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Association of *eNOS* gene polymorphisms with renal disease in Caucasians with type 2 diabetes

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ABSTRACT

Aim: In this study we investigated if the −786T > C, the VNTR intron 4 a/b and the 894G > T (Glu298Asp) polymorphisms in the *eNOS* gene were associated with renal disease in 617 type 2 diabetic Caucasian-Brazilians. These polymorphisms were also examined in 100 Caucasian healthy blood donors.

Methods: Genotyping of *eNOS* polymorphisms was performed by PCR or PCR-RFLP and haplotype frequencies were estimated using a Bayesian method. Logistic regression analysis was done to test for association of *eNOS* polymorphisms with susceptibility to renal involvement (microalbuminuria, macroalbuminuria or end-stage renal disease). This analysis was carried out assuming three diferent genetic models for the minor allele, adjusting for possible effect modifiers.

Results: Genotype and allele frequencies in patients with renal disease were not significantly different from those of patients with normoalbuminuria and healthy blood donors for all *eNOS* polymorphisms. Likewise, there were no differences in haplotype frequencies among healthy blood donors and type 2 diabetic patients with or without renal involvement ($P > 0.05$ for all comparisons).

Conclusion: No associations between the −786T > C, the VNTR intron 4 a/b and the 894G > T (Glu298Asp) polymorphisms in the *eNOS* gene and renal disease were observed in type 2 diabetic Caucasian-Brazilians.

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1. Introduction

Diabetic nephropathy (DN) is the leading cause of chronic kidney disease in patients starting renal replacement therapy and it is associated with increased cardiovascular mortality. Although poor glycemic control and arterial hypertension are strong risk factors for this complication [1], several lines of evidence have suggested that its development also depends on genetic factors [1–3]. The endothelial nitric oxide synthase (*eNOS*) has been considered to be a potential candidate gene for susceptibility to DN since chromosome 7q35 was indicated as a candidate region containing genes for susceptibility to this complication of diabetes in Pima Indians [4] and in Caucasians and non-Caucasians [5].

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Endothelium-derived nitric oxide (NO) plays a key role in the regulation of vascular tone. It exerts vasoprotective effects by scavenging superoxide radicals and suppressing platelet aggregation, leukocyte adhesion and smooth muscle cell proliferation [6]. Therefore, a reduction in basal NO release may predispose individuals to vascular abnormalities, such as hypertension and atherosclerosis. On the other hand, overproduction can also damage cells and tissues because NO increases the accumulation of reactive oxygen species, which in turn leads to atherogenesis [6,7]. In the endothelium, NO is mainly synthesized by the *eNOS* isoform, a constitutive enzyme whose expression is regulated by several factors, such as cytokines and smoking [7].

Variants of the *eNOS* gene have been shown to modify its expression or activity [8], thus leading to reduced or excessive NO production and consequently contributing to many pathological processes. Among the several polymorphisms identified in the *eNOS* gene, three have been subjects of intensive research in relation to microvascular complications of diabetes, namely, the −786T > C substitution in promoter region (rs2070744), the tandem repeat of 27 bp in intron 4 (VNTR intron 4 a/b) and the 894G > T (Glu298Asp) missense substitution in exon 7 (rs1799983). These polymorphisms have been found to be associated with different stages of DN, ranging from an increase of albuminuria up to end-stage renal disease (ESRD) in diabetic patients on hemodialysis [9–22]. However, several other authors have not found any associations of *eNOS* polymorphisms with DN [23–35]. Moreover, a recent meta-analysis of candidate gene population-based association studies relating variants of *eNOS* gene to the risk of presenting DN or the so-called diabetes leading to severe nephropathy (DSN) showed no association of *eNOS* polymorphisms with both DN and DSN in type 2 diabetes, except for 894G > T variant [36].

Therefore, considering that DN is a complex disease with multifactorial etiology and that *eNOS* polymorphisms show a marked population variability in their distributions, the aim of this study was to evaluate whether the −786T > C, the VNTR intron 4 a/b and the 894G > T (Glu298Asp) polymorphisms in the *eNOS* gene are associated with renal disease (micro-, macro-albuminuria and end-stage renal disease) in Caucasian-Brazilians with type 2 diabetes.

2. Subjects, materials and methods

2.1. Subjects

This case–control study was carried out on 617 unrelated Caucasian-Brazilian type 2 diabetic patients participating in a multicentric study in the Brazilian State of Rio Grande do Sul. All Caucasian-Brazilians were subjects of European origin (mainly from Portugal, Spain, Italy, and Germany). Type 2 diabetes was diagnosed according to the American Diabetes Association criteria [37]. Cases were defined by increased urinary albumin excretion (UAE) (micro- and macro-albuminuria) or ESRD. Controls were patients with normoalbuminuria and known diabetes duration of at least 10 years.

Patients underwent a standardized clinical and laboratory evaluation that consisted of a questionnaire, physical examination, and laboratory tests. Weight and height were used to calculate body mass index (BMI) (kg/m^2). Blood pressure was measured after a 5-min rest in the sitting position using a standard mercury sphygmomanometer. Arterial hypertension was defined as blood pressure levels ≥140/90 mmHg, and patients being treated with antihypertensive medication whose blood pressure was lower than 140/90 mmHg were also considered hypertensive. Details about the onset of smoking and the cessation of smoking in ex-smokers were recorded with a questionnaire. Patients who were ex- or current-smokers were considered as having a positive history of smoking and they were compared with those who never smoked.

Assessment of DR was performed by ophthalmoscopic examination through dilated pupils. DR was graded as absent, non-proliferative, or proliferative [38]. In relation to renal status, cases were defined based on the UAE in at least two of three consecutive 24-h timed or random spot sterile urine collections for 76% and 24% of the patients, respectively. Patients were classified as having normoalbuminuria (UAE <20 μg/min or <17 mg/l) (n = 241), microalbuminuria (UAE 20–199 μg/min or 17–174 mg/l) (n = 171), macroalbuminuria (UAE ≥200 μg/min or >174 mg/l) (n = 95), or ESRD by the presence of chronic renal disease treated by dialysis when other causes of proteinuria or renal disease were ruled out (n = 110).

Venous blood samples were collected for biochemical analyses after a 12-h fast. Glycated hemoglobin was measured by an ion-exchange HPLC procedure (reference range: 4.7–6.0%). Serum creatinine concentrations were determined by Jaffé's reaction and AER by immunoturbidimetry (Sera-Pak immuno microalbuminuria; Bayer, Tarrytown, USA). Total cholesterol, HDL cholesterol and triglycerides were measured by standard enzymatic methods.

In order to estimate the allele frequencies in the general population, the −786T > C, the VNTR intron 4 a/b and the 894G > T polymorphisms were also examined in 100 Caucasians recruited among volunteer healthy blood donors (50 females, 50 males; mean age, 46.4 ± 9.6 years) from the Hospital de Clínicas de Porto Alegre (Porto Alegre, Brazil). All subjects participating in this study provided written informed consent, the protocol for which was approved by all hospital ethics committees.

2.2. *eNOS* genotyping

DNA was extracted from peripheral blood leukocytes by a salting out procedure [39]. Gene fragments containing the −786T > C (rs2070744) and the 894G > T (Glu298Asp) (rs1799983) variant sites in the *eNOS* gene were amplified by polymerase chain reaction (PCR) using the primers and conditions as previously described by Tanus-Santos et al. [40]. The amplification products were digested with the appropriate restriction enzymes under the conditions recommended by the manufacturer (MBI Fermentas, St. Leon-Rot., Germany). The digested fragments were then separated by electrophoresis in 6% polyacrylamide gels, followed by ethidium bromide staining and direct visualization under ultraviolet light. To improve genotyping accuracy, samples with known genotypes were used in each batch as positive controls to evaluate the completeness of PCR product

Table 1 – PCR primers, reaction conditions and restriction enzymes for the genotyping of *eNOS* polymorphisms.

Polymorphism	Primer sequence	Annealing temperature for PCR	PCR product (bp)	Restriction enzyme	Restriction enzyme digest fragment (bp)	Reference
−786T > C	5′-TGGAGAGTGCTGGTGTACCCCA-3′ 5′-GGCTCCACCCCCACCCTGTC-3′	60 °C	180	*MspI*	T: 140 + 40 C: 90 + 50 + 40	[40]
VNTR intron 4 a/b	5′-AGGCCCTATGGTAGTGCCTTT-3′	58 °C	393 (*a* allele)			[13]
	5′-TCTCTTAGTGCTGTGGTCAC-3′		420 (*b* allele) 447 (*c* allele)	–	–	
894G > T (Glu298Asp)	5′-AAGGCAGGAGACAGTGGATGGA-3′	59 °C	248	*Eco24I* (*BanII*)	G: 163 + 85	[40]
	5′-GCCAGTCAATCCCTTTGGTGCTCA-3′				T: 248	

digestion. The VNTR polymorphism in intron 4 was detected by PCR using primers and conditions that were previously described [13], and the alleles were identified according to the length of bands after separation of PCR fragments in ethidium bromide-stained 2% agarose gels. Genotyping was performed by laboratory personnel who were unaware of clinical characteristics of the patients and the details of genotyping method are shown in Table 1.

2.3. *Statistical analyses*

Comparisons between diabetic patients with or without renal involvement were accomplished using the unpaired Student's *t*-test for normally distributed variables or the Mann–Whitney *U*-test for variables with a skewed distribution (SPSS for Windows, version 10.0). Allele frequencies were determined by gene counting, and departures from the Hardy–Weinberg equilibrium were verified using the χ^2-test. The χ^2-test and Fisher's exact test, whichever appropriate, were used to evaluate the allele and genotype distributions among groups of subjects.

Logistic regression analysis was done to test for association of *eNOS* polymorphisms with type 2 diabetes, adjusting for gender and age, and renal disease (comparison between diabetic patients with renal involvement and diabetic patients with normoalbuminuria), adjusting for demographic and clinical variables that could be possible effect modifiers. These analyses were carried out assuming three diferent genetic models for the minor allele (allele contrast, dominant and recessive models), as proposed by some investigators [41]. Power calculations (PEPI program, version 4.0 [42]) showed that this study had a power of approximately 80% at a significance level of 0.05 to detect an odds ratio of 1.65 for the three *eNOS* polymorphisms (patients with renal disease compared to those with normoalbuminuria), under a dominant model (genotypes carrying at least one minor allele comparing to homozygote for major allele).

Table 2 – Clinical and demographic characteristics of type 2 diabetic patients according to the presence of renal disease.

	Renal disease		*P*-value
	Without (n = 241)	With (n = 376)	
Gender (% male)	35.3	57.2	0.001
Age (years)	62.0 ± 9.4	60.4 ± 9.7	0.047
Duration of diabetes (years)	16.7 ± 6.8	15.0 ± 9.1	0.003
Body mass index (kg/m^2)	28.2 ± 4.4	28.7 ± 5.0	0.366
Hypertension (%)	75.3	85.2	0.004
Systolic blood pressure (mmHg)	143 ± 23	148 ± 25	0.072
Diastolic blood pressure (mmHg)	85 ± 13	87 ± 14	0.420
Antihypertensive medications (%)			
ACEi	52.9	57.7	0.566
Diuretic	40.0	53.2	0.068
Beta-blocker	32.9	23.7	0.165
Calcium antagonists	20.0	30.8	0.099
Positive history of smoking (%)	40.0	47.9	0.069
Therapy for diabetes (% insuline use)	35.0	50.0	0.001
Glycated hemoglobin (%)	6.7 ± 1.7	6.6 ± 1.9	0.529
Serum creatinine (μmol/L)	80 (35–168)	97 (35–1193)	<0.001
Total cholesterol (mmol/L)	5.45 ± 1.09	5.52 ± 1.26	0.586
HDL cholesterol (mmol/L)	1.16 ± 0.27	1.08 ± 0.29	0.001
Triglycerides (mmol/L)	1.64 (0.39–7.44)	1.85 (0.50–16.60)	0.017
Diabetic retinopathy (%)	47.7	78.6	<0.001

Data are reported as mean ± SD, median (range), or percentage. *P*-values were obtained by the unpaired Student's *t*, Mann-Whitney *U*, or χ^2 tests, as appropriate. n = number of individuals. ACEi: angiotensin-converting-enzyme inhibitor. HDL: high-density lipoprotein. Patients who were ex- or current-smokers were considered as having a positive history of smoking.

Table 3 – Genotype and allele frequencies of *eNOS* polymorphisms in healthy blood donors and type 2 diabetic patients with or without renal disease, assuming three diferent genetic models.

Polymorphism	Blood donors	Diabetes	Diabetes	
			Without renal disease	With renal disease
−786T > C	*n* = 100	*n* = 617	*n* = 241	*n* = 376
Genotypes				
TT	42 (42.0)	233 (37.8)	93 (38.6)	140 (37.2)
TC	46 (46.0)	264 (42.8)	104 (43.2)	160 (42.6)
CC	12 (12.0)	120 (19.4)	44 (18.2)	76 (20.2)
P-value	–	0.202[a]	0.364[b]	0.168[c]; 0.830[d]
Alleles				
T	130 (65.0)	730 (59.2)	290 (60.2)	440 (58.5)
C	70 (35.0)	504 (40.8)	192 (39.8)	312 (41.5)
P-value	–	0.137[a]	0.273[b]	0.113[c]; 0.605[d]
Odds ratio (95% CI) for the C allele				
Allele contrast model				
Univariate	–	1.28 (0.93–1.77)[a]	1.23 (0.86–1.76)[b]	1.32 (0.94–1.85)[c]; 1.07 (0.84–1.36)[d]
Dominant model				
Univariate	–	1.20 (0.78–1.84)[a]	1.15 (0.72–1.85)[b]	1.22 (0.78–1.91)[c]; 1.06 (0.76–1.48)[d]
Multivariate	–	1.20 (0.68–1.85)[a]	1.02 (0.54–1.93)[b]	1.07 (0.62–1.83)[c]; 0.91 (0.60–1.37)[d]
Recessive model				
Univariate	–	1.77 (0.94–3.34)[a]	1.64 (0.82–3.25)[b]	1.86 (0.97–3.57)[c]; 1.13 (0.75–1.71)[d]
Multivariate	–	1.62 (0.79–3.34)[a]	1.90 (0.76–4.76)[b]	1.78 (0.83–3.79)[c]; 1.13 (0.70–1.83)[d]
VNTR intron 4 a/b	*n* = 100	*n* = 583	*n* = 233	*n* = 350
Genotypes				
bb	67 (67.0)	405 (69.5)	168 (72.1)	237 (67.7)
ba	30 (30.0)	158 (27.1)	59 (25.3)	99 (28.3)
aa	3 (3.0)	16 (2.7)	5 (2.2)	11 (3.1)
bc	–	4 (0.7)	1 (0.4)	3 (0.9)
P-value	–	0.881[a]	0.688[b]	0.976[c]; 0.678[d]
Alleles				
a	36 (18.0)	190 (16.3)	69 (14.8)	124 (17.7)
b	164 (82.0)	972 (83.4)	396 (85.0)	573 (81.9)
c	–	4 (0.3)	1 (0.2)	3 (0.4)
P-value	–	0.755[a]	0.510[b]	0.922[c]; 0.456[d]
Odds ratio (95% CI) for the *a* allele				
Allele contrast model				
Univariate	–	0.89 (0.59–1.35)[a]	0.89 (0.65–1.20)[b]	0.99 (0.64–1.52)[c]; 1.25 (0.90–1.75)[d]
Dominant model				
Univariate	–	0.86 (0.55–1.35)[a]	0.76 (0.46–1.27)[b]	0.93 (0.58–1.49)[c]; 1.22 (0.84–1.76)[d]
Multivariate	–	0.81 (0.47–1.39)[a]	0.63 (0.31–1.26)[b]	0.89 (0.50–1.57)[c]; 1.28 (0.83–2.00)[d]
Recessive model				
Univariate	–	0.91 (0.26–3.18)[a]	0.70 (0.16–3.01)[b]	1.05 (0.29–3.83)[c]; 1.49 (0.51–4.33)[d]
Multivariate	–	1.49 (0.23–9.80)[a]	2.05 (0.16–26.05)[b]	1.53 (0.21–10.92)[c]; 1.48 (0.41–5.40)[d]
894G > T (Glu298Asp)	*n* = 100	*n* = 609	*n* = 235	*n* = 374
Genotypes				
GG	47 (47.0)	294 (48.2)	118 (50.2)	176 (47.1)
GT	48 (48.0)	261 (42.9)	95 (40.4)	166 (44.4)

TT	5 (5.0)	54 (8.9)	22 (9.4)	32 (8.5)
P-value	–	0.351[a]	0.253[b]	0.471[c]; 0.628[d]
Alleles				
G	142 (71.0)	849 (69.7)	331 (70.4)	518 (69.3)
T	58 (29.0)	369 (30.3)	139 (29.6)	230 (30.7)
P-value	–	0.774[a]	0.955[b]	0.696[c]; 0.711[d]
Odds ratio (95% CI) for the T allele				
Allele contrast model				
Univariate	–	1.06 (0.76–1.50)[a]	1.03 (0.70–1.50)[b]	1.09 (0.76–1.55)[c]; 1.06 (0.82–1.37)[d]
Dominant model				
Univariate	–	0.95 (0.62–1.45)[a]	0.88 (0.55–1.40)[b]	1.00 (0.64–1.55)[c]; 1.14 (0.82–1.57)[d]
Multivariate	–	0.95 (0.57–1.56)[a]	0.79 (0.42–1.49)[b]	1.01 (0.60–1.72)[c]; 1.18 (0.79–1.76)[d]
Recessive model				
Univariate	–	1.85 (0.72–4.74)[a]	1.96 (0.72–5.34)[b]	1.78 (0.67–4.68)[c]; 0.91 (0.51–1.60)[d]
Multivariate	–	1.37 (0.46–4.06)[a]	1.48 (0.36–6.15)[b]	1.30 (0.41–4.06)[c]; 0.84 (0.44–1.63)[d]

Genotype and allele frequencies are shown as number (%). *n* = number of individuals. *P*-values were obtained using the χ^2 or Fisher's exact test, as appropriate. Odds ratios (ORs) with 95% confidence intervals (95% CI) were obtained by logistic regression analysis. Multivariate logistic regression analysis was carried out controlling for gender and age for comparisons between type 2 diabetic patients *vs*. healthy blood donors, type 2 diabetic patients without renal involvement *vs*. healthy blood donors and type 2 diabetic patients with renal involvement *vs*. healthy blood donors. For comparison between type 2 diabetic patients with renal disease *vs*. patients with normoalbuminuria, multivariate logistic regression model was adjusted for gender, duration of diabetes, smoking (ever smoked = 1, never smoked = 0), systolic blood pressure, insulin therapy (yes = 1, no = 0) and tryglicerides levels. Allele contrast model: Minor allele *vs*. major allele. Dominant model: genotypes carrying the minor allele *vs*. homozygous genotype for major allele. Recessive model: homozygous genotype for the minor allele *vs*. genotypes carrying major allele. As the *c* allele (VNTR intron 4a/b polymorphism) was very rare, carriers of this allele were excluded from the logistic regression analysis.

[a] Type 2 diabetic patients *vs*. healthy blood donors.

[b] Type 2 diabetic patients without renal disease *vs*. healthy blood donors.

[c] Type 2 diabetic patients with renal disease *vs*. healthy blood donors.

[d] Type 2 diabetic patients with renal disease *vs*. patients with normoalbuminuria.

Table 4 – Haplotype frequencies of *eNOS* polymorphisms in healthy blood donors and type 2 diabetic patients with or without renal disease.

Haplotype	Blood donors		Diabetes				Diabetes					
	Frequencies	S.E.	Frequencies	S.E.	Z-score	P-value	Without renal disease		With renal disease		Z-score	P-value
							Frequencies	S.E.	Frequencies	S.E.		
n	200		1222				474		748			
−786T/4b/894G	98 (49.0)	0.009	555 (45.4)	0.004	0.87	0.384	227 (48.0)	0.006	325 (43.5)	0.005	1.48	0.139
−786T/4b/894T	26 (13.0)	0.009	117 (9.6)	0.004	1.35	0.176	41 (8.6)	0.005	77 (10.3)	0.005	0.88	0.378
−786T/4a/894G	8 (4.0)	0.008	50 (4.1)	0.003	0.00	>0.999	15 (3.1)	0.005	36 (4.8)	0.004	1.31	0.192
−786T/4a/894T	–	–	–	–	–	–	–	–	–	–	–	–
−786C/4b/894G	9 (4.5)	0.008	100 (8.2)	0.004	1.68	0.093	39 (8.2)	0.005	64 (8.5)	0.005	0.08	0.937
−786C/4b/894T	31 (15.5)	0.009	248 (20.3)	0.004	1.49	0.137	97 (20.5)	0.006	152 (20.3)	0.005	0.01	0.991
−786C/4a/894G	27 (13.5)	0.009	147 (12.0)	0.003	0.48	0.628	52 (11.0)	0.005	92 (12.3)	0.005	0.60	0.552
−786C/4a/894T	1 (0.5)	0.005	5 (0.4)	0.002	0.00	>0.999	3 (0.6)	0.002	2 (0.3)	0.002	0.34	0.736
Permutation test *P*-value	–	–	0.127[a]	–			0.103[b]	–	0.181[c] 0.634[d]	–		

Haplotype frequencies are shown as number (%). *n* = number of chromosomes. S.E. = standard error. PHASE program version 2.1 was used to estimate the haplotype frequencies and to compare groups of subjects (cases and controls), computing *P*-values by a case–control permutation test. Individual haplotypes were compared between cases and controls using Z-score in PEPI program version 4.0. As the *c* allele (intron 4 VNTR) was rare, carriers of this allele were excluded from the haplotype analysis and the VNTR intron 4 a/b polymorphism was considered as a bi-allelic marker.

[a] Type 2 diabetic patients *vs.* healthy blood donors.

[b] Type 2 diabetic patients without renal disease *vs.* healthy blood donors.

[c] Type 2 diabetic patients with renal disease *vs.* healthy blood donors.

[d] Type 2 diabetic patients with renal disease *vs.* patients with normoalbuminuria.

The linkage disequilibrium between all pairs of *loci* was calculated and expressed in terms of D' and r^2 [43]. Haplotype frequencies were estimated by a Bayesian method using PHASE version 2.1 [44,45]. We also used the PHASE program to compare the distribution of different *eNOS* haplotypes between groups of subjects through permutation analyses of 1000 random replicates. Individual haplotypes were compared between the groups by Z-score, using PEPI program. A P-value < 0.05 was considered as statistically significant.

3. Results

3.1. Clinical and demographic characteristics

The clinical and demographic characteristics of type 2 diabetic patients according to the presence or absence of renal involvement are summarized in Table 2. Caucasian-Brazilians with renal disease were more often male, younger, with a shorter duration of diabetes, higher prevalence of hypertension, insulin therapy, and retinopathy, higher levels of serum creatinine and triglycerides and lower levels of HDL cholesterol as compared to patients with normoalbuminuria.

3.2. Genotype and allele frequencies

The genotype frequencies were in agreement with those predicted by the Hardy–Weinberg equilibrium for all *eNOS* polymorphisms in both type 2 diabetic patients and healthy blood donors, except for the −786T > C polymorphism, in which there was a lower frequency of heterozygotes than expected among diabetic patients (expected frequency = 48.0% *vs.* observed frequency = 43.5%, $P = 0.015$). As shown in Table 3, there were no statistically significant differences when genotype and allele frequencies for the −786T > C, VNTR intron 4 a/b and 894G > T polymorphisms were compared among healthy blood donors, type 2 diabetic patients, diabetic patients with normoalbuminuria and patients with renal involvement.

In order to test for an association of *eNOS* polymorphisms with type 2 diabetes and renal disease, logistic regression analyses were carried out assuming three diferent genetic models for the minor allele. However, no relationship of the three *eNOS* polymorphisms with type 2 diabetes or renal involvement was observed. Even after adjusting for demographic and clinical variables, the results were only slightly modified (Table 3). Considering that the inclusion of patients with microalbuminuria in the group of subjects with renal disease might result in underestimating the magnitude of an association, all statistical procedures were repeated excluding these patients from analyses. Again, results remained almost identical to those previously obtained.

3.3. Haplotype analysis

Based on two different measures of linkage disequilibrium (LD), D' and r^2, it could be inferred that the three *eNOS* polymorphisms were in weak LD among healthy blood donors ($D' = 0.658$ and $r^2 = 0.177$, for −786T > C *vs.* VNTR intron 4 a/b; $D' = 0.326$ and $r^2 = 0.081$, for −786T > C *vs.* 894G > T; $D' = -0.885$ and $r^2 = 0.070$, for VNTR intron 4 a/b *vs.* 894G > T). Despite some moderate D' values, the r^2 values were very low. Likewise, in type 2 diabetic patients the *eNOS* polymorphisms were also in weak LD ($D' = 0.584$ and $r^2 = 0.098$, for −786T > C *vs.* VNTR intron 4 a/b; $D' = 0.486$ and $r^2 = 0.153$, for −786T > C *vs.* 894G > T; and $D' = -0.834$ and $r^2 = 0.057$, for VNTR intron 4 a/b *vs.* 894G > T).

Next, using a Bayesian method to estimate the frequency of different haplotypes composed of the three studied *eNOS* polymorphisms, we investigated whether a specific haplotype is associated with type 2 diabetes or with renal disease. As the *c* allele of the VNTR polymorphism in intron 4 was very rare (only 4 out of 583 diabetic patients and none of the healthy blood donors carried this allele in heterozygosis), carriers of the *c* allele were excluded from haplotype analysis and the VNTR intron 4 a/b polymorphism was considered as a bi-allelic marker. A total of seven haplotypes resulting from the three *eNOS* polymorphisms was observed in both type 2 diabetic patients and healthy blood donors (Table 4). The haplotype frequencies in diabetic patients were not significantly different from those of healthy blood donors. Moreover, among diabetic patients, the haplotype frequencies were also similar between subjects with or without renal involvement and they were not different from those of healthy blood donors (Table 4).

4. Discussion

In the present study, no associations between the −786T > C, the VNTR intron 4 a/b and the 894G > T (Glu298Asp) polymorphisms in the *eNOS* gene and the presence or severity of renal disease were observed in Caucasian-Brazilians with type 2 diabetes. As a matter of fact, elucidating the role of *eNOS* polymorphisms in the development or progression of kidney disease in type 2 diabetes has proven to be a challenging area of investigation, with many studies reporting controversial results [9–36].

In relation to the −786T > C polymorphism in the promoter region, the C allele was found to be associated with a greater degree of albuminuria in European American families [15], with an increased risk of DN in North Asian Indians [9] and with ESRD in Japanese [11]. However, three studies did not observe an association of this polymorphism with renal insufficiency in Tunisians [14], Japanese [30] and North and South Asian Indians [22] with type 2 diabetes, as also observed in the meta-analysis by Zintzaras et al. [36] and in the present study for Caucasian-Brazilians.

The VNTR intron 4 a/b variant has been the most studied *eNOS* polymorphism in the pathogenesis of renal insufficiency in both diabetic and nondiabetic patients, predominantly in Asian populations. Some authors have found an association of the *a* allele with ESRD in Poles [13] and Brazilians from the Southeast region [12], with progression of chronic renal failure in the Japanese [10], and with DN in the Japanese [17] and North Asian Indians [9]. However, most studies have observed that the VNTR in intron 4 is not related to the different stages of DN (from increased albuminuria up to ESRD treated by dialysis) in African-American [25], German [24], Polish [27], Hellen (from Greece and Cyprus) [28], Tunisian [14], North and

South Asian Indian [22], Han Chinese [29], and Japanese [11,16,18,26,31–33,35] type 2 diabetic patients. In our study, the VNTR intron 4 a/b polymorphism was not associated with renal disease in Caucasian-Brazilians from the Southern region, which also corroborates the results of the meta-analysis by Zintzaras et al. [36].

Of the three *eNOS* variants analyzed in the present study, the 894G > T (Glu298Asp) polymorphism in exon 7 has provided the most controversial results in relation to renal outcomes in type 2 diabetic patients. The T allele was found to be associated with an increased risk of deterioration of renal function in Korean [19], ESRD in Indonesian [21] and Japanese [16,18,20], and DN in North Asian Indian [9] and Tunisian [14] type 2 diabetic patients. Also, the T allele was shown to be weakly associated with DN and DSN in patients with type 2 diabetes and it was strongly associated with DSN in East Asians in the meta-analysis [36]. In contrast, the GG genotype was recently associated with an increased risk of chronic renal insufficiency (CRI) in a South Asian Indian population with type 2 diabetes, whereas such an association was not observed among North Asian Indians in the same study [22]. Moreover, several other authors did not find any association of the polymorphism in exon 7 with renal insufficiency in African American [25], European American [15], Finnish [34], Australian [23], and Japanese [30,32] type 2 diabetic patients, as also observed in the present study for Caucasian-Brazilians.

Among the several factors which could have contributed to the discrepancies between the above-mentioned studies, the definition of outcome itself and the criteria of inclusion seem to be a major factor. For instance, considering only the tens of studies reported in the Japanese population, it is worthwhile to note that in those which analyzed the association of *eNOS* polymorphisms in relation to ESRD as the main endpoint, with the sample study composed of patients with and without diabetes (~30–40% diabetic patients in most reports), and comparing both diabetic and nondiabetic patients on hemodialysis to healthy controls, a trend towards a positive association was observed [10,11,16,18,20,35]. On the other hand, a trend towards no association was detected in those studies that utilized DN as the outcome (the entire study sample composed of diabetic patients) [26,30–33]. This trend was also observed in two studies by the same Polish research group [13,27]. First, Ksiazek et al. [27] reported that the frequency of the *a* allele (VNTR intron 4 a/b polymorphism) was similar among diabetic patients with or without DN, although both groups of patients presented a higher frequency of this allele compared to healthy controls. Later, Buraczynska et al. [13] observed an increased frequency of the *a* allele among ESRD patients, both diabetic and nondiabetic, in comparison to healthy controls.

In the present study, 21% of the subjects with renal impairment did not have DR, which suggests that other renal disease could be the cause of increased UAE other than DN [46]. In the study by Christensen et al. [46], 31% of patients with overt proteinuria and normal fundoscopy who were submitted to kidney biopsy based on clinical indication did not have DN (13.7% had glomerulonephritis and 17.6% normal glomerular structure). Considering this proportion, in our sample only 6.3% of cases (30% of 21% without DR) would have nondiabetic nephropathy. This is a very small number of subjects that probably did not have an important effect in the final results and conclusions. As a matter of fact, we repeated the analyses with only those with increased albuminuria and DR, and the results remained virtually the same (data not shown).

Apart from this, ethnic differences have often been evoked as being partly responsible for the discrepancies between studies regarding the association of *eNOS* polymorphisms with ESRD and DN [14,21,24,28,36]. However, even reports from the same country have presented conflicting results, as reported in North Asian Indians [9,22]. In fact, population studies have shown marked interethnic differences in the distribution of *eNOS* variants [40,47]. Therefore, the linkage disequilibrium observed between the *eNOS* polymorphisms may be responsible, at least in part, for the controversial findings observed between these polymorphisms and DN.

In this context, the most recent studies have investigated the effect of different haplotypes in order to obtain a more comprehensive analysis of the role of *eNOS* gene polymorphisms in the susceptibility to DN [9,14,22]. Unfortunately, the findings remain unclear. For instance, Ahluwalia et al. [9] reported that the haplotype with all the major alleles (−786T/4b/894G) was found to be associated with a decreased risk of DN in North Asian Indians, whereas Ezzidi et al. [14] reported that the −786T/4b/894T haplotype was associated with an increased risk of DN in Tunisians. Moreover, Tiwari et al. [22] observed an excess of the haplotype carrying the 894G allele (−786T/4b/894G) in type 2 diabetic patients with CRI as compared to patients without this condition in a South Indian population. The authors hypothesized that CRI subjects carrying the 894T allele might not have survived, possibly due to the premature mortality caused by cardiovascular events, with the 894G allele conferring greater survival ability to comorbid complications. On the other hand, Shin Shin et al. [19] found that, in a retrospective cohort study of Koreans with overt nephropathy, deterioration of renal function was faster and renal survival was lower in patients with the 894GT genotype than in those with the GG genotype.

In the present study, the haplotype frequencies of patients with renal involvement were not different from those with normoalbuminuria. In our study, although the −786C, 4a and 894T alleles were as frequent as in other Caucasian populations [40,47,48], the haplotype frequencies differed from those observed in Brazilian Caucasians from the Southeast region [47] but were similar to those found in Caucasians from the United States [40], indicating that our population is less admixed than Caucasians from the Southeast region.

Apart from this, DN is a disorder of multifactorial inheritance [1–3]. Thus, the existence of gene-environment interactions may explain the discrepancy of results among different studies, as already recognized in literature [36]. Moreover, it is also possible that the *eNOS* polymorphisms exert only a weak to moderate effect on the development of this complication. Thus, one possible limitation of the present study is that a factor with a small effect would require a bigger sample size to be detected. This limitation, however, does not seem to be a contributing factor in the present study, since we obtained an approximately 80% statistical power of detecting an odds ratio as low as 1.65 for any of the three *eNOS* polymorphisms. By comparison, an order of magnitude lower than has been found in most studies of DN or ESRD [9,12,13,15,17,18,20–22].

In conclusion, no association of the −786T > C, the VNTR intron 4 a/b or the 894G > T (Glu298Asp) polymorphisms in the *eNOS* gene with renal disease was observed in Caucasian-Brazilians with type 2 diabetes. Likewise, no association between *eNOS* polymorphisms and the presence of type 2 diabetes itself was observed. Considering that studies designed to investigate the relationship of *eNOS* polymorphisms in the development of renal dysfunction have shown very controversial results, it is early to define its role in the pathogenesis of this diabetic complication. Further large, multiethnic and prospective studies should be performed to clarify the relationship of the −786T > C, VNTR intron 4 a/b and 894G > T (Glu298Asp) polymorphisms in the *eNOS* gene with kidney disease in type 2 diabetic patients.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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