The joint effect of the endothelin receptor B gene (*EDNRB*) polymorphism rs10507875 and nitric oxide synthase 3 gene (*NOS3*) polymorphism rs869109213 in Slovenian patients with type 2 diabetes mellitus and diabetic retinopathy

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ABSTRACT

Increasing evidence suggests that endothelin and nitric oxide synthase genes and their products exert biological effects on the vasculature via the nitric oxide or endothelin pathway. The aim of the study was to evaluate the association of rs10507875 and rs869109213 (alone or in interaction) with diabetic retinopathy (DR) in subjects with type 2 diabetes mellitus (T2DM). We genotyped the single nucleotide polymorphism rs10507875 of the endothelin receptor B gene (*EDNRB*) and variable number tandem repeats rs869109213 of the nitric oxide synthase 3 gene (*NOS3*) in 270 Slovenian patients with DR and T2DM and 256 controls with T2DM without clinical signs of DR. The genotyping was performed using either real-time polymerase chain reaction (PCR) or standard PCR. We found a significant association between the genotypes of *NOS3* rs869109213 polymorphism and the risk of DR in the co-dominant model (4a4b genotype; 1.99-fold increased risk [1.09-3.65]; 95% confidence interval [CI]; p = 0.02), co-dominant model (4a4a genotype; 4.16-fold increased risk [1.03-16.74]; 95% CI; p = 0.04), and dominant model (4a4a and 4a4b genotypes; 2.22-fold increased risk [1.26-3.92]; 95% CI; p = 0.01) compared to the 4b4b genotype. Moreover, the joint effect of the two polymorphisms on DR risk was greater than the individual effect of each polymorphism in the analyzed genetic models. Additionally, adjusted odds ratio showed an increased risk in dominant × dominant (4.15-fold [1.40-12.26]; 95% CI; p = 0.01) and recessive × dominant (2.24-fold [1.25-4.01]; 95% CI; p = 0.02) genotype combinations of the two polymorphisms. In conclusion, our results indicate that *NOS3* rs869109213 polymorphism alone or in a combination with *EDNRB* rs10507875 polymorphism may be associated with DR in Slovenian patients with T2DM.

KEY WORDS: Nitric oxide synthase 3; NOS3; endothelin receptor B; EDNRB; diabetic retinopathy; DR; type 2 diabetes mellitus; T2DM; polymorphism; genetic model of inheritance

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INTRODUCTION

Most diabetic patients, especially those with poor glycemic control, develop diabetic retinopathy (DR), which remains the major cause of new-onset blindness among the working population in developed countries [1]. Both genetic and environmental factors are involved in the development of DR [2].

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DR is largely asymptomatic, and when the loss of vision develops, retinal vascular pathology may be at the advanced stage [3]. Endothelial dysfunction in diabetes is very likely to have a major effect on the retinal microcirculation because the circulation in the retina depends entirely on endothelium-mediated autoregulation, due to the absence of extrinsic innervation [4]. Relatively few studies have investigated the relationship between endothelial function and DR, with differing results [3,5,6].

The nitric oxide synthase 3 (*NOS3*) and endothelin 1 (ET-1; *EDN1)* genes and their products play an important role in the regulation of endothelial function [6]. In addition, the

modulation of vasomotor tone is affected by the actions of endothelin receptor A (ET_A) and ET_B [7]. ET_B has a dual role in the regulation of vascular tone. The activation of ET_B located on the endothelium stimulates the production of NO and indirectly has a vasorelaxant effect on the underlying smooth muscles, whereas ET_B located in the vascular smooth muscles causes vasoconstriction [8]. When ET-1 binds to ET_B located on the endothelial cells [ECs] (maximum 20% of the total ET-1 produced by ECs), the NOS3 activity is enhanced [9,10]. Stimulation of NOS3 may serve as an inhibitory feedback mechanism to prevent excessive and potentially damaging ET-1 signaling [11].

Although rs10507875 polymorphism of the ET_B gene (*EDNRB*) is not in the coding region (GRCh37.p13, Ensembl, April 2017), previous studies indicated an association between the SNPs in the non-coding regions of the *EDNRB* and the regulation of transcription [12,13].

Despite the constitutive expression of *NOS3* gene, numerous stimuli regulate its expression at the transcriptional, posttranscriptional, and posttranslational levels. Recently, the posttranscriptional regulation of the *NOS3* gene by small interfering RNAs (siRNAs) was reported [14]. This may consequently decrease the NO production leading to lower NOS3 levels [14].

The aim of this study was to evaluate the association of rs10507875 of the *EDNRB* and rs869109213 of the *NOS3* gene (alone or in interaction) with DR in subjects with Type 2 diabetes mellitus (T2DM).

MATERIALS AND METHODS

Patients

In this case-control retrospective study we evaluated 526 unrelated Caucasians with T2DM of at least 10-year duration and a defined ocular status, admitted to the general hospitals in Murska Sobota, Maribor, Slovenj Gradec, Ljubljana, and Izola. The patients had not been monitored for glycemic control. DR screening was performed at the Eye Clinic, University Medical Center in Ljubljana, between December 2005 and September 2014. After an informed consent was obtained, a detailed interview was performed. To avoid the confounding effect of impaired kidney function, patients with overt nephropathy were not included in the study.

T2DM was diagnosed according to the current American Diabetes Association diagnostic criteria [15]. Fundus examination was performed by a senior ophthalmologist after pupil dilation with 2.5% tropicamide and phenylephrine, using a slit lamp biomicroscope with non-contact lens and electronically documented using a fundus camera with a 50-degree angle (Topcon-TRC 40-IX, Tokyo, Japan). DR staging was determined according to the Early Treatment Diabetic Retinopathy Study (ETDRS) retinopathy severity scale [16]. The study group (DR) included 270/526 patients with T2DM and DR, while the control group consisted of 256/526 individuals with T2DM who had no clinical signs of DR.

DNA extraction

DNA extraction was performed at the Laboratory for Molecular Genetics, Institute of Histology and Embryology, Medical Faculty in Ljubljana. Genomic DNA was extracted from 200 µL of whole blood using QIAgen DNA Blood Mini Kit and QIACube robot workstation (Qiagen GmbH, Hilden, Germany), according to the blood and body fluid spin protocol V3.

Single nucleotide polymorphism (SNP) selection

Based on the latest data available in the Ensembl Genome Browser for vertebrate genomes we selected two polymorphisms: rs10507875, a SNP located at chromosome 13 in the intron 7 of *EDNRB* gene and rs869109213, variable number of tandem repeats (VNTRs) 27 base pairs (bp) long, positioned in the intron 4 close to the 5' end of *NOS3* gene, at chromosome 7. The G allele of rs10507875 results in a non-coding variant (Sequence Ontology, Ensembl) of unknown functional significance (as determined by variant effect predictor of Ensembl, GRCh38.p7 and UCSC Genome Browser on Human, GRh38/hg38). Similarly, the functional role of rs869109213 in DR is controversial.

Polymerase chain reaction (PCR)

PCR for rs10507875 was performed on a StepOne Real-Time PCR System (Applied Biosystems Inc., Massachusetts, USA), according to the manufacturer's instructions. The real-time PCR reactions were set up in a final volume of 5 µL using KASP by Design (KBD) Assay mix (LGC Genomics, Middlesex, UK), containing: 2.5 µL of KASP Master Mix, 0.07 µL of KBD mix, 3.39 µL of nuclease-free water, and 15 ng of genomic DNA. The PCR amplification was carried out under the following cycling conditions: Step 1 - 94°C for 15 minutes; Step 2 - Touchdown protocol at 94°C for 20 seconds, 61°C for 60 seconds, 61-55°C (drop - 0.6°C/per cycle) for 60 seconds, and repeating 9 times the cycles at 94°C for 20 seconds and 61°C for 60 seconds, achieving the annealing temperature of 55°C; step 3 - repeating 25 times cycles at 94°C for 10 seconds and 55°C for 60 seconds.

rs869109213 was analyzed using the method of Wang et al. [17]. DNA samples were subjected to conventional PCR using primers 5-AGG CCC TAT GGT AGT GCC TTT-3 (forward), and 5-TCT CTT AGT GCT GTG GTC AC-3 (reverse). The PCR reaction was performed in a final volume of 10 µL containing: 2.0 µL of 5 × Go Taq® reaction

buffer (Promega, Wisconsin, USA), 0.4 μL of each primer (TIB MOLBIOL GmbH, Germany), 0.2 μL of 10 mM deoxynucleotide triphosphates (dNTP) Mix (Promega, Wisconsin, USA), 0.5 μL of 25 mM $MgCl_2$ (Promega, Wisconsin, USA), 0.5 U Go Taq DNA polymerase (Promega, Wisconsin, USA), 5.9 μL of nuclease-free water, and 25 ng of genomic DNA. After the initial denaturation at 95°C for 5 minutes, 30 cycles were conducted: 94°C for 60 seconds, annealing at 56°C for 45 seconds, and at 72°C for 2 minutes. The final extension lasted for 5 minutes at 72°C. The PCR products were analyzed by electrophoresis in a 3% agarose gel and visualized with SYBR safe DNA gel stain (Thermo Fisher Scientific Inc., Massachusetts, USA). The wild-type 4b allele generated a 420 bp product, while the mutant 4a allele generated a 393 bp product.

Statistical analysis

Statistical analyses were conducted using IBM SPSS Statistics for Windows, Version 20.0. (IBM Corp., Armonk, NY, USA). Continuous variables were compared by unpaired Student's t-test, while Chi-square test was used to compare discrete variables. Data were expressed as mean ± standard deviation [SD] (continuous variables) or as the number and percentage of patients (categorical variables). All variables that showed significant differences in the univariate analysis were analyzed together in a binary logistic regression analysis. A value of $p < 0.05$ was considered statistically significant. The deviation from Hardy–Weinberg equilibrium (HWE) was assessed by the Fisher's exact test (http://ihg.gsf.de/) [18]. The joint effects of the two polymorphisms were analyzed in a logistic regression model, where different combinations of the recessive (Rec) and dominant (Dom) models were considered. Logistic regression was used to compute odds ratio (OR) by adjusting for potential confounders, including the duration of diabetes, insulin therapy, glycated hemoglobin (HbA_{1c}), blood pressure level, body mass index (BMI), cholesterol, and triglyceride concentration (Table 1).

RESULTS

The demographic and clinical data of 526 enrolled patients with T2DM are shown in Table 1. Out of the 526 patients, 256 patients had no clinical signs of DR (controls), and the remaining 270 had DR. There were no significant differences between the groups with respect to the age, sex, history of hypertension, and smoking status. However, a statistically significant difference was observed in the following parameters: Duration of diabetes, insulin treatment, HbA_{1c}, BMI, systolic/diastolic blood pressure, low-density lipoprotein (LDL), high-density lipoprotein (HDL), total cholesterol levels, and triglycerides. The duration of diabetes in patients with DR was at least 5 years longer compared to controls. A significantly higher proportion of T2DM patients with DR required insulin treatment compared to diabetics without DR. T2DM patients with DR had higher HbA_{1c}, systolic/diastolic blood pressure, total cholesterol, and LDL cholesterol levels, whereas BMI and HDL cholesterol levels were significantly lower in DR compared to control group.

TABLE 1. Clinical and laboratory characteristics of T2DM patients with and without DR (controls) for minimum 10 years

Characteristics	Patients (n=270)	Controls (n=256)	*p*
Age (years)	64.1±9.7	65.7±9.6	0.06
Sex – Male (%)	141 (52.2)	132 (51.6)	0.9
Duration of T2DM (years)	18.8±7.3	13.3±3.6	<0.001
Patients on insulin therapy (%)	197 (73.8)	112 (45.0)	<0.001
HbA1c (%)*	8.1±1.4	7.4±1.1	<0.001
Systolic blood pressure (mmHg)	144±20	141±17	0.01
Diastolic blood pressure (mmHg)	84±9	82±10	0.04
BMI (kg/m^2)	28.5±4.4	29.4±3.8	0.02
History of hypertension (%)	212 (78.5)	193 (75.4)	0.4
Total cholesterol (mmol/L)	4.9±1.2	4.7±1.1	0.01
LDL cholesterol (mmol/L)	2.9±0.9	2.7±0.8	0.01
HDL cholesterol (mmol/L)	1.1±0.2	1.2±0.3	<0.001
Triglycerides (mmol/L)	1.9±0.8	1.7±0.7	0.02
Smokers (%)	25 (9.5)	17 (7.0)	0.3

The values represent mean±SD. Bold indicates statistically significant results. The average value for HbA1c is provided. T2DM: Type 2 diabetes mellitus; DR: Diabetic retinopathy; HbA1c: Glycated hemoglobin; BMI: Body mass index; LDL: Low-density lipoprotein; HDL: High-density lipoprotein

The distribution of genotype and allele frequencies in DR and control groups is shown in Table 2.

The population data, including genotype and allele frequencies, for rs10507875 were published in the 1000 genomes project Phase 3 (for populations of European ancestry a allele = 84.8%, g allele = 15.2%). However, no similar reports are available for rs869109213. The average allele frequencies for rs869109213 in our study population (526 patients with T2DM) were 83.6% for 4b and 16.4% for 4a allele.

The two polymorphisms were in HWE in both DR (rs10507875: $p = 0.22$; rs869109213: $p = 0.06$) and control (rs10507875: $p = 0.09$; rs869109213: $p = 0.09$) groups.

The genotype and allele distribution of rs10507875 and rs869109213 polymorphisms differed significantly between DR and control group (Table 2). Following these observations, we used a logistic regression analysis to evaluate whether these polymorphisms were independently associated with DR after adjusting for the duration of T2DM, insulin therapy, HbA_{1c}, BMI, total cholesterol, LDL and HDL levels, and triglycerides. The 4a4a and 4b4a genotypes of rs869109213 polymorphism were associated with an increased risk of DR compared with the 4b4b genotype ($OR_{co\text{-}dominant(Co\text{-}dom)}1$ = 1.99-fold, 95% confidence interval [CI] = 1.09-3.65, $p = 0.02$; $OR_{Co\text{-}dom}2$ = 4.16-fold [1.03-16.74]; 95% CI; $p = 0.04$). The association of rs869109213

with DR was also observed when we applied Dom model (OR_{Dom} = 2.22-fold, 95% CI = 1.26-3.92, p = 0.01). On the contrary, no association was observed between rs10507875 and DR in any of the tested models (Table 3).

Finally, logistic regression analysis was used for the evaluation of the joint effect of two polymorphisms. The results indicated that the joint effect of rs869109213 and rs10507875 on DR risk was greater than the individual effect of each polymorphism in the analyzed genetic models. After adjusting for the confounding variables found relevant in the univariate analysis, we demonstrated the strength of association with $OR_{Rec \times Dom}$ of 2.24-fold ([1.25-4.0] 95% CI, p = 0.007) for the individuals with genotypes 4a4a/4a4b (rs869109213) and AA/AG (rs10507875) compared to carriers of 4b4b (rs869109213) and AA/AG (rs10507875). The genotypes 4a4a/4a4ba (rs869109213) in a combination with GG/AG (rs10507875), compared to the reference 4b4b (rs869109213) and AA (rs10507875), pointed out a significantly increased risk for DR in Dom × Dom combination. The OR was 4.15-fold (1.40-12.26), 95% CI, p = 0.01. Moreover, the carriers of 4a4a/4a4ba (rs869109213) and AA (rs10507875) compared with the aforementioned reference had $OR_{Dom \times Dom}$ of 2.18-fold (1.14-4.16), 95% CI, p = 0.02 (Figure 1).

DISCUSSION

In this case-control study, we showed that rs869109213 of the *NOS3* gene alone and in a combination with rs10507875 of the *EDNRB* gene is associated with DR. Since it was characterized in the mid-1990s, many polymorphic sites have been described in the human *NOS3* gene, including the selected VNTR. However, previous studies on the association between rs869109213 and the risk for DR among T2DM patients reported conflicting and inconclusive results, which might be due to interethnic differences in the distribution of *NOS3* polymorphisms [14,19].

TABLE 2. Distribution of rs10507875 and rs869109213 genotypes and alleles in T2DM patients with and without DR (controls) for minimum 10 years

Gene	Polymorphism	Genotype/allele	Patients n=270 (%)	Controls n=256 (%)	*p*
EDNRB	rs10507875 intron 1; g. 37411 A>G	GG	9 (3.3)	5 (2.0)	0.03
		AG	65 (24.1)	41 (16.0)	
		AA	196 (72.6)	210 (82.0)	
		G	83 (15.4)	51 (10.0)	0.008
		A	457 (84.6)	461 (90.0)	
NOS3	rs869109213 intron 4; g. 11112_11138 GAAGTCTAGACCTGCTGCAGGGGTGAG [4][5][3]	4a4a	16 (5.9)	7 (2.7)	0.006
		4b4a	77 (28.5)	50 (19.5)	
		4b4b	177 (65.6)	199 (77.7)	
		4a	109 (20.2)	64 (12.5)	0.001
		4b	431 (79.8)	448 (87.5)	

Bold indicates statistically significant results. T2DM: Type 2 diabetes mellitus; DR: Diabetic retinopathy; EDNRB: Endothelin receptor type B; NOS3: Nitric oxide synthase 3

TABLE 3. Association between *EDNRB* and *NOS3* polymorphisms and the risk of DR in T2DM patients

Gene/polymorphism	Model	Number of patients/controls	OR* (95% CI)	*p**
EDNRB/rs10507875 (NG_011630.2:g. 37411 A > G)	Codominant:			
	GG versus AA (reference)	9/5 versus 196/210	1.64 (0.41-6.62)	0.49
	AG versus AA (reference)	65/41 versus 196/210	1.88 (0.99-3.57)	0.05
	Dominant:			
	GG + AG versus AA (reference)	74/196 versus 48/208	1.84 (1.01-3.35)	0.05
	Recessive:			
	GG versus AG + AA (reference)	9/5 versus 261/256	1.47 (0.37-5.88)	0.59
NOS3/rs869109213 (NG_11992.1:g. 11112_11138 GAAGTCTAGAC CTGCTGCAGG] GGTGAG[4][5][3])	Codominant:			
	4a4a versus 4b4b (reference)	16/7 versus 177/199	4.16 (1.03-16.74)	0.04
	4a4b versus 4b4b (reference)	77/50 versus 177/199	1.99 (1.09-3.65)	0.02
	Dominant:			
	4a4a + 4a4b versus 4b4b (reference)	93/57 versus 177/199	2.22 (1.26-3.92)	0.01
	Recessive:			
	4a4b + 4b4b versus 4a4a (reference)	54/249 versus 16/7	3.60 (0.90-14.37)	0.07

*Adjusted for the duration of diabetes, insulin therapy, HbA1c, BMI, total cholesterol, LDL, HDL levels, and triglycerides. Bold indicates statistically significant results. DR: Diabetic retinopathy; T2DM: Type 2 diabetes mellitus; EDNRB: Endothelin receptor type B; NOS3: Nitric oxide synthase 3; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; BMI: Body mass index; HbA1c: Glycated hemoglobin

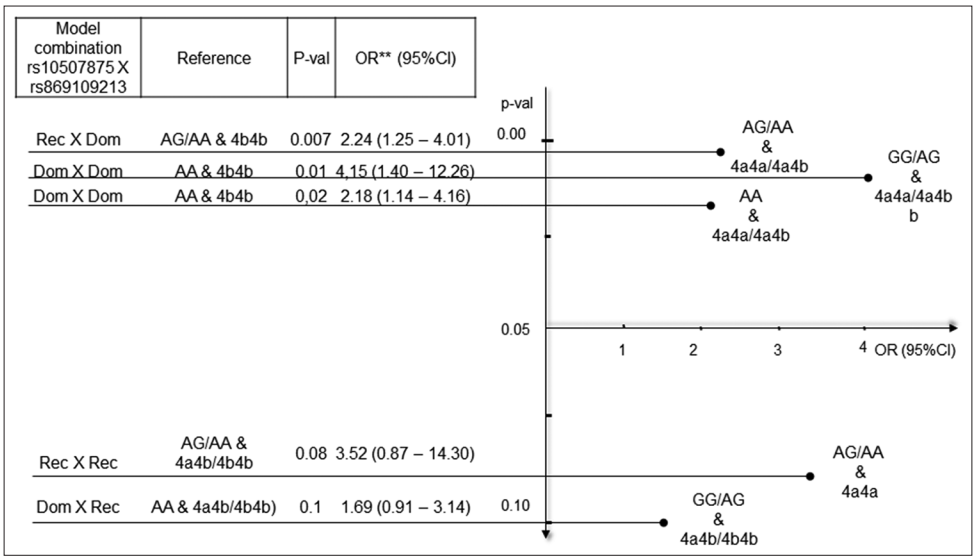

FIGURE 1. The joint effect of rs10507875 and rs869109213 on the risk for diabetic retinopathy. **Adjusted odds ratio for low-density lipoprotein, high-density lipoprotein, triglycerides, glycated hemoglobin, body mass index, diastolic pressure, insulin therapy, duration of diabetes, and arterial hypertension (AH). Rec: Recessive; Dom: Dominant.

Here we confirmed an association between the 4a4a homozygotes of rs869109213 and the occurrence of DR in Slovenian (Caucasians) patients with T2DM. As expected, the frequencies of genotype 4a4a in Slovenian patients with T2DM ($\%_{patients}$ = 5.9, $\%_{controls}$ = 2.7) did not differ from those reported in various populations with diabetes (2.1-16%) [20]. Assuming the standard models of inheritance, our T2DM patients with either 4a4a genotype (co-dominant model), 4a4b (co-dominant model), or 4a4a+4a4b (dominant model) had a significantly higher risk of DR compared to the carriers of 4b4b genotype (wild-type).

Several other authors reported the association between the *NOS3* rs869109213 and DR in Caucasians with T2DM [19,21]. For example, Cheema et al. [21] found that the 4a4a genotype of rs869109213 was an independent protective factor for DR and was associated with a low risk of DR. Moreover, the NO levels in their T2DM patients with 4b4a and 4a4a genotypes were significantly higher in comparison with other genotypes. The authors suggested that *NOS3* rs869109213 might be a good candidate as a biomarker of DR in Asian North Indian patients with T2DM [21].

To the best of our knowledge, this is the first study to demonstrate an association between *EDNRB* rs10507875 and DR in Caucasians with T2DM. The minor, G, allele occurred more frequently in our diabetic patients with DR compared to controls. However, we found no significant association between the risk of DR and rs10507875 in T2DM patients, using the standard genetic models.

A mutation in the *EDNRB* gene was associated with Hirschsprung's disease and hearing loss [12]. Moreover, SNPs linked to rs10507875 with a very high linkage disequilibrium (D' = 1 and $r^2 > 0.95$ for populations of European ancestry: rs12720174, rs7317759, rs17068554, rs4885492, rs12720167, rs7333867, rs17068547, rs4884073, rs3027111, and rs12585038) have been associated with other disease phenotypes, such as: Contraction of the vascular smooth muscle cells, vasoconstriction, negative regulation of cellular protein metabolic process, endothelin receptor (EDNR) signaling, and EDNR activity (GRCh37.p13, Ensembl, April 2017) (http://grch37.ensembl.org/Homo_sapiens/Variation/HighLD?db=core;r=13:78516754-78517754;v=rs10507875;vdb=variation;vf=5876751#373514_tablePanel). Furthermore, MacClellan et al. observed a significant association of rs10507875 with ischemic stroke in Caucasian women [22].

Hein et al. [23] suggested that ET_B-mediated vasoconstriction in retinal ischemia and diabetes might be enhanced through a mechanism that selectively regulates the *EDNRB* expression [23].

In this study, we selected the two polymorphisms as potential risk factors of DR in T2DM patients, based on the role of ligand/receptor relationship and the importance of endothelial function in regulating vascular blood flow. Interestingly, our results indicated that the combination of rs10507875 and rs869109213 could affect the susceptibility for DR in T2DM patients. The occurrence of DR was 2.2-fold higher in Dom × Rec and Dom × Dom combinations. Although rs10507875 was not significantly associated with DR in this study, the inclusion of the GG genotype in Dom × Dom combination (rs10507875 × rs869109213) further increased the risk for DR, with $OR_{Dom \times Dom}$ of 4.15.

The limitations of our study are the relatively small sample size, duration of T2DM in controls, and the lack of a direct biochemical evidence such as the level of gene and protein

expression. The duration of diabetes in our control group was relatively long (10-15 years). We selected this period based on the fact that around 20% of T2DM patients, aged 20-74 years, develop DR after more than 5 years of the onset of T2DM, with majority of the patients developing DR after the first two decades of the onset of diabetes [24]. Thus, it is possible that some of the T2DM patients were inadequately excluded from the control group due to the short follow-up period. Nevertheless, this probably does not affect our results significantly, as T2DM duration was used as a covariate in the logistic regression analysis.

DR is a multifactor disease possibly determined by multiple genes. For accurate determination of reliable DR genetic markers a higher number of patients from diverse populations is required.

Generally, the *NOS3*/*EDNRB* gene activity and the effect of polymorphisms on gene expression are difficult to analyze. Namely, the tissue-specific activity of *EDNRB* and rapid diffusion of NO (present in subnanomolar concentrations in cells), away from the location of protein synthesis, make the *in vivo* measuring complicated, often with contradictory results [8,25].

Further evidence is necessary to consolidate our observations and to confirm the joint effect of rs869102213 and rs10507875 on the risk of DR in T2DM patients.

CONCLUSION

Our results indicate that *NOS3* rs869109213 polymorphism alone or in a combination with *EDNRB* rs10507875 may be associated with DR in Slovenian patients with T2DM.

To the best of our knowledge, this is the first report on the joint effect of rs10507875 and rs869109213 on the risk of DR in T2DM patients.

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DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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