

Original Article

Association of endothelial nitric oxide synthase gene intron 4 polymorphism with end-stage renal disease

MARIA H BELLINI,^{1,2} MÁRCIA N FIGUEIRA,¹ MICHELLY F PICCOLI,¹ JÚLIO T MARUMO,² MAYSA S CENDOROGLO,¹ MIGUEL C NETO,¹ MARIA A DALBONI,¹ MARCELO C BATISTA,¹ MIGUEL Â GOES¹ and NESTOR SCHOR¹

¹Division of Nephrology and Geriatrics, Department of Medicine, Universidade Federal de São Paulo, and

²Instituto de Pesquisas Energéticas e Nucleares, Cidade Universitária IPEN-CNEN/SP, São Paulo, SP, Brazil

SUMMARY:

Background: Nitric oxide (NO) released from endothelial cells is related to the maintenance of physiological vascular tone. The impairment of endothelial NO generation brought about by gene polymorphism is considered one of the deterioration factors in progressive renal disease. In the endothelial nitric oxide synthase (eNOS) intron 4 polymorphism, the presence of the *aa* genotype has been associated with cardiovascular and renal disease. The aim of this study was to investigate the presence of eNOS gene intron 4 polymorphism in patients with end-stage renal disease (ESRD).

Methods: A total of 114 patients and 94 controls were studied. DNA specimens were extracted from blood and amplified by polymerase chain reaction. The alleles were separated by agarose gel electrophoresis. Genotype distribution and allele frequencies were compared between groups using the chi-squared test.

Results: Statistical analysis revealed that the frequency of the eNOS4 genotype *aa* was significantly different in ESRD patients and in controls ($P = 0.016$, OR = 2.07, CI 95%: 1.14–3.74). There was also a statistically significant difference between ESRD patients and controls regarding allele carriers ($P = 0.004$; OR = 2.26; CI 95%: 1.29–3.96). When the frequencies of allele carriers in the diabetic nephropathy group and in the control group were compared, a significant difference was found ($P = 0.034$, OR = 2.28; CI 95%: 1.04–5.00).

Conclusion: This study showed a strong correlation between eNOS4a polymorphism and end-stage renal disease.

KEY WORDS: 27-bp repeats in intron 4, chronic renal failure, diabetes mellitus, endothelial nitric oxide synthase, end-stage renal disease, gene polymorphism.

Endothelial dysfunction – defined as the impaired ability of vascular endothelium to stimulate vasodilation – plays a major role in the development of various pathological conditions which predispose to cardiovascular disease, such as hypercholesterolemia, hypertension, type 2 diabetes and chronic renal failure.¹

Nitric oxide (NO), an important intracellular messenger molecule, plays a major role in maintaining homeostasis.^{2,3} NO is synthesized from L-arginine by three NO synthase (NOS) isoforms: neuronal (nNOS), inducible (iNOS) and

endothelial NOS (eNOS). The NOS isoforms share approximately 50% homology and are encoded by different genes.^{4–6} nNOS and eNOS are constitutively expressed mainly in the nervous system and the vascular endothelium, respectively. Upon stimulation and under basal conditions, they synthesize a small amount of NO in a calcium-dependent manner. In contrast, iNOS is induced when stimulated by microbial endotoxins or certain proinflammatory cytokines, producing a greater amount of NO in a calcium-independent manner.⁴

Human eNOS is encoded by a gene located on chromosome 7q35-36 comprising a 26 exons-25 introns and its predominant form has 133 kDa.⁷ The enzymatic activity of the constitutively expressed eNOS is controlled by intracellular Ca^{2+} levels and other cofactors. After being released in endothelial cells, NO diffuses rapidly through cell membranes and relaxes neighbouring vascular smooth muscle cells through the production of guanosine 3',5'-cyclic

Correspondence: Dr Maria H Bellini, Instituto de Pesquisas Energéticas e Nucleares, Cidade Universitária IPEN-CNEN/SP, Avenue. Lineu Prestes, 2242, 05508-900, São Paulo, SP, Brazil. Email: mhmarumo@ipen.br

Accepted for publication 14 January 2007.



Fig. 1 Schematic representation of the eNOS gene. Exons (empty boxes); introns (filled boxes). eNOS, endothelial nitric oxide synthase.

monophosphate (cGMP). In addition, NO inhibits platelet activation, regulates angiogenesis and controls microvascular permeability.^{8,9} NO is synthesized in the kidney and plays an important role in regulating renal haemodynamics and function. There is evidence that NO is generated not only in the vascular endothelium, but also in other renal cells.^{10,11}

Over the last few years, several polymorphisms of the eNOS gene (*NOS3*) have been identified, and their association with various diseases has been explored.^{1,12} In particular, a single-nucleotide polymorphism (SNP) in the promoter region (T⁻⁷⁸⁶C), a SNP in exon 7 (Glu298Asp) and the variable number of tandem repeats (VNTR) in intron 4 have received most of the attention, because of their association with cardiovascular and renal diseases.^{1,13,14}

In the 27-bp repeat of intron 4, two alleles have been identified, the larger of which, eNOS4b, has 5 tandem 27-bp repeats (GAAGTCTAGACCTGCTGC(A/G)GGGGTGAG) and the smaller, eNOS4a, has four repeats (Fig. 1). The eNOS4a allele has been found to be more frequent in end-stage renal disease (ESRD).^{8,12,13,15}

The aim of this study was to investigate possible correlations between eNOS intron 4 polymorphism and ESRD in Brazilian patients.

MATERIALS AND METHODS

A total of 114 patients with ESRD were included in this study (66 men and 48 women, mean age = 53.8 years). Sixty of the patients were in treatment with dialysis and 53 without dialysis. In 107 of the patients, the cause of the renal failure was established as being: hypertensive nephropathy ($n = 43$), diabetic nephropathy (DN, $n = 37$), glomerulonephritis ($n = 13$), interstitial nephritis ($n = 6$), polycystic kidney disease ($n = 6$) and systemic lupus erythematosus ($n = 2$). Healthy control subjects ($n = 94$) with no clinical signs of vascular or renal disease and no family history of renal disease were recruited among blood donors and hospital staff (52 men and 42 women, mean age = 52.7 years). Our study group, patients and controls, was very heterogeneous and included people of different ethnic origins and admixtures. All subjects signed an informed consent form, and the studies were approved by the local University Ethics Committee on human research.

Genomic DNA

Genomic DNA was isolated from peripheral blood leucocytes using a DNA extraction kit (QIAamp DNA Blood Mini Kit (250) from Qiagen, Valencia, CA, USA).

Determination of eNOS genotype

Two oligonucleotide primers that flank the region of the 27-bp repeat sequence in intron 4 of the eNOS gene were used for polymerase chain

reaction (PCR) amplification. The eNOS primers were synthesized by Integrated DNA Technologies, Inc. (IDT, Santa Clara, CA, USA). The forward primer was 5'-AGGCCCTATGGTAGTGCCTT-3' and the reverse primer was 5'-TCTCTTAGTGCTGTGGTCAC-3' (2). Genomic DNA (100 ng) was amplified by PCR using 10 μ L of Master Mix (Eppendorf, Hamburg, Germany), and 10 pmol of each primer, in a final reaction volume of 25 μ L. The reaction mixture was heated to 94°C for 6 min for denaturation and then subjected to 35 cycles at 94°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 2 min, followed by a final extension step at 72°C for 7 min. The PCR products were analysed by electrophoresis in 2.5% agarose gels stained with ethidium bromide.

Statistical analysis

Normally distributed data are presented as means \pm standard deviation (SD). The distribution of the genotypes was assessed for deviation from the Hardy-Weinberg equilibrium by using the chi-squared (χ^2) test. Genotype distribution and allele frequencies were also compared between groups using the χ^2 -test. Student's *t*-test was used to compare the mean time with ESRD between genotypes. Multiple logistic regression analysis was used to determine the relationship between eNOS gene polymorphism and ESRD.

RESULTS

The genotypes of 208 individuals, 114 ESRD patients and 94 healthy controls, were investigated for eNOS gene polymorphisms. Clinical characteristics of the study group are summarized in Table 1. There were no significant differences between groups regarding age and gender.

The genotypes of the group were clearly determined by PCR. Fragments containing 420 bp (eNOSb allele) and 393 bp (eNOSa allele) can be visualized in Figure 2.

No significant deviation from the Hardy-Weinberg equilibrium was observed in either the control or the case population ($\chi^2 = 0.186$ and 0.02, respectively).

The genotype distribution in patients and controls is shown in Table 2. The ESRD group presented eNOSa allele frequency of 34.2%, while in the control group it was 20.7%, a statistically significant difference ($P = 0.002$). The 12.2% frequency of the eNOS *a/a* genotype in the ESRD patients was also significantly different from the value found for the normal controls (5.3%) ($P = 0.012$). Multiple logistic regression analysis for correcting the influence of age and sex revealed that the frequency of the eNOS *a/a* genotype was significantly higher in the ESRD patients than in the controls ($P = 0.016$, OR = 2.07, 95% CI: 1.14–3.74). The difference in frequency of allele carriers was equally significant ($P = 0.004$; OR = 2.26, 95% CI: 1.29–3.96).

Table 1 Clinical characteristics of studied subjects

	ESRD patients	Controls	P
Age (years)	53.8 ± 13.7	52.7 ± 14.9	0.575†
Sex (M/F)	66/48	52/42	0.709‡
Serum creatinine (mg/dL)	6.30 ± 4.2	0.96 ± 0.17	<0.001
Urea (mg/dL)	106.94 ± 39.9	26.5 ± 6.95	<0.001
Glucose	137 ± 64.7	Not applicable	
Cholesterol	202 ± 59.7	Not applicable	
Hypertension (%)	37.7	0	
Diabetes (%)	32.5	0	

Data are expressed as mean ± SD, except where percents are shown. †Student's *t*-test. ‡Chi-squared test. ESRD, end-stage renal disease; F, female; M, male.

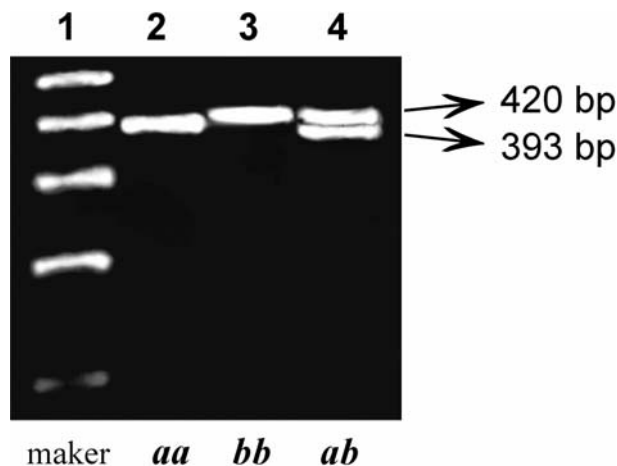


Fig. 2 Analysis of the variable number of tandem repeats polymorphism in intron 4 of the eNOS (endothelial nitric oxide synthase) gene. The 420-bp band indicates five 27-bp repeats, and the 393-bp band indicates four 27-bp repeats: lane 1, size of the molecular marker: 100 pb; lane 2, wild type (4aa); lane 3, four repeats homozygote (4bb); lane 4, four and five repeats heterozygote (4a/b).

Despite the small size of the sample of patients with DN, the authors calculated the genotype and allele frequencies and compared them with the control group. The frequency of the eNOSa allele in the DN group was 36.5%, higher than in the control group (20.7%) ($P = 0.008$). Furthermore, the frequency of eNOSa carriers (eNOSaa + eNOSab) was higher in diabetic patients (56.8%) than in controls (36.2%) ($P = 0.032$). Multiple logistic regression analysis for correcting the influence of age and sex revealed a significant difference in the frequencies of eNOSa carriers (eNOSaa + eNOSab) between DN and control groups ($P = 0.034$, OR = 2.28; CI 95%: 0.05–0.83) (Table 3). In contrast, there was no significant difference in the frequencies of eNOS genotypes between DN and non-DN patients ($P = 0.6593$).

When the genotype and allele frequencies of hypertensive patients (9.3%) and controls (5.3%) were compared, no significant difference was found ($P = 0.237$).

Comparing the distribution of the eNOS intron 4 genotype in our controls with the data reported previously for other study populations (Table 4), it is clear that the a variant genotype is associated with ethnicity. χ^2 -analysis showed a significant difference in the genotype distribution between our Brazilian group and data reported for Polish ($P = 0.025$) and Japanese ($P < 0.001$) populations. It is interesting to note that there was also a significant difference in genotype distribution between our multiethnic control group and Brazilian blacks ($P = 0.032$),¹⁶ however, there was no significant differences among our control group and Brazilian whites, African Americans and Caucasian Americans.

DISCUSSION

Chronic renal diseases are highly variable, even among individuals with the same underlying cause of renal injury or degree of functional impairment. Interindividual variability is a typical phenomenon of complex diseases and reflects the multifactorial nature of the biologic mechanisms involved in the underlying disease process.^{17–20}

Genetic factors may contribute to endothelial dysfunction that is present in several pathologies including renal diseases.¹⁹ A polymorphism of the 27-bp repeat in intron 4 of the eNOS gene was reported to have a correlation with terminal renal failure in several populations.^{8,10,12–15,20}

In the present study, the authors analysed the distribution of genetic variants of the eNOS intron 4 polymorphism in a multiethnic Brazilian group of 114 patients and 94 healthy controls. The frequency of the a allele in eNOS intron 4 and eNOS 4a allele carriers was significantly higher in patients than in controls ($P = 0.004$, OR: 2.26, 95% CI: 1.29–3.96), indicating that the eNOS 4a allele may be one of the risk factors for ESRD in Brazilian patients.

Our data are consistent with the results reported previously in the literature for different populations. Buraczynska *et al.*¹⁰ studied the distribution of eNOS intron 4 in 706 patients and 321 controls from Poland. The frequencies of genotypes NOSaa, NOSab and NOSbb in patients (46%, 35% and 58.5%, respectively) and controls (1%, 25% and 74%, respectively) were statistically different. Wang *et al.*¹³ and Nagase *et al.*⁸ studied the distribution of eNOS intron 4 in Japanese haemodialysis patients. Both authors found a

Table 2 Distribution of eNOS intron 4 polymorphism in cases and controls, and multiple logistic regression analysis

Category	ESRD patients n (%)	Controls n (%)	P-value	OR (95% CI)†
Aa	14 (12.2)	5 (5.3)	0.016	2.07 (1.14–3.74)
Ab	50 (43.9)	29 (30.9)		
Bb	50 (43.9)	60 (63.8)		
Alleles				
A	78 (34.2)	39 (20.7)		
B	150 (65.8)	149 (79.3)		
A allele carriers (<i>aa + ab</i>)	64 (56)	34 (36.2)	0.004	2.26 (1.29; 3.96)
Non-carriers (<i>bb</i>)	50 (43.8)	60 (63.8)		

†Odds ratios were adjusted for age and sex. eNOS, endothelial nitric oxide synthase; ESRD, end-stage renal disease.

Table 3 Distribution of eNOS intron 4 polymorphism in diabetic nephropathy (DN) patients and controls, and multiple logistic regression analysis

Category	DN patients n (%)	Controls n (%)	P-value	OR (95% CI)†
Aa	6 (16.2)	5 (5.3)	0.034	2.28 (1.04; 5.00)
Ab	15 (40.5)	29 (30.9)		
Bb	16 (43.3)	60 (63.8)		
Alleles				
A	27 (36.5)	39 (20.7)		
B	47 (63.5)	149 (79.3)		
Alleles				
A allele carriers (<i>aa + ab</i>)	21 (56.8)	34 (36.2)		
Non-carriers (<i>bb</i>)	16 (43.2)	60 (63.8)		

†Odds ratios were adjusted for age and sex.

Table 4 Distribution of genotypes and frequency of allele a in different healthy populations

Population	Genotypes			Allele a	P-value†	Reference
	<i>a/a</i> n (%)	<i>a/b</i> n (%)	<i>b/b</i> n (%)			
Brazilian (n = 94)	5 (5.3)	29 (30.9)	60 (63.8)	0.207		This study
Brazilian blacks (n = 134)	17 (27)	53 (48.7)	64 (24.3)	0.320	0.032	Marroni <i>et al.</i> ¹⁶
Brazilian Whites (n = 154)	4 (2.6)	42 (27.3)	108 (70.1)	0.179	0.408	Marroni <i>et al.</i> ¹⁶
African American (89)	6 (6)	38 (38%)	45 (45)	0.265	0.191	Tanus-Santos <i>et al.</i> ¹⁷
Caucasian American (n = 100)	1 (1)	30 (30)	69 (69)	0.160	0.209	Tanus-Santos <i>et al.</i> ¹⁷
Polish (n = 321)	4 (1)	81 (25)	236 (74)	0.140	0.025	Buaczynska <i>et al.</i> ¹⁰
Japanese (n = 248)	0 (0.0)	47 (19.0)	201 (81.0)	0.095	<0.001	Nagase <i>et al.</i> ⁸

†P-value obtained by chi-squared test.

significantly higher frequency of the eNOS_a allele in the ESRD patients. A strong association between the eNOS gene intron 4 polymorphism and plasma levels of nitric oxide derivatives (NO_x) was found by Tsukada *et al.*²¹ The mean plasma NO_x levels of subjects who were homozygous for allele *a* were 20% lower than those of subjects with allele *b*. Reduced production of NO may contribute to progression of glomerular damage via systemic or intraglomerular hypertension.¹⁰

In our study, the frequency of eNOS_a allele carrier genotypes (eNOS_{4aa} and eNOS_{4ab}) was higher in diabetic patients than in healthy controls ($P = 0.032$). Multiple logistic regression analysis revealed that the carrier genotypes were significantly correlated to the development of chronic renal failure ($P = 0.034$, OR: 2.28, 95% CI: 1.04–5.00). An association between the eNOS_a allele and diabetes has already been observed in other populations, but the mechanism responsible for this association is so far not

known.^{10,12,22,23} In fact, an abnormal renal vasomotor tone is present in the early stage of diabetes mellitus. In their case-control and family-based studies, Zanchi *et al.*¹² have demonstrated clearly that DNA sequence differences in eNOS have an influence on the risk of advanced nephropathy in type 1 diabetes. These authors hypothesized two molecular mechanisms to explain the correlation between the eNOS intron 4 polymorphism and DN. The first one assumes that the eNOS_a and eNOS_b alleles themselves play a role in tissue-specific regulation of eNOS expression. The intron structure may have a function in splicing and consequently in the maturation of the RNA. The second possible explanation would be a lack of biological function of the eNOS_a allele. Asakimori *et al.*²⁴ studied the frequency of eNOS intron 4 polymorphism in non-diabetic and DN patients undergoing haemodialysis. They found a significantly higher frequency of intron 4 allele *a* in both non-diabetic and diabetic patients.

The authors compared the genotype distribution of eNOS variants in our control group with those of control groups reported in previous publications (Table 4). The authors found a marked difference in the frequency of eNOS_a among our multiethnic group and the Polish, the Japanese and also the group of Brazilian blacks studied by Marroni *et al.*¹⁶

In conclusion, this study shows that the eNOS_a allele frequency is significantly higher in ESRD patients than in controls. This result suggests that the eNOS gene polymorphism may be used as a genetic marker to evaluate the susceptibility to chronic renal failure, even in multiethnic populations. The participation of other genes, as well as of environmental factors, must, however, be considered in the progression of renal diseases.

REFERENCES

1. Wang XL, Wang J. Endothelial nitric oxide synthase gene sequence variations and vascular disease. *Mol. Genet. Metab.* 2000; **4**: 241–451.
2. Wang XL, Sim AS, Badenhop RF, McCredie RM, Wilcken DE. A smoking-dependent risk of coronary artery disease associated with a polymorphism of the endothelial nitric oxide synthase gene. *Nat. Med.* 1996; **1**: 41–5.
3. Noiri E, Satoh H, Taguchi J *et al.* Association of eNOS Glu298Asp polymorphism with end-stage renal disease. *Hypertension* 2002; **40**: 535–40.
4. Morishita T, Tsutsui M, Shimokawa H *et al.* Nephrogenic diabetes insipidus in mice lacking all nitric oxide synthase isoforms. *Proc. Natl. Acad. Sci. USA* 2005; **30**: 10616–21.
5. Fagan KA, Morrissey B, Fouty BW *et al.* Upregulation of nitric oxide synthase in mice with severe hypoxia-induced pulmonary hypertension. *Respir. Res.* 2001; **5**: 306–513.
6. Devuyst O. Variable renal disease progression in autosomal dominant polycystic kidney disease: A role for nitric oxide. *J. Nephrol.* 2003; **3**: 449–52.
7. Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: Structure, function and inhibition. *Biochem. J.* 2001; **3**: 593–615.
8. Nagase S, Suzuki H, Wang Y *et al.* Association of eNOS gene polymorphisms with end stage renal diseases. *Mol. Cell. Biochem.* 2003; **244**: 113–18.
9. Dusserre N, L'Heureux N, Bell KS *et al.* PECAM-1 interacts with nitric oxide synthase in human endothelial cells: Implication for flow-induced nitric oxide synthase activation. *Arterioscler. Thromb. Vasc. Biol.* 2004; **10**: 1796–802.
10. Buraczynska M, Ksiazek P, Zaluska W, Nowicka T, Ksiazek A. Endothelial nitric oxide synthase gene intron 4 polymorphism in patients with end-stage renal disease. *Nephrol. Dial. Transplant.* 2004; **9**: 2302–6.
11. Kurata H, Takaoka M, Kubo Y *et al.* Protective effect of nitric oxide on ischemia/reperfusion-induced renal injury and endothelin-1 overproduction. *Eur. J. Pharmacol.* 2005; **11**: 232–9.
12. Zanchi A, Moczulski DK, Hanna LS, Wantman M, Warram JH, Krolewski AS. Risk of advanced diabetic nephropathy in type 1 diabetes is associated with endothelial nitric oxide synthase gene polymorphism. *Kidney Int.* 2000; **2**: 405–13.
13. Wang Y, Kikuchi S, Susuki H, Nagase S, Koyama A. Endothelial nitric oxide synthase gene polymorphism in intron 4 affects the progression of renal failure in non-diabetic renal diseases. *Nephrol. Dial. Transplant.* 1999; **14**: 2898–902.
14. Miyamoto Y, Saito Y, Kajiyama N *et al.* Endothelial nitric oxide synthase gene is positively associated with essential hypertension. *Hypertension* 1998; **1**: 3–8.
15. Yokoyama K, Tsukada T, Matsuoka H, Hara S, Yamada A, Kawaguchi Y. High accumulation of endothelial nitric oxide synthase (eNOS): A gene polymorphism in patients with end-stage renal disease. *Nephron* 1998; **3**: 360–61.
16. Marroni AS, Metzger IF, Souza-Costa DC *et al.* Consistent inter-ethnic differences in the distribution of clinically relevant endothelial nitric oxide synthase genetic polymorphisms. *Nitric Oxide* 2005; **3**: 177–82.
17. Tanus-Santos JE, Desai M, Flockhart DA. Effects of ethnicity on the distribution of clinically relevant endothelial nitric oxide variants. *Pharmacogenetics* 2001; **8**: 719–25.
18. McClellan MW, Flanders DW. Risk factors for progressive kidney disease. *J. Am. Soc. Nephrol.* 2003; **14**: S65–S70.
19. Ghiadoni L, Cupisti A, Huang Y *et al.* Endothelial dysfunction and oxidative stress in chronic renal failure. *J. Nephrol.* 2004; **4**: 512–19.
20. Freedman BI, Yu H, Anderson PJ, Roh BH, Rich SS, Bowden DW. Genetic analysis of nitric oxide and endothelin in end-stage renal disease. *Nephrol. Dial. Transplant.* 2000; **11**: 1794–800.
21. Tsukada T, Yokoyama K, Arai T *et al.* Evidence of association of the eNOS gene polymorphism with plasma NO metabolite levels in humans. *Biochem. Biophys. Res. Commun.* 1998; **1**: 190–93.
22. Neugebauer S, Baba T, Watanabe T. Association of nitric oxide synthase gene polymorphism with an increased risk for progression to diabetic nephropathy in type 2 diabetes. *Diabetes* 2000; **49**: 500–503.
23. Miller JA, Floras JS, Zinman B, Skorecki KL, Logan AG. Abnormalities in the renal and vascular responses to LBNP in humans with early diabetes. *Am. J. Physiol.* 1994; **266**: R442–50.
24. Asakimori Y, Yorioka N, Yamamoto I *et al.* Endothelial nitric oxide synthase intron 4 polymorphism influences the progression of renal disease. *Nephron* 2001; **2**: 219–23.