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Protective role of 27bp repeat polymorphism in intron 4 of eNOS gene in lacunar infarction

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Abstract

Association of the three potential endothelial nitric oxide synthase gene (eNOS) polymorphisms (T-786C in promoter region, G894T in exon 7 and tandem 27-bp repeats in intron 4) with an increased risk of lacunar infarction (LI) were investigated. Genotypes of 70 patients and 81 healthy controls were determined through PCR with or without RFLP. Flow-mediated dilatation (FMD) was performed to assess endothelial-dependent vasodilatation, whereas the endothelial-independent vasodilatation was assessed with nitroglycerin (NTG). Genotype distribution was significantly different between LI patients and controls for intron 4aa (alleles for four repeats), genotype frequency being 1.4% and 16.0%, respectively (odds ratio for additive effect, 0.47; 95% CI, 0.28–0.81; $p = 0.006$). Haplotypes with the intron 4aa polymorphism were significantly higher in controls when compared with the LI group ($p = 0.001$). Diminished FMD but normal NTG response confirmed that patients with LI have generalized endothelial dysfunction. Intron 4aa genotype of eNOS gene seems to be protective for isolated LI and the effect was potentiated by the absence of 786C polymorphism in any allele of the promoter region.

Keywords: *eNOS gene, lacunar infarction, cerebral small vessel disease, polymorphisms, intron, VNTR*

Introduction

Lacunar infarction (LI) accounts for ~25% of all ischemic strokes worldwide and has a heterogeneous aetiology. Patients with LI carry an increased risk of death, recurrent strokes and cognitive dysfunction in the long term [1]. One of the current hypotheses proposes that LI is a consequence of endothelial dysfunction in cerebral penetrating arteries and genetic factors may predispose to LI by acting on the endothelium [2–4]. Discovery of the predisposing genes may therefore provide insight to the pathophysiology of LI and help development of therapeutic interventions that can improve endothelial function.

Independent of conventional vascular risk factors, a genetic component has long been thought to be present in ischemic stroke [5]. Recent advances have led to the identification of specific genetic loci on endothelial nitric oxide synthase (eNOS) that may increase vulnerability to cerebral ischemia [6–8]. eNOS catalyses the formation of nitric oxide (NO) from L-arginine. NO is a powerful vasodilatory and anti-aggregant agent and plays an important role in maintaining vascular integrity and regulation of cerebral circulation [9,10]. Most NO actions are mediated via cyclic guanosine monophosphate (cGMP) pathway [11]. Insufficient endothelial NO synthesis leads to impaired cerebral autoregulation,

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endothelial dysfunction and may promote development of microatheroma at the origin of perforating arteries, which predisposes to thrombosis and LI [3,12].

The eNOS gene is located on chromosome 7q35–36 and consists of 26 exons spaning 21 kb [13–15]. Several polymorphisms have been identified in the eNOS gene. These include a promoter (T-786C), a variable number of tandem 27-bp repeats in intron 4 and a G894T in exon 7 polymorphisms. Only a few and conflicting reports have been published on the potential relationship between polymorphisms of eNOS gene and cerebral small-vessel disease (SVD) that underlies most of the LIs [6–8,16–19]. Most of these studies, except one [6], investigated only single polymorphisms in their cohort. However, interactions between polymorphisms on the same chromosome are important in determining the disease risk. Therefore, multilocus haplotypes should be tested to overcome the limitations caused by using single polymorphisms in genetic association studies [20].

In the present study, we investigated whether the three polymorphisms of the eNOS gene (tandem 27-bp repeats in intron 4, T-786C in the promoter region and G894T in exon 7) and haplotypes bearing them are independent risk factors for isolated symptomatic LI in the Turkish population. We chose SVD patients with isolated LI but not with ischemic leukoaraiosis because the two conditions likely have differences in aetiopathogenesis, although the small penetrating brain arteries are affected in both. We also evaluated any potential functional significance of the allelic eNOS polymorphisms observed by using a flow-mediated dilatation test in the brachial artery, which is an established non-invasive method to assess endothelial NO-dependent vasodilation. Unfortunately, vascular NO generation cannot be measured directly because most endogenous NO rapidly oxidizes to nitrite (NO^{2-}) and finally to the stable form, nitrate (NO^{3-}). On the other hand, measuring plasma or urinary nitrite/nitrate in humans requires strict control of confounding variables such as diet, medication, smoking and concurrent infection [21], although recent studies have shown that plasma nitrite can reflect endogenous NO formation [22,23].

Subjects and methods

Study population

Seventy consecutive patients with symptomatic, isolated LI admitted to Hacettepe University Hospital within a 2 year-period were studied. Isolated LI was defined as a single focal lesion in a clinically relevant location presented with a classical lacunar syndrome and without (or with mild) leukoaraiosis on brain magnetic resonance imaging (MRI). All patients were evaluated with electrocardiography, echocardiography, brain MRI and imaging of the carotid and vertebral arteries with duplex ultrasonography or MR angiography. Patients were excluded if they had subcortical infarction ≥ 1.5 cm in diameter, cortical infarction of any size, a potential cardiac source of embolism, large-vessel atherothrombosis or other rare causes of lacunar infarcts (vasculitis, vasculopathies, etc.) [20].

Eighty-one healthy controls free of symptomatic cerebrovascular disease, vascular risk factors, history of myocardial infarction or peripheral vascular disease were enrolled in the study. Sampling was stratified to provide a distribution of age and sex similar to that in the patient group. The study protocol was approved by the ethical committee of Hacettepe University Faculty of Medicine (LUT 02/37) and informed consent was obtained from all participants.

Molecular analysis

Genomic DNA was isolated from the whole blood by the salting out method [24]. Polymerase Chain Reactions (PCR) were carried out for T-786C in the promoter region, G894T in exon 7 and tandem 27-bp repeats in intron 4 by using specific primers and conditions.

Genotyping

- *Tandem 27-bp repeats in intron 4*: Sense and antisense primers 5′- AGG CCC TAT GGT AGT GCC TTT-3′ and 5′- TCT CTT AGT GCT GTG GTC AC-3′ were used for PCR. PCR was performed in a 40 µl reaction volume containing 400 ng of template DNA, 10 pmol/µl of each primer, 0.2 mM of each dNTP, 4 µl of $10 \times$ PCR buffer, 2.5 mM of $MgCl_2$ and 3 U of Taq DNA Polymerase (Fermentas Life Sciences, Germany). The PCR reaction mixtures were heated to 94°C for 30 s for denaturation and underwent 35 cycles at 94°C for 30 s of denaturation, 63°C for 30 s of annealing and 72°C for 1 min of extension by using ICycler (BioRad). Finally, extension was conducted at 72°C for 5 min. The tandem 27-bp repeats was determined by separating intron 4aa (four repeats of 27 bp) (393 bp) and intron 4bb (five repeats of 27 bp) (420 bp) DNA fragments on a 3% NuSieve agarose gel [25]. Intron 4ab indicates four repeats of 27 bp in one allele while five repeats of 27 bp in the other allele.
- *T-786C polymorphism in promoter region*: The sense 5′- TGG AGA GTG CTG GTG TAC CCC A -3′ and antisense 5′- GCC TCC ACC CCC ACC CTG TC-3′ primers were used in a total volume of 50 µl, containing 400 ng of template DNA, 6.25 pmol/µl of each primer, 0.25 mM of each dNTP, 5 µl of $10 \times$ PCR buffer, 1.5 mM of $MgCl_2$ and 3 U of Taq DNA Polymerase (Fermentas Life Sciences, Germany). The reaction conditions used were: one step of denaturation at 94°C

for 5 min followed by 40 cycles comprising 1 min at 94°C for denaturation, 1 min at 61°C for annealing, 1 min at 72°C for extension and one step of extension at 72°C for 5 min using ICycler (BioRad). PCR products were checked on a 2% agarose gel. Amplified products were digested with *Msp*I (Fermentas Life Sciences, Germany) for 3 h at 37°C. Resulting fragments of 140 bp and 40 bp for the wild type allele (TT) or 90 bp, 50 bp and 40 bp in the case of polymorphic allele (CC) were determined by separating them on a 2% agarose gel [25].

- *G894T polymorphism in exon 7*: The sequence of the sense and antisense primers were 5′- AAG GCA GGA GAC AGT GGA TGG A-3′ and 5′- CCC AGT CAA TCC CTT TGG TGC TCA-3′, respectively. PCR reactions were performed in a total volume of 50 µl, containing 400 ng of template DNA, 6.25 pmol/µl of each primer, 0.25 mM of each dNTP, 10 µl of 10 × PCR buffer, 1.5 mM of $MgCl_2$ and 3 U of Taq DNA Polymerase (Fermentas Life Sciences, Germany) with the same cycles as described above for the promoter region. The resulting 268 bp fragment was digested with *Mbo*I (Fermentas Life Sciences, Germany) for 3 h at 37°C, producing 178 bp and 90 bp fragments (polymorphic allele) (TT) or no digestion (wild type) (GG). Restriction sites were confirmed on 2% agarose gel [25].

Functional analysis

Flow-mediated dilatation (FMD). FMD was performed to assess endothelial-dependent vasodilation in 23 patients and 16 genotyped controls. FMD was assessed non-invasively by B-mode high resolution ultrasound imaging (GE-Vingmed Ultrasound AS, Horten, Norway). Each patient fasted and was not allowed to use any medication or smoke for at least 12 h before the study. Subjects were studied in a quiet, temperature-controlled room and rested for 30 min before any measurement. A 7.0-MHz linear array transducer was placed at 3–5 cm proximal to the antecubital fossa in the right arm. After a straight brachial artery segment was visualized, baseline right brachial artery diameter was measured at end-diastole. After measuring baseline values, a sphygmomanometric cuff was placed distally to the brachial artery (forearm) and inflated (50 mmHg above systolic pressure) for 5 min. The brachial artery was scanned continuously for 60–90 s after cuff deflation and the vessel's maximal diameter was measured during reactive hyperemia. FMD was taken as the percentage change of an artery's baseline diameter [26]. For assessment of the reproducibility, 19 scans were selected randomly for repeated measurements by the same observer and by an independent observer.

To measure the endothelium-independent vasodilation reflecting vascular smooth muscle function, an exogenous NO donor, nitroglycerin (NTG) sublingual tablet (0.4 mg) was given to the same 23 patients and 16 genotyped controls to determine the maximum obtainable vasodilator response, using the aforementioned technique. Images were obtained 4 min after sublingual NTG. The NTG-induced dilatation was calculated as the percentage increase in arterial diameter after NTG vs the corresponding rest value (NTD% = [(brachial artery diameter (BAD) after NTG – baseline BAD)/(baseline BAD)].

Statistical analysis

We used a chi-square test to compare distribution of categorical variables between cases and controls. Quantitative variables are presented as mean ± SD and qualitative variables as percentages. Odds ratios (OR) with 95% confidence intervals (CI) were presented to estimate the risk of LI associated with the presence of the analysed polymorphisms. ORs greater than 1 signify increased risk, while those less than 1 show a protective effect of the related polymorphism. Different models at each locus were tested by coding genotypes at each position as follows: 1 (−786TT, intron 4bb, 894GG), 2 (−786CT, intron 4ab, 894GT), 3 (−786CC, intron 4aa, 894TT). The dominant model compared genotypes 3 and 2 vs 1, while the recessive model compared genotype 3 vs 1 and 2. The additive model compared the groups separately, assuming a trend in LI risk with increasing number of polymorphisms. These analyses were performed with SPSS for Windows. Haplotype analysis was performed with FASTEHPLUS, a program based on estimating haplotypes. Mantel-Haenzsel test was used to assess homogeneity of ORs with promoter-intron interaction. The multivariate analysis was performed using the logistic regression to calculate ORs associated with eNOS polmorphisms adjusted for age and gender. Linkage disequilibrium coefficients (D') were calculated using the 2LD program.

Results

Subject characteristics

Typical differences for conventional cerebrovascular risk factors were observed between cases and controls (Table I). Lacunar infarction was higher in patients with increased age, male sex and the presence of hypertension, diabetes or hypercholesterolemia.

Genotype distribution of eNOS polymorphisms

The electrophoretic results for the three eNOS gene polymorphisms; T-786C in the promoter region, G894T in exon 7, tandem 27-bp repeats in intron

Table I. Subject characteristics.

Characteristics	Controls ($n=81$)	Lacunar infarction ($n=70$)
Age	40.9 ± 12.5	63.5 ± 10.5^a
Male sex	38 (46.9)	44 (62.9)^b
Hypertension	0 (0.0)	38 (54.3)^a
Diabetes	0 (0.0)	14 (20.0)^a
Hypercholesterolemia	0 (0.0)	21 (30.0)^a

$^a p < 0.001$, $^b p < 0.10$ vs controls; mean $\pm$ SD for age and mean for male sex, values in parentheses denote percentage for categorical variables.

4, are shown in Figure 1 as examples. The tandem 27-bp repeats was determined by separating intron 4aa (four repeats of 27 bp) (393 bp) and intron 4bb (five repeats of 27 bp) (420 bp) DNA fragments on a 3% NuSieve agarose gel. Intron 4ab indicates a heterozygote patient carrying four repeats of 27 bp in one allele while five repeats of 27 bp in the other allele. In Figure 1B , G894T polymorphism in exon 7 was determined after digesting with *Mbo*I, producing 178 bp and 90 bp fragments (polymorphic allele) (TT) or no digestion (wild type) (GG). The figure shows a homozygote patient carrying the polymorphism and a homozygote control for wild alleles. Figure 1C indicates a homozygote and a heterozygote case for T-786C polymorphism and a control. After digestion with *Msp*I resulting fragments of 90 bp, 50 bp and 40 bp in the case of polymorphic allele (CC) or 140 bp and 40 bp for the wild type allele (TT) were seen.

Genotyping was successfully performed in at least 92.7% of the cases for any given polymorphism. The three loci were in linkage disequilibrium with each other ($p < 0.0005$). Table II presents genotype distribution and allele frequencies of the three eNOS gene polymorphisms in the LI and controls. Seventy patients were enrolled for the intronic region, whereas 67 patients were studied for the promoter and exon 7 polymorphisms. The control group consists of 81 cases for intron 4 and exon 7, however 79 controls were studied for the promoter region. The difference in numbers is a result of the cases in which the gene regions could not be amplified in repeated PCRs. The intron 4aa genotype was significantly less ($p = 0.006$) in frequency (1.4%) when compared with controls (16.0%). The CC genotype of the promoter region was observed to be significantly higher ($p = 0.035$) in frequency in the LI (13.4%) compared to controls (3.8%). No significant differences were observed in genotype distributions of the other alleles between LI cases and controls.

Table III shows the results of univariate analysis for the risk of LI by the -786C allele of promoter, intron 4aa and 894T allele of exon 7. Univariate analyses revealed that the recessive phenotype for the promoter region polymorphism (CC) was a risk

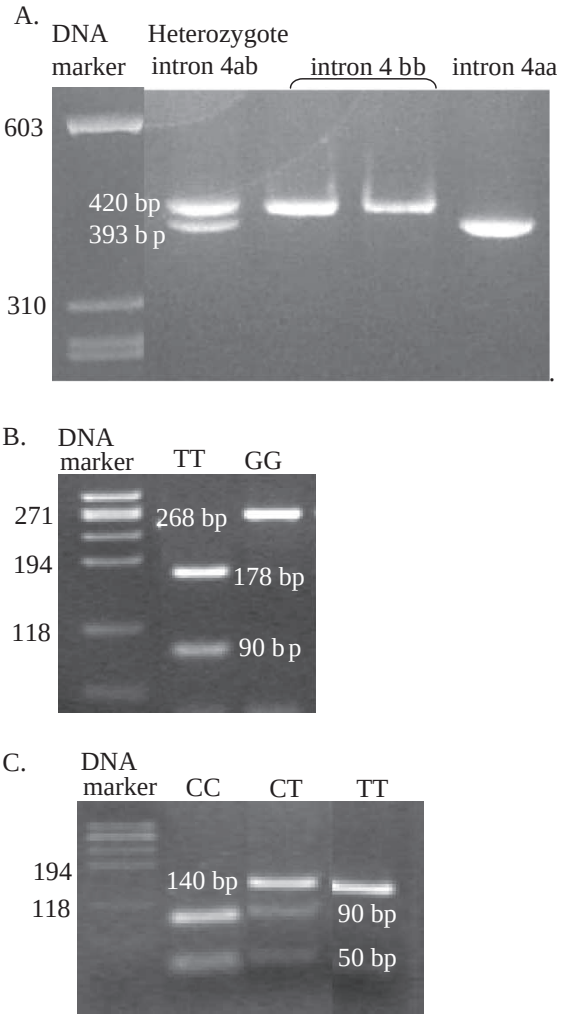

Figure 1. The polymorphic regions of the eNOS gene. DNA marker was ØX174 DNA/BsuRI9(HaeIII) (Fermentas Life Sciences, Germany) (A) tandem 27-bp repeats in intron 4. Lane 1, DNA marker; lane 2, a heterozygote patient; lanes 3 and 4, two patients with intron 4bb; lane 5, a patient with intron 4aa polymorphism. (B) G894T in exon 7. Lane 1, DNA marker; lane 2, a homozygote patient carrying the polymorphic allele; lane 3, a control carrying wild type allele. (C) T-786C in promoter region. Lane 1, DNA marker; lane 2, a homozygote patient carrying a polymorphic allele; lane 3, a heterozygote patient carrying both the polymorphic and wild type alleles; lane 3, a control carrying wild type allele.

factor for the disease (OR: 2.93, i.e. nearly 3-times increased risk) and the 4aa genotype for the intron region had a protective effect (OR: 0.51, i.e. nearly 2-times decreased risk). Also, the protective effect of intron 4aa polymorphism was increased (OR: 0.37, 95% CI: 0.18–0.77) significantly in the absence of the CC phenotype of the promoter region ($p = 0.019$, Mantel-Haenzsel test). Multivariate analyses controlling for age and gender showed that the intron 4a allele remained independently associated with LI (dominant model OR: 0.58; 95% CI, 0.35–0.97; $p = 0.04$; additive model: OR, 0.59; 95% CI, 0.37–0.95; $p = 0.03$). However, after adjustment for gender

Table II. Genotype distribution of eNOS polymorphisms.

		Controls		Lacunar infarction	
		n	%	*n*	%
Promoter (T-786C)	TT	35	44.3	25	37.3
	CT	41	51.9	33	49.3
	CC	3	3.8	9	13.4
Total (*n*)		79		67	
Intron 4	aa	13	16.0	1	1.4
	ab	24	29.6	20	28.6
	bb	44	54.3	49	70.0
Total (*n*)		81		70	
Exon 7 (G894T)	GG	22	27.2	23	34.3
	GT	47	58.0	36	53.7
	TT	12	14.8	8	11.9
Total (*n*)		81		67	

and age, the promoter region polymorphism presence was no more a significant predictor of increased disease risk. These multivariate results show that the age and gender imbalance between the controls and LI cases does not affect our finding of the protective effect of intron 4aa polymorphism, demonstrated by the adjusted ORs of less than 1.

Haplotype analysis

Regarding the three loci, all eight potential haplotypes (combinations of T/C for promoter, a/b for intron 4 and G/T for exon 7 regions) were represented. There was a significant difference in haplotype distribution in isolated LI ($p = 0.001$), where the C-a-T and C-a-G haplotypes were less frequently seen in LI patients.

Functional significance of polymorphisms: Forearm blood flow levels and NTG test

FMD levels were 10.4 ± 2 (mean $\pm$ SE) in controls and 5.1 ± 1 in patients with LI ($p = 0.035$). When a cut-off of 10% was used, 77.8% of patients with LI had lower FMD levels as opposed to 31.8% of controls ($p = 0.006$, chi-square test). This cut-off was 77.8% sensitive and 68.8% specific in detecting LI. There were no significant differences in FMD levels when the polymorphism sub-groups were compared with each other (all p's > 0.30, Chi-square test). The mean intraobserver variability for measurement of flow-mediated vasodilatation was 1.7% (SD 0.8%) and the mean interobserver variability was found to be 2.2% (SD 0.9%). The response to NTG (nitroglycerin-mediated dilatation [NMD]) was not significantly different between both groups ($14.5 \pm 4.3\%$ vs $15.6 \pm 5.1\%$, $p = 0.63$), indicating that the source of dilatory dysfunction in patients with LI is the endothelium, but not the vascular smooth muscle.

Table III. Odds ratios associated with polymorphisms.

Allele	Odds ratio for risk of lacunar infarct
−786C (Promoter)	
Additive	1.55 (0.91–2.64)
Dominant	1.33 (0.73–3.16)
Recessive	2.93 (1.02–15.2)[a]
4aa (Intron)	
Additive	0.47 (0.28–0.81)[b]
Dominant	0.51 (0.26–1.0)[c]
894T (Exon)	
Additive	0.78 (0.47–1.30)
Dominant	0.71 (0.35–1.44)
Recessive	0.78 (0.30–2.04)

[a] $p = 0.047$, [b] $p = 0.006$, [c] $p = 0.0495$, all other p's > 0.34.

Discussion

Our findings suggest that intron 4aa genotype of eNOS gene is protective for isolated symptomatic LI in Turkish patients and this effect is potentiated by the absence of -789C polymorphism in the promoter region. The protective effect of intron 4aa genotype for isolated LI was sustained after adjustment for age and gender. These findings are consistent with the hypothesis proposing a role of endothelial dysfunction in cerebral SVD, which is possibly related to a deficient production of the endothelium-derived NO, as suggested by an impaired FMD in these patients. The CC genotype in the promoter region was determined to be associated with 2.9-times higher LI risk, but was insignificant when age and gender adjustment was performed in our cohort.

Conflicting data exist in the literature concerning the association of the eNOS polymorphisms and SVD. Hou et al. [8] reported the intron 4aa polymorphism as a risk factor for all stroke types in Chinese, whereas Yahashi et al. [19] found no evidence for this polymorphism as a genetic risk factor for cerebrovascular diseases including lacunar stroke in Japanese. On the other hand, Hassan et al. [6] found the same polymorphism to be protective in isolated LI in British population. It is possible that the frequency of eNOS polymorphisms vary noticeably among different ethnic groups. 4aa variant in intron 4 was found to be more common in African-Americans (26.5%) than in Caucasians (16.0%) or Asians (12.9%), so inter-ethnic differences also exist in the distribution of eNOS genetic variants [27,28]. Moreover, environmental factors may modify the influence of eNOS variants on disease risk. For example, the highly salted Japanese diet may mask the contribution of eNOS polymorphism to LI risk by substantially increasing the prevalence of hypertension [29].

To date, nine studies investigating the role of eNOS gene polymorphisms in different diseases in Turkish population have been reported [30–38]. However, there are no reports investigating tandem 27-bp repeats of intron 4 in stroke patients in Turkey and, in the only eNOS polymorphism study, Akar et al.

[35] found no correlation between G894T (exon 7) polymorphism and stroke. Our results for frequency of polymorphisms in controls are consistent with the nine eNOS polymorphism studies performed in Turkish population, whereas minor differences exist, possibly reflecting regional ethnic differences, variation in recruitment criteria used and limited sample sizes.

Although we found an association with intron 4 locus and SVD, it could be possible that this locus acts simply as a marker for another functional polymorphism including those we have not studied. In fact, it has been reported that eNOS haplotypes modulate NO formation [39,40]. Wang [5] explored the underlying molecular mechanism by site-directed mutagenesis and found that the intron 4 polymorphism affects the transcription efficiency in a haplotype-specific fashion in linkage disequilibrium with the T-786C polymorphism in the promoter region. As discussed by the authors, one polymorphism may be in linkage disequilibrium with a regulatory polymorphism on the same haplotype [41]. In our study, with an increased frequency of C allele in T-786C promoter region polymorphism, the association of intron 4aa polymorphism with SVD became stronger (OR representing LI risk in absence of four repeats goes from 1.96 to 2.70). An increased transmission of the −786C intron 4a suggests that the regulatory polymorphism may be located on this haplotype. This haplotype could have a particular functional role. One possibility is that the tandem 27-bp repeats in intron 4 may have a *cis* regulatory role enhancing transcription activity at the T-786C promoter region [5]. Similar to our results, Hassan et al. [6] also found that the intron 4aa was protective against isolated LI. They also proposed that the protective effect of the intron 4aa could be mediated through changes in eNOS promoter activity. However, it is also possible that the tandem 27-bp repeats in intron 4 could affect mRNA stability and, hence, enzyme levels. Supporting this idea, Dosenko et al. [42] measured eNOS mRNA and enzyme activity in isolated platelets and found that the enzyme activity was lower in carriers of intron 4aa genotype compared to normal homozygotes.

The G894T variant has been shown to be functional, yielding a protein that is more susceptible to proteolytic cleavage and thus leads to lower eNOS levels *in vitro* [43]. However, conflicting results exist regarding clinical significance of this finding; Cam et al. [34] reported that the G894T polymorphism in exon 7 was associated with coronary artery disease in the Turkish population. In contrast, Akar et al. [35] and Afrasyap and Ozturk [36] found no significant effect of G894T polymorphism on the risk and extent of coronary heart disease in another Turkish cohort. Discrepancy may stem from the nature of sampling variability in case control study design and/or it reflects geographical difference or from confounding environmental risk factors (e.g. smoking). The characteristics of haplotypes may also show differences between different populations. For example, Elbaz et al. [7] reported 894G to be a risk factor for lacunar stroke, but us and others [6,12] have not found such an association.

We used the established, non-invasive technique, FMD in brachial artery as a functional test to assess the effect of eNOS polymorphisms on the endothelium [44]. A principal mediator of FMD is the endothelium-derived NO. Ischemia provokes the endothelium to release NO with subsequent vasodilatation that can be imaged and quantified as an index of endothelial function [26]. eNOS and the subsequent generation of NO is believed to account for FMD, since treatment with NOS inhibitors abolish FMD in a variety of arterial vessels [45]. Yet, it was recently shown that blood vessels from mice genetically engineered to lack the eNOS enzyme (eNOS knockout mice) still respond to shear stress by vasodilatation [46]. So, other endothelial mediators than NO may also contribute to FMD. Although the exact molecular mechanism underlying the endothelial dysfunction in patients with LI remains to be determined for future studies, we found that FMD in LI patients was significantly lower compared to those with healthy controls, as very recently reported by two other groups as well [47,48], confirming that patients with LI have generalized endothelial dysfunction. However, we found no significant differences in FMD levels when the polymorphism subgroups were compared with each other. Although the later finding is most likely a reflection of the small sample size used during the functional assessment in this study, this may also suggest that the genetic predisposition becomes clinically significant only after development of SVD under the influence of cardiovascular risk factors that disrupt the endothelial function. This finding is not surprising because, had the polymorphism caused major eNOS malfunction, it would lead to a familial form of SVD that would probably appear earlier in life and be associated with generalized cardiovascular problems. We therefore conclude that the tandem 27-bp repeats in intron 4 may have functional consequences only in arteries already afflicted with dysfunctional endothelium. Penetrating brain arterioles may be particularly dependent on high throughput NO synthesis and, hence, vulnerable to endothelial dysfunction and eNOS polymorphisms [49].

In conclusion, intron 4aa genotype of eNOS gene appears to be protective against isolated LI. Further studies with larger sample sizes are needed to confirm the interaction between intron 4aa and other eNOS gene polymorphisms on isolated LI. Our findings from genotyping and forearm blood flow studies strongly support the hypothesis that the endothelial

dysfunction plays an important role in the pathogenesis of LI. Future studies could help determine the molecular mechanisms of the arteriolopathy underlying LI and may have implications in the treatment and prevention of LI.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Norrving B. Long-term prognosis after lacunar infarction. Lancet Neurol 2003;2:238–245.

[2] Bamford JM, Warlow CP. Evolution and testing of the lacunar hypothesis. Stroke 1988;19:1074–1082.

[3] Hassan A, Markus HS. Genetics and ischaemic stroke. Brain 2000;123:1784–1812.

[4] Briley D, Brown MM, Thomas DJ, Markus HS. Markers of endothelial dysfunction in lacunar infarction and ischaemic leukoaraiosis. Brain 2003;126:424–432.

[5] Wang MM. Genetics of ischemic stroke: future clinical applications. Semin Neurol 2006;26:523–530.

[6] Vallance P, Bamford J, Markus H. Endothelial nitric oxide gene haplotypes and risk of cerebral small-vessel disease. Stroke 2004;35:654–659.

[7] Elbaz A, Poirier O, Moulin T, Chedru F, Cambien F, Amarenco P. Association between the Glu298Asp polymorphism in the endothelial constitutive nitric oxide synthase gene and brain infarction. The GENIC investigators. Stroke 2000;31:1634–1639.

[8] Hou L, Osei-Hyiaman D, Yu H, Ren Z, Zhang Z, Wang B, Harada S. Association of a 27-bp repeat polymorphism in ecNOS gene with ischemic stroke in Chinese patients. Neurology 2001;56:490–496.

[9] Iadecola C, Pelligrino DA, Moskowitz MA, Lassen NA. Nitric oxide synthase inhibition and cerebrovascular regulation. J Cereb Blood Flow Metab 1994;14:175–192.

[10] White RP, Deane C, Vallance P, Markus HS. Nitric oxide synthase inhibition in humans reduces cerebral blood flow but not the hyperemic response to hypercapnia. Stroke 1998;29:467–472.

[11] Moncada S, Higgs EA. Nitric oxide and the vascular endothelium. Handb Exp Pharmacol 2006;176:213–254.

[12] Markus HS, Lythgoe DJ, Ostegaard L, O'Sullivan M, Williams SC. Reduced cerebral blood flow in white matter in ischaemic leukoaraiosis demonstrated using quantitative exogenous contrast based perfusion MRI. J Neurol Neurosurg Psychiatry 2000;69:48–53.

[13] Janssens SP, Shimouchi A, Quertermous T, Bloch DB, Bloch KD. Cloning and expression of a cDNA encoding human endothelium-derived relaxing factor/nitric oxide synthase. J Biol Chem 1992;267:14519–14522.

[14] Shi XM, Tsui LC, Schappert KT. Structure and chromosomal localization of the human constitutive endothelial nitric oxide synthase gene. J Biol Chem 1993;268:17478–17488.

[15] Nadaud S, Bonnardeaux A, Lathrop M, Soubrier F. Gene structure, polymorphism and mapping of the human endothelial nitric oxide synthase gene. Biochem Biophys Res Commun 1994;198:1027–1033.

[16] Lange LA, Macko RF, Bashera MJ, Meyers DA, Cole JW, Kittner SJ. Promoter polymorphisms in the nitric oxide synthase 3 gene are associated with ischemic stroke susceptibility in young black women. Stroke 2005;36:1848–1851.

[17] Nasreen S, Nabika T, Shibata H, Moriyama H, Yamashita K, Masuda J, Kobayashi S. T-786C polymorphism in endothelial NO synthase gene affects cerebral circulation in smokers: possible gene-environmental interaction. Arterioscler Thromb Vasc Biol 2002;22:605–610.

[18] Somogyvari F, Kondacs A, Szabo M, Fodor L, Bodor A, Gati I, Wittman I, Melegh B. Endothelial nitric oxide synthase gene interactions and the risk of ischaemic stroke. Acta Neurol Scand 2005;111:29–33.

[19] Yahashi Y, Kario K, Shimada K, Matsuo M. The 27-bp repeat polymorphism in intron 4 of the endothelial cell nitric oxide synthase gene and ischemic stroke in a Japanese population. Blood Coagul Fibrinolysis 1998;9:405–409.

[20] Davidson S. Research suggests importance of haplotypes over SNPs. Nat Biotechnol 2000;18:1134–1135.

[21] Jungersten L, Edlund A, Petersson AS, Wennmalm A. Plasma nitrate as an index of nitric oxide formation in man: analyses of kinetics and confounding factors. Clin Physiol 1996;16:369–379.

[22] Kleinbongard P, Dejam A, Lauer T, Rassaf T, Schindler A, Picker O, Scheeren T, Gödecke A, Schrader J, Schulz R, Heusch G, Schaub GA, Bryan NS, Feelisch M, Kelm M. Plasma nitrite reflects constitutive nitric oxide synthase activity in mammals. Free Radic Biol Med 2003;35:790–796.

[23] Metzger IF, Sertorio JTC, Tanus-Santos JE. Relationship between systemic nitric oxide metabolites and cyclic GMP in healthy male volunteers. Acta Physiol 2006;188:123–127.

[24] Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988;16:1215.

[25] Tanus-Santos JE, Desai M, Deak LR, Pezzullo JC, Abernethy DR, Flockhart DA, Freedman JE. Effects of endothelial nitric oxide synthase gene polymorphisms on platelet function, nitric oxide release, and interactions with estradiol. Pharmacogenetics 2002;12:407–413.

[26] Creager MA, Deanfield J, Drexler H, Gerhard-Herman M, Herrington D, Vallance P, Vita J, Vogel R. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. J Am Coll Cardiol 2002;39:257–265.

[27] Marroni AS, Metzger IF, Souza-Costa DC, Nagassaki S, Sandrim VC, Correa RX, Rios-Santos F, Tanus-Santos JE. Consistent interethnic differences in the distribution of clinically relevant endothelial nitric oxide synthase genetic polymorphisms. Nitric Oxide 2005;12:177–182.

[28] Tanus-Santos JE, Desai M, Flockhart DA. Effects of ethnicity on the distribution of clinically relevant endothelial nitric oxide variants. Pharmacogenetics 2001;11:719–725.

[29] Araki S, Sugiomoto T, Nishio Y, Maegawa H, Koya D, Haneda M, Kashiwagi A. Enhanced sodium sensitivity and disturbed circadian rhythm of blood pressure in essential hypertension. J Hypertension 2006;24:1627–1632.

[30] Karasneh JA, Hajeer AH, Silman A, Worthington J, Ollier WE, Gul A. Polymorphisms in the endothelial nitric oxide synthase gene are associated with Behcet's disease. Rheumatology 2005;44:614–617.

[31] Oksel F, Keser G, Ozmen M, Aksu K, Kitapcioglu G, Berdeli A, Doganavsargil E. Endothelial nitric oxide synthase gene Glu298Asp polymorphism is associated with Behcet's disease. Clin Exp Rheumatol 2006;24:S79–S82.

[32] Berdeli A, Gurkan A, Emingil G, Atilla G, Kose T. Endothelial nitric oxide synthase Glu298Asp gene polymorphism in periodontal diseases. J Periodontol 2006;77:1348–1354.

[33] Dayi SU, Ciloglu F, Aksu H, Asilturk R, Cagil A. The relationship between endothelial nitric oxide synthase gene polymorphism (T-786 C) and coronary artery disease in the Turkish population. Heart Vessels 2006;21:285–290.

[34] Cam SF, Sekuri C, Tengiz I, Ercan E, Sagcan A, Akin M, Berdeli A. The G894T polymorphism on endothelial nitric

oxide synthase gene is associated with premature coronary artery disease in a Turkish population. Thrombosis Res 2005;116:287–292.
[35] Akar N, Akar E, Deda G, Sipahi T. No association between Glu/Asp polymorphism of NOS3 gene and ischemic stroke. Neurology 2000;55:460–461.
[36] Afrasyap L, Ozturk G. NO level and endothelial NO synthase gene polymorphism (Glu298Asp) in the patients with coronary artery disease from the Turkish population. Acta Biochim Biophys Sin (Shanghai) 2004;36:661–666.
[37] Kara N, Senturk N, Gunes SO, Bagci H, Yigit S, Turanli AY. Lack of evidence for association between endothelial nitric oxide synthase gene polymorphism (glu298asp) with Behcet's disease in the Turkish population. Arch Dermatol Res 2006;297:468–471.
[38] Alasehirli B, Demiryurek S, Arica E, Gursoy S, Demiryurek AT. No evidence for an association between the Glu298Asp polymorphism of the endothelial nitric oxide synthase gene and fibromyalgia syndrome. Rheumatol Int 2007;27:275–280.
[39] Metzger IF, Sertório JT, Tanus-Santos JE. Modulation of nitric oxide formation by endothelial nitric oxide synthase gene haplotypes. Free Radic Biol Med 2007;43:987–992.
[40] Metzger IF, Souza-Costa DC, Marroni AS, Nagassaki S, Desta Z, Flockhart DA, Tanus-Santos JE. Endothelial nitric oxide synthase gene haplotypes associated with circulating concentrations of nitric oxide products in healthy men. Pharmacogenet Genom 2005;15:565–570.
[41] Kim JU, Chang HK, Lee SS, Kim JW, Kim KT, Lee SW, Chung WT. Endothelial nitric oxide synthase gene polymorphisms in Behcet's disease and rheumatic diseases with vasculitis. Ann Rheum Dis 2003;62:1083–1087.

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[42] Dosenko VE, Zagoriy VY, Haytovich NV, Gordok OA, Moibenko AA. Allelic polymorphism of endothelial NO-synthase gene and its functional manifestations. Acta Biochim Pol 2006;53:299–302.
[43] Tesauro M, Thompson WC, Rogliani P, Qi L, Chaudhary PP, Moss J. Intracellular processing of endothelial nitric oxide synthase isoforms associated with differences in severity of cardiopulmonary diseases: cleavage of proteins with aspartate vs. glutamate at position 298. Proc Natl Acad Sci USA 2000;97:2832–2835.
[44] Sorensen KE, Celermajer DS, Spiegelhalter DJ, Georgakopoulos D, Robinson J, Thomas O, Deanfield JE. Non-invasive measurement of human endothelium dependent arterial responses: accuracy and reproducibility. Br Heart J 1995;74:247–253.
[45] Joannides R, Haefeli WE, Linder L, Richard V, Bakkali EH, Thuillez C, Luscher TF. Nitric oxide is responsible for flow-dependent dilatation of human peripheral conduit arteries in vivo. Circulation 1995;91:1314–1319.
[46] Shesely EG, Koller A, Kaley G. Enhanced release of prostaglandins contributes to flow-induced arteriolar dilation in eNOS knockout mice. Circ Res 1999;85:288–293.
[47] Pretnar-Oblak J, Sabovic M, Pogacnik T, Sebestjen M, Zaletel M. Flow-mediated dilatation and intima-media thickness in patients with lacunar infarctions. Acta Neurol Scand 2006;113:273–277.
[48] Chen PL, Wang PY, Sheu WH, Chen YT, Ho YP, Hu HH, Hsu HY. Changes of brachial flow-mediated vasodilation in different ischemic stroke subtypes. Neurology 2006;67:1056–1058.
[49] Kimura M, Dietrich HH, Dacey RG, Jr. Nitric oxide regulates cerebral arteriolar tone in rats. Stroke 1994;25:2233–2234.