associated with all diabetic microvascular complications, although the results were inconsistent across several studies [31-37]. Our study also showed a more frequent TT (ValVal) genotype accounting for the less frequent CC (AlaAla) genotype in DN+ group, but the difference was not statistically significant. There was no significant difference in oxidative stress level in relation to the three *SOD2* C47T genotypes.

CAT has a predominant role in controlling the concentration of H_2O_2. It is present in peroxisomes of various cells and in cytoplasm of macrophages and erythrocytes. The enzyme consists of four identical subunits, which form a rigid, stable double shaped tetramer. It is encoded by the *CAT* gene – the C-262T polymorphism being located in its promoter region. In patients with DN, CAT activity in erythrocytes is decreased [38]. When exposed to hyperglycemic

Genotype	DN+ group	DN- group	Significance (P)	8-OHdG level	Significance (P)
SOD2 Val16Ala TT	24/87 (27.6%)	34/109 (31.2%)		14.93±5.79	
SOD2 Val16Ala CT	43/87 (49.4%)	54/109 (49.5%)	0.766	13.86±3.2	0.491
SOD2 Val16Ala CC	20/87 (23.0%)	21/109 (19.3%)		13.06±2.52	
CAT C262T TT	4/88 (4.5%)	9/107 (8.4%)		13.82±4.21	
CAT C262T CT	36/88 (40.9%)	34/107 (31.8%)	0.291	13.41±2.61	0.616
CAT C262T CC	48/88 (54.5%)	64/107 (59.8%)		14.61±5.08	
p22 phox C242T TT	10/88 (11.4%)	15/108 (13.9%)		13.74±4.33	
p22 phox C242T CT	31/88 (35.2%)	44/108 (40.7%)	0.530	13.72±2.51	0.649
p22 phox C242T CC	47/88 (53.4%)	49/108 (45.4%)		14.87±6.09	
MPO T-764C CC	4/88 (4.5%)	2/108 (1.9%)		25.34±12.91	
MPO T-764C CT	21/88 (23.9%)	34/108 (31.5%)	0.311	14.00±2.70	0.000
MPO T-764C TT	63/88 (71.6%)	72/108 (66.7%)		13.52±3.46	
GSTP1 Ile105Val GG	6/87 (6.9%)	13/108 (12.0%)		13.39±1.95	
GSTP1 Ile105Val AG	41/87 (47.1%)	40/108 (37.0%)	0.251	13.88±3.21	0.827
GSTP1 Ile105Val AA	40/87 (46.0%)	55/108 (50.9%)		14.45±5.52	
GSTT1 -	56/88 (63.6%)	70/109 (64.2%)	0.932	14.24±3.53	0.935
GSTT1 +	32/88 (36.4%)	39/109 (35.8%)		14.08±4.34	
GSTM1 -	19/88 (21.6%)	29/109 (26.6%)	0.414	14.24±5.16	0.801
GSTM1 +	69/88 (78.4%)	80/109 (73.4%)		13.95±2.96	

Table 4. Genotype distributions and genotype status in comparison to 8-OHdG level.

conditions (*in vitro*), *CAT* mRNA expression in blood cells was significantly decreased. *CAT* -262T promoter variant has been associated with higher transcriptional activity and increased erythrocyte CAT level, offering protective effect. Previous studies associated C-262T polymorphism to diabetic neuropathy but not DN or DR [35,39,40]. Our study found no association between CAT C-262T and DN and no genotype – 8-OHdG serum level correlation.

P22 phox gene encodes a subunit of NADPH oxidase, which is an important source of ROS in phagocytes and less in non-phagocytic cells. There is an entire family of NADPH oxidases, among which only Nox2 and Nox4 (also termed Renox or renal oxidase) are found in kidney cells [25]. Diabetic animal models suggest that Nox4 is the major source of ROS during early stages of diabetes, mediating renal hypertrophy and increased fibronectin expression [41]. All Noxes appear to have an obligatory need for p22phox. The gene coding the p22 subunit is polymorphic. The C242T (His72Tyr; rs4673) polymorphism showed significance regarding the association with DN. This polymorphism substitutes histidine by tyrosine in the potential heme-binding sites, offering explanation of its functional role. Both alleles were previously associated to higher risk of DN [42-44]. In patients with coronary artery disease, T allele has proven to have a protective effect on coronary risk [45]. Our study showed no significant correlation, although TT genotype was more frequent in DN- group and CC genotype in DN+ group. There was no genotype – oxidative stress marker level association.

A functional SNP was also previously described for *MPO*: the G-463A substitution (rs2333227), located in the promoter region - the binding site of a SP1 transcription factor - thus conferring lower transcriptional activation due to disruption of the binding site [46]. Hypothetically, the -463A variant relates to reduced MPO activity and thus lower ROS production. It was consequently expected to relate to reduced cancer risk, but studies showed inconsistent results, offering weak support for this biologically plausible hypothesis [47-49]. Due to technical difficulties, a *MPO* T-764C (rs2243828) was tested instead - genotyping concordance between the two SNPs was 100% [http://snp500cancer.nci.nih.gov, 48,49]. To our knowledge, this polymorphism has previously not been tested in relation to diabetes

or DN. We observed no difference in genotype frequencies between both groups. There was, however, a significantly higher oxidative stress level associated with genotype CC.

Of the GST isoforms, deletion polymorphisms exist for *GSTM1* and *GSTT1* genes, whereas homozygous individuals with the "null allele" lack the respective enzyme function. In the GSTP subfamily, two *GSTP1* alleles (*GSTP1a* and *GSTP1b*) have been described that differ in a single base pair (A/G) and result in an amino acid substitution that alters the enzyme function (*GSTP1* Ile105Val). So far, studies relating these polymorphisms to diabetic microvascular complications are contradictory. *GSTT1* polymorphism has been described as a risk factor for diabetic end stage renal disease and associated with premature vascular morbidity, progression of DN and DR in T2D [50-52], but other studies have found no correlation with T2D [53] and DN in T1D patients [35]. *GSTM1* polymorphism has been significantly associated with T2D but not with DN [35,53-55]. *GSTP1* polymorphism has been correlated with T2D [55] and DN [54]. Considering these diverse and often contradictory results it seems reasonable that we found no significant difference in oxidative stress levels between the potential genotypes and no significant genotype difference between the two groups tested. There are however, limitations in this interpretation regarding *GSTM1* and *GSTT1* genotypes common to other studies published. Both genotypes were only assessed by "+/-" PCR method (one or two copies *vs*. absence of the risk allele) although copy number variations are correlated with altered enzyme activity – it was previously proposed analysis in a dose-dependent manner would better describe disease outcome association [22]. In addition, there was no significant difference in serum 8-OHdG levels between DN+ and DN- groups in our specific, well managed, but size-limited population [28], possibly due to exclusion of patients with overt nephropathy.

In conclusion, despite the commonly recognized link between oxidative stress and diabetes and its complications we found no association between the selected polymorphisms and DN. However, we confirmed an association between oxidative stress level and MPO *T-764C* genotype, which was tested in relation to DN for the first time.

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