Genetic polymorphisms in genes encoding antioxidant enzymes are associated with diabetic retinopathy in type 1 diabetes

Short running title: Polymorphisms associated with T1D retinopathy

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Submitted 8 May 2009 and accepted 7 September 2009.

This is an uncopyedited electronic version of an article accepted for publication in *Diabetes Care*. The American Diabetes Association, publisher of *Diabetes Care*, is not responsible for any errors or omissions in this version of the manuscript or any version derived from it by third parties. The definitive publisher-authenticated version will be available in a future issue of *Diabetes Care* in print and online at http://care.diabetesjournals.org.
Objective: Oxidative stress plays an important role in the development of microangiopathic complications in type 1 diabetes. We investigated polymorphic markers in genes encoding enzymes regulating production of reactive oxygen species in association with diabetic retinopathy (DR) or diabetic nephropathy (DN).

Research design and methods: One hundred and twenty four patients with type 1 diabetes were investigated in this case-control study. All subjects were matched for sex, age and duration of diabetes. Genotyping was conducted using real-time PCR for p.Val16Ala polymorphism in MnSOD gene and c.C(-262)T in the promotor region of CAT gene. Multiplex PCR method was used for determination of GSTM1 and GSTT1 polymorphic deletions. Fluorescence labeled PCR amplicons and fragment analysis was used for assessing the number of pentanucleotide (CCTTT)n repeats in iNOS.

Results: A positive association of MnSOD genotype Val/Val (OR = 2.49, 95% CI = 1.00-6.16, P = 0.045) and GSTM1-1 genotype (OR = 2.63, 95% CI = 1.07-6.47, P = 0.031) with diabetic retinopathy but not with diabetic nephropathy was demonstrated. Additionally, the combination of the two genotypes conveyed an even higher risk (OR = 4.24, 95% CI = 1.37-13.40, P = 0.009). No other investigated genetic polymorphisms were associated with either DR or DN.

Conclusions: Selected polymorphisms in genes encoding MnSOD and GSTM1 could be added to a panel of genetic markers for identification of individuals with type 1 diabetes at an increased risk for developing DR.
Several studies have suggested that reactive oxygen species (ROS) are implicated in the aetiology of type 1 diabetes (1) as well as in development of severe microangiopathic complications like diabetic retinopathy (DR) and diabetic nephropathy (DN) (2). Chronic extracellular hyperglycaemia in diabetes stimulates ROS production and increases oxidative stress (3). The oxidation of high levels of glucose inside diabetic cells produces more electron donors (NADH and FADH₂) and increases the electron transfer, thereby generating superoxide (4).

Excess generation of ROS like superoxide (O₂⁻●), hydrogen peroxide (H₂O₂), hydroxyl radical (•OH) and reactive nitrogen species (RNS), such as nitric oxide (NO), oxidize target cellular proteins, nucleic acids or membrane lipids and damage their cellular structure and function (4). Hyperglycaemia stimulates the expression of inducible nitric oxide synthase (iNOS) and increases production of NO, an intracellular second messenger (2). Increased NO generation accompanied by the superoxide overproduction favours the formation of peroxynitrite, a highly reactive oxidant (5). Evidence suggests that ROS also regulate the expression of genes encoding for proteins involved in inflammation, immune response and cell death (6).

Antioxidant enzymes like manganese superoxide dismutase (MnSOD) and catalase (CAT) directly eliminate ROS, while glutathione-S-transferases (GSTs) detoxify cytotoxic secondary metabolites of ROS. Together they represent protective mechanism against the damage caused by the oxidative stress. Most of the enzymes involved in the defence against oxidative stress are polymorphic.

Associations between MnSOD Val16Ala single nucleotide polymorphism (SNP) and DN (7) or DR in type 2 diabetes (8) and polyneuropathy (DPN) in type 1 diabetes (9) emphasize the importance of polymorphism in these genes. Individuals carrying the homozygous TT or heterozygous CT genotype in the promoter region of the CAT gene have significantly higher enzyme activity which offer a degree of protection against development of DPN (10). Human GSTM1 and GSTT1 polymorphic deletion are associated with the age of onset of type 1 diabetes (11). Variable expression of the human iNOS gene polymorphic pentanucleotide (CCTTT)n repeat is associated with different autoimmune diseases including type 1 diabetes (12). All described polymorphisms could add to the interindividual variability in patients with type 1 diabetes for the development of microangiopathic complications.

The aim of our study was to evaluate the association of polymorphic markers in genes encoding antioxidant enzymes which share a common detoxification pathway (MnSOD, CAT, GSTM1 and GSTT1) or regulate ROS production (iNOS) with DR or DN in a cohort of patients with type 1 diabetes.

**RESEARCH DESIGN AND METHODS**

**Subjects:** Individuals with type 1 diabetes were recruited from the national register of childhood-onset type 1 diabetes. Diagnosis of type 1 diabetes was based on WHO/ADA definition of diabetes (13). All patients were screened prospectively for the presence of DR by annual dilated eye examination and fundus photography performed by an ophthalmologist experienced in diagnosing the presence of DR (14). The presence of DN was screened annually by assessing microalbuminuria in a random urine specimen with a dipstick (Micral-test, Roche Diagnostics, Mannheim, Germany) and
confirmed if positive by the determination of the urine albumin/creatinine ratio from a second morning urine specimen. A value of >100 mg/g repeated on at least two out of three measurements was considered diagnostic for DN (15).

The study population consisted of 124 unrelated individuals with type 1 diabetes (70 male and 52 female patients, median age 27.12). All patients were treated with basal-bolus insulin regimen with at least four daily injections or an insulin pump. They were divided in two groups: patients with DR or DN (N=62, case subject) and patients without complications after at least 11 years of duration of type 1 diabetes (N=62, control subject). Both groups were matched by sex, age and duration of diabetes. The clinical characteristics of participating patients are shown in Table 1. Mean HbA1c was calculated from all available values over the observation period with typically 3 to 4 values per patient annually. All of the participating patients were of Caucasian origin and had type 1 diabetes onset before 16 years of age. Samples for DNA extraction were obtained during a routine annual screening for diabetes-related conditions. This population based case-control study was approved by the National Medical Ethics Committee. All participating patients gave their written informed consent.

**MATERIALS AND METHODS**

Genomic DNA was extracted from peripheral blood (10ml) using a FlexiGene DNA isolation kit (Qiagene GmbH, Hilden, Germany) according to the recommended protocol.

**MnSOD and CAT genotyping:** For MnSOD Val16Ala polymorphism and CAT C-262T promotor polymorphism real-time PCR genotyping was conducted on ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). TaqMan SNP Genotyping Assays were used to determine the regions encompassing polymorphic sites. For detection of Val16Ala (ref SNP ID: rs4880) assay ID:C_8709053_10, and for CAT C-262T (ref SNP ID: rs1001179) assay ID:C_11468116_10 were used. The reaction mixture (5µl) contained 0.125 µl of TaqMan SNP genotyping assay, 2.5 µl of TaqMan Universal PCR Master Mix and 100 ng of extracted genomic DNA. Thermo-cycling program was performed according to the manufacturer’s recommendations.

**GSTT1 and GSTM1 genotyping:** GSTT1 and GSTM1 polymorphic deletions were identified using a multiplex PCR-based method with three sets of primers. For GSTT1 polymorphism the sequences of the forward and reverse primers were 5'-ATG TGA CCC TGC AGT TGC-3' and 5'-GAG ATG ATG TGA GGA CCA GTA AGG AA'-3. For GSTM1 polymorphic deletion the forward and reverse primers were 5'-GCT TCA CGT GTT ATG GAG GTT -3’ and 5'-GAG ATG ATG AAG TTC AGA-3’. A third set of primers were 5’-GAA GAG CCA AGG ACAGGT AC-3’ and 5’-CAA CTT CAT CCA CGT TCA CC-3’ used for co-amplification of the human β-globin gene, an internal control of positive amplification in the case of the presence of deletion in both genes. Such genotyping approach did not allow as detecting heterozygous carriers of GSTM1 or GSTT1 deletion, so GSTM1-0 or GSTT1-0 genotype group included only patients homozygous for GSTM1 or GSTT1 deletion. GSTM1-1 or GSTT1-1 genotype group included homozygous and heterozygous carriers of the functional allele.

**iNOS genotyping:** The iNOS microsatellite marker located in the promotor region, 2.5 kb upstream of the transcription start site, was amplified by PCR combined with fluorescence labelled primers as described previously (16). Amplification product sizes were estimated using capillary electrophoresis with ABI PRISM 310 automated sequencer and GeneMapper.
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Allele distribution of pentanucleotide (CCTTT)n repeats were assessed accordingly to the different length of amplicons.

**Statistical methods:** SPSS version 16.0 software (SPSS Inc., Chicago, IL) was used. The sample size and power estimation suggested conventional statistical methods for comparison between case and control subjects and 86% power to detect median size affect (0.3-0.5) with an alpha level of 0.05. The null-hypothesis that there is no association between the genotype and the development of DR or DN in type 1 diabetes were tested for each polymorphic gene locus and P values less then 0.05 were considered statistically significant. Correction for potential multiple testing errors was performed using Bonferroni method. Testing for deviation from Hardy-Weinberg equilibrium using HWE calculator was performed and observed genotype frequencies of the studied polymorphisms were in agreement with HWE ($\chi^2=0.291, P=0.864$ for MnSOD and $\chi^2=0.828, P=0.363$ for GSTM1).

Independent $t$ test was performed to compare the continuous parameters between cases and controls. Levene's test for equality of variances suggested which statistic analysis is more appropriate and which P value should be considered. Genotype and allele frequencies between study groups were compared by Pearson Chi-square and/or Fischer exact test. Strength of association between polymorphisms in genes encoding antioxidant enzymes and development of complication in diabetes was estimated using odds ratios (OR) and 95% confidence interval (CI). The binary logistic regression analysis was applied to check on the calculated odds ratios, adjusted for independent variables such as age, sex, age at onset and duration of diabetes.

**RESULTS**

Clinical characteristics of patients are presented in Table 1. No significant differences were observed between both groups regarding age, age at onset, duration of diabetes or mean HbA1c.

The MnSOD genotype Val/Val was significantly more frequent in patients with DR, carrying a 2.49-fold higher risk for development of DR (OR=2.49, 95% CI=1.00-6.16, $P=0.045$, Table 2). Genotype GSTM1-1 was significantly more frequent in patients with DR, carrying a 2.63-fold higher risk for the development of DR (OR=2.63, 95% CI=1.07-6.47, $P=0.031$, Table 2). Results of the Pearson Chi-square test revealed no significant differences in allele and genotype frequencies for CAT and GSTT1 polymorphism in association with DR or DN.

Nine alleles for the iNOS microsatellite ranging from 176 to 216 bp (8 to 16 pentanucleotide repeats) were observed (Table 3). No significant difference in overall allelic distribution was detected between patients with DR versus controls ($\chi^2=1.738$, $df=8$, $P=0.988$) or between patients with DN versus controls ($\chi^2=7.736$, $df=8$, $P=0.460$). Allele 196 (12 repeat allele) proved to be the most frequent allele in our study population (31.2% of all observed alleles). We detected slightly increased risk for DR in patients carrying allele 196 (OR=2.19, 95% CI=0.88-5.49, $P=0.089$) but it did not reach statistical significance.

Interaction between different genotypes in genes encoding for antioxidant enzymes was investigated (Table 4). The carriers of both GSTM1-0 and GSTT1-0 null genotypes, which result in a complete lack of enzyme activity, did not have an increased risk for development of microangiopathic complication in type 1 diabetes. Patients with combination of MnSOD Val/Val genotype and GSTM1-1 normal genotype had significantly higher risk for retinopathy than patients lacking this particular combination (OR=4.24, 95% CI=1.37-13.40, $P=0.009$).
CONCLUSIONS

We hypothesized that genetic variability of enzymes regulating oxidative stress could be involved in development of microangiopathic complications in people with type 1 diabetes.

The MnSOD Val/Val genotype was associated with a 2.49-fold higher risk for DR in our cohort of patients with type 1 diabetes. Val/Val genotype combined with smoking is associated with DN in type 1 diabetes (7). We did not find an association between Val/Val and DN in our cohort of patients with type 1 diabetes where none reported smoking. MnSOD Val16Ala polymorphism is also associated with DR (8) and DN (17) in type 2 diabetes. In vitro studies show that amino acid substitution of Ala with Val modifies helical structure of the signal sequence and alters the import of MnSOD enzyme into the mitochondrial matrix (18). Less efficient import decreases MnSOD concentration inside mitochondria and results in overproduction of superoxide, which is considered to be a causal link between hyperglycaemia and metabolic pathways involved in vascular complications in diabetes (4). Studies on mice, treated with streptozotocin, show that MnSOD overexpression in mitochondria play a significant protective role in development of DR (19).

A significant association between the presence of the functional GSTM1 gene and development of DR was observed in our study. This suggested that deletion in the GSTM1 gene had a protective role for DR. A large number of studies on GSTM1-0 and/or GSTT1-0 null genotypes report an increased risk for development and progression of rheumatoid arthritis and asthma (20,21). However, one study reports an association of GSTM1 gene deletion with protection from development of type 1 diabetes in a group of 14-20 years old children (11). The protective role of GSTM1 null genotype in the development of DR in type 1 diabetes has to the best of our knowledge not been described yet. One possible explanation for the protection role is that the absence of the GSTM1 gene may up-regulate other antioxidant enzymes including MnSOD (22). However, all explanations are speculative and need to be confirmed in further studies.

When studying synergistic effect of different polymorphism, we observed a 4.24-fold higher risk for development of DR in patients with type 1 diabetes carrying MnSOD Val/Val and normal GSTM1-1 genotype. Evidence of interaction between MnSOD and GST genes is reported in a case-control study of rheumatoid arthritis (23), but has to our knowledge not been described in patients with type 1 diabetes and DR.

The association studies that evaluate the impact of genotype on disease progression are usually limited by the fact that more chronic complication will develop with longer follow up. Considering that the duration of diabetes is an important risk factor for microangiopathic complications our results must be interpreted with caution. In most patients with type 1 diabetes the earliest signs of diabetic complications occur after 5-10 years and the highest incidence (between 75%-95%) occur after 10 years (24). Control subjects in our study were without diabetic complication for a mean of 17.9 years after the onset of type 1 diabetes. Another limitation of our study was a relatively small number of participants. However, the studied population was homogenous and power estimation showed that our study had a 86% power to detect median size affect (0.3-0.5) with an alpha level of 0.05.

Other genetic polymorphisms involved in the hyperglycaemia induced cell damage could influence our results. Notably advanced glycation end products (AGE) modify ROS formation through AGE receptors and thus influence the production of
growth factors and cytokines by affected cells (4). Our results thus represent only a part of the complex pathobiologic network of DR or DN.

Finally, poor glycemic control increases the risk for DN or DR. Moreover, a limited period of poor glycemic control can have a prolonged effect on the incidence of DR ("metabolic memory") as demonstrated by the EDIC cohort follow up (25). Our groups with and without DR had similar mean HbA1c. However, possible periods of poor glycemic control in individual patients during the observation period could influence our results.

In conclusion, we found a statistically significant association between MnSOD Val/Val and GSTM1-1 genotype and development of DR in type 1 diabetes. Additionally, a combination of both MnSOD Val/Val and GSTM1-1 genotypes further increased the risk for DR. Testing for genetic markers in genes encoding antioxidant enzymes either individually or in combination could be added to a genetic panel for identifying patients at higher risk for developing DR in clinical practice.

ACKNOWLEDGMENTS
We thank Mrs Jurka Feran for her expert technical assistance and dr. Maruša Debeljak and dr. Barbka Repič Lampret for their advices. We are also grateful to Petra Bohanec Grabar from the Institute of Biochemistry, Medical Faculty, University of Ljubljana for the help with real-time PCR. This work was supported in part by the Slovenian Research Agency, grants no. J3-9663 and no. P3-0343.

Disclosure: The authors declare no conflict of interest.
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Table 1: Characteristics of patients with type 1 diabetes with chronic complications (DR or DN case subjects) and without chronic complications (control subjects)

|                        | Control subjects(N=62) | Case subjects(N=62) | P       |
|------------------------|------------------------|---------------------|---------|
| Age (years)            | 26.8 ± 5.5             | 27.4 ± 5.8          | 0.072   |
| Sex (male/female)      | 35/27                  | 35/27               | -       |
| Age at onset (years)   | 8.5 ± 4.4              | 7.3 ± 3.4           | 0.257   |
| Duration of diabetes (years) | 17.9 ± 5.6      | 19.3 ± 5.8          | 0.087   |
| Mean HbA1c             | 8.1 ± 1.1              | 8.2 ± 1.0           | 0.748   |

Data are given as means ± SD (standard deviation). Patient groups were matched by sex, age, age at onset and duration of diabetes. P values are result of t test for comparison of continuous parameters between both groups.

Table 2: Genotype frequencies of MnSOD, GSTM1, GSTT1 and CAT polymorphisms in patients with type 1 diabetes with retinopathy (DR subjects) or nephropathy (DN subjects)

|          | DR/control                        | P     | OR   | 95% CI | DN/control                       | P     | OR   | 95% CI |
|----------|------------------------------------|-------|------|--------|----------------------------------|-------|------|--------|
| MnSOD    |                                    |       |      |        |                                  |       |      |        |
| genotype Ala/Ala | 18.8 / 28.3     | 0.290 | 1.71 | 0.63-4.63 | 18.9 / 28.7     | 0.253 | 1.73 | 0.67-4.45 |
| genotype Ala/Val | 46.9 / 54.3     | 0.530 | 0.77 | 0.34-1.74 | 62.2 / 48.3     | 0.157 | 1.76 | 0.80-3.86 |
| genotype Val/Val | 34.4 / 17.4    | 0.045 | 2.49 | 1.00-6.16 | 18.9 / 23.0     | 0.615 | 0.78 | 0.30-2.05 |
| CAT      |                                    |       |      |        |                                  |       |      |        |
| genotype CC | 64.5 / 52.7     | 0.255 | 0.61 | 0.26-1.43 | 62.2 / 52.9     | 0.346 | 0.68 | 0.31-1.51 |
| genotype CT | 32.3 / 41.8     | 0.35  | 0.66 | 0.28-1.57 | 32.4 / 42.4     | 0.303 | 0.65 | 0.29-1.47 |
| genotype TT | 3.2 / 5.5      | 0.614 | 1.74 | 0.19-15.54 | 5.4 / 4.7       | 0.870 | 0.86 | 0.15-4.94 |
| GSTM1    |                                    |       |      |        |                                  |       |      |        |
| genotype GSTM1-1 | 75.0 / 46.7     | 0.031 | 2.63 | 1.07-6.47 | 59.5 / 58.6     | 0.931 | 1.03 | 0.47-2.26 |
| genotype GSTM1-0 | 25.0 / 53.3     | -     | 1.00 | -       | 40.5 / 41.4     | -     | 1.00 | -       |
| GSTT1    |                                    |       |      |        |                                  |       |      |        |
| genotype GSTT1-1 | 81.2 / 75.0     | 0.472 | 1.44 | 0.53-3.95 | 81.1 / 74.7     | 0.443 | 1.45 | 0.56-3.76 |
| genotype GSTT1-0 | 18.8 / 25.0     | 0.000 | 2.19 | 0.88-5.49 | 18.9 / 25.3     | 0.000 | 2.19 | 0.88-5.49 |

Odds ratio (OR) and 95% confidence interval (CI) were calculated from crosstabulation 2x2 table and risk estimate of one genotype versus the rest of all others genotypes comparing patients with type 1 diabetes with DR or DN and without DR or DN.

Table 3: Allele frequencies of iNOS (CCTTT)n gene polymorphism in patients with type 1 diabetes with diabetic retinopathy (DR subject) or diabetic nephropathy (DN) and control subjects

| Number of (CCTTT) repeats | Size (base pairs) | DR subjects N (%) | Control subjects N (%) | DN subjects N (%) | Control subjects N (%) |
|---------------------------|-------------------|-------------------|------------------------|-------------------|------------------------|
| 8                         | 176               | 1.8               | 1.6                    | 0                 | 2.4                    |
| 9                         | 181               | 3.6               | 3.3                    | 1.4               | 4.2                    |
| 10                        | 186               | 10.7              | 13.6                   | 12.2              | 13.3                   |
| 11                        | 191               | 19.6              | 23.4                   | 31.1              | 18.7                   |
| 12*                       | 196               | 35.7              | 29.9                   | 29.7              | 31.9                   |
| 13                        | 201               | 16.1              | 17.4                   | 16.2              | 17.5                   |
| 14                        | 206               | 8.9               | 8.2                    | 8.1               | 8.4                    |
| 15                        | 211               | 3.6               | 2.2                    | 1.4               | 3.0                    |
| 16                        | 216               | 0                 | 0.5                    | 0                 | 0.6                    |

Four patients were excluded from the analysis due to insufficient PCR amplification of the iNOS microsatellite locus. P values are result of Pearson Chi square test comparing allele versus all the other alleles.

*OR=2.19, 95% CI=0.88-5.49, P=0.089
Table 4: Genotype frequencies for gene gene interactions between patients with type 1 diabetes with diabetic retinopathy (DR subject) or without retinopathy (control subjects)

|                               | DR subject | Control subject | P    | OR   | 95% CI       |
|-------------------------------|------------|-----------------|------|------|--------------|
|                               | N=32       | N=92            |      |      |              |
| **GSTM1-1 and GSTT1-1**       |            |                 |      |      |              |
| Carriers                      | 53.1       | 35.9            | 0.087| 2.03 | 0.89-4.58    |
| Non carriers                  | 46.9       | 64.1            |      |      |              |
| **MnSOD Val/Val and GSTM1-1** |            |                 |      |      |              |
| Carriers                      | 41.7       | 14.3            | 0.009| 4.24 | 1.37-13.40   |
| Non carriers                  | 58.3       | 85.7            |      |      |              |
| **MnSOD Val/Val and GSTT1-1** |            |                 |      |      |              |
| Carriers                      | 30.8       | 17.4            | 0.154| 2.11 | 0.75-5.97    |
| Non carriers                  | 69.2       | 82.6            |      |      |              |

Odds ratio (OR) and 95% confidence interval (CI) were calculated from crosstabulation 2x2 table composing carrier versus non carrier of particular genotype among patients with type 1 diabetes with retinopathy and without retinopathy.