Clinical Endocrinology (2008) **68**, 542–546 doi: 10.1111/j.1365-2265.2007.03089.x

ORIGINAL ARTICLE

Endothelial nitric oxide synthase Glu298Asp, 4b/a, and T-786C polymorphisms in type 2 diabetic retinopathy

Intissar Ezzidi*, Nabil Mtiraoui*, Manel Ben Hadj Mohamed*, Touhami Mahjoub*, Maha Kacem† and Wassim Y. Almawi‡

*Research Unit of Haematological and Autoimmune Diseases, Faculty of Pharmacy, Monastir, Center University, Tunisia, †Nephrology and Internal Medicine Service – EPS F. Bourguiba, Monastir, Tunisia, ‡Department of Medical Biochemistry, College of Medicine & Medical Sciences, Arabian Gulf University, Manama, Bahrain

Summary

Objective The possible association between the endothelial nitric oxide (eNOS) gene T-786C (promoter region), 27-bp repeat 4b/4a (intron 4), and Glu298Asp (exon 7) polymorphisms with diabetic retinopathy (DR) was investigated.

Design A retrospective case-control study.

Patients A total of 872 type 2 diabetes (T2DM) patients were studied, of whom 383 presented with preproliferative/proliferative retinopathy (DR group), and 489 with absent/mild retinopathy (DWR group).

Measurements Glu298Asp and T-786C genotyping was carried out by PCR-RFLP analysis, while 4b/4a was assessed by PCR. Genotype distribution was compared using the χ^2-test, and the contributions of the polymorphisms to DR were analysed by haplotype analysis and multivariate regression analysis.

Results Lower prevalence of mutant 4a ($P = 0{\cdot}011$), and heterozygous 4b/4a ($P = 0{\cdot}042$) were seen in the DR compared to the DWR groups; the allele and genotype distribution of the Glu298Asp and T-786C polymorphisms were comparable between DR and DWR groups. Three-loci haplotype analysis demonstrated significant association between eNOS variants and DR, with protective [haplotype 122 (Glu298/4a/-786C)], and susceptible haplotypes [haplotypes 112 (Glu298/4b/-786C) and 222 (Asp298/4a/-786C)] identified. Multivariate regression analysis confirmed the association between haplotypes 122 ($P = 0{\cdot}015$); 112 ($P = 0{\cdot}027$), and 222 ($P = 0{\cdot}048$) and DR, after controlling for potential covariates (including age, sex, age of disease onset; HbA1c; hypertension, total cholesterol).

Conclusions This study identifies genetic variation at the eNOS locus as genetic risk factor for diabetic retinopathy, which may serve as a useful marker of increased susceptibility to the risk of retinopathy.

Correspondence: Wassim Y. Almawi, Department of Medical Biochemistry, College of Medicine & Medical Sciences, Arabian Gulf University, PO Box 22979, Manama, Bahrain. Tel.: +973 39717118; Fax: +973 17271090; E-mail: wyalmawi@yahoo.co.uk

(Received 16 June 2007; returned for revision 17 August 2007; finally revised 29 August 2007; accepted 24 September 2007)

Introduction

Diabetic retinopathy (DR) is a major cause of blindness among diabetic adults,[1] and is aggravated by poor glycaemic control.[2,3] Several mechanisms are reportedly involved in the DR-associated malfunction of the blood–retinal barrier (BRB), including induction of inflammatory processes,[4] altered endothelial cell junctions and viability,[5] and central retinal venous congestion.[5,6] Endothelial dysfunction induced by reduced nitric oxide (NO) availability, and consequently increased reactive oxygen species production, reportedly impaired ocular haemodynamics, suggesting a role for NO in DR pathogenesis.[7]

NO is a pleiotropic molecule, which regulates several aspects of vascular tone, including inhibition of platelet aggregation, down-regulation of leucocyte adherence,[8] and suppression of smooth muscle cell proliferation.[8,9] NO is produced by three nitric oxide synthase (NOS) isoforms: neuronal NOS, inducible NOS, and endothelial NOS (eNOS or NOS3).[8,9] Low NO concentrations induced by eNOS are necessary for maintaining endothelial function, while attenuation of NO production induced by eNOS gene mutations resulted in endothelial dysfunction, and precipitated atherogenic events, including those associated with T2DM.[10,11]

Several eNOS gene polymorphisms have been identified, of which the T-786C (promoter region), Glu298Asp (exon 7), and the 27-bp repeat 4b4a (intron 4) polymorphisms are the most investigated, and are associated with cardiovascular diseases, hypertension, and vascular disorders.[10–13] A limited number of studies have examined the possible association between these eNOS polymorphisms and DR, with inconsistent results. For example, the (mutant)-786C[12,13] and 4a[12] alleles were associated with DR in some studies, while others reported no such association of either T-786C[14,15] or 4b/4a.[14–16] Others suggested that the 4b, but not 4a, allele was associated with a high risk of DR in type 1 diabetes.[17] Here we compared the distribution of the three eNOS polymorphisms in Tunisian T2DM patients with (DR) or without (DWR) retinopahy, together with their possible association

© 2007 The Authors
Journal compilation © 2007 Blackwell Publishing Ltd

Table 1. Clinical characteristics of study subjects

Characteristic	DWR group (489)	DR group (383)	*P*
Gender (Male : female)	227:262	169:214	0·537†
Age at study (years)	59·9 ± 9·7	60·9 ± 10·9	0·141‡
Mean BMI (kg/m^2)	27·6 ± 5·2	28·0 ± 5·6	0·206‡
Diabetes duration (years)	10·7 ± 5·6	11·2 ± 4·6	0·248‡
Age of onset (years)	47·4 ± 11·0	47·7 ± 10·9	0·720‡
Systolic BP (mmHg)	137·7 ± 30·0	142·0 ± 24·2	0·073‡
Diastolic BP (mmHg)	81·0 ± 13·0	81·0 ± 12·2	0·954‡
Glucose (mmol/l)	13·0 ± 5·3	12·5 ± 5·1	0·134‡
HbA1c (%)	9·8 ± 3·8	9·4 ± 3·3	0·124‡
HDL (mmol/l)	1·0 ± 0·3	1·1 ± 0·4	0·490‡
LDL (mmol/l)	3·8 ± 1·3	3·8 ± 1·4	0·743‡
Total cholesterol (mmol/l)	5·1 ± 1·3	5·5 ± 1·6	< 0·001‡
Triglycerides (mmol/l)	1·5 ± 1·1	2·1 ± 1·4	< 0·001‡

†Pearson's χ^2-test; ‡Student's *t*-test.

with DR. Here we demonstrate the presence of DR-susceptible and DR-protective eNOS haplotypes in the population studied.

Subjects and methods

Subjects

This was a retrospective case-control study involving 872 unrelated adult Tunisian T2DM patients (476 female and 396 male subjects), recruited from the outpatient endocrinology service of Farhat Hached University Hospital (Sousse, Tunisia), and Fattouma Bourguiba University Hospital (Monastir, Tunisia). The study was carried out in accordance with the guidelines of the Helsinki Declaration of 1975, and had the approval of the University of Monastir Ethics Committee, and written informed consent was obtained from all participants. T2DM diagnosis was based on clinical features, and none of the patients had ever had ketoacidosis. Initial T2DM treatment included diet and/or oral antidiabetic drugs, and subjects who required insulin had been treated with oral drugs for at least 2 years (Table 1).

Demographic details were obtained on all subjects; these included age, gender, BMI, age at onset and duration of diabetes, first-degree family history of diabetes, history of chronic diabetes complications, and treatment of diabetes. The historical information was verified from clinic records where available. Venous blood samples were collected after an overnight fast to measure plasma glucose, HbA1c, and serum lipids. Hypertension was defined as seated blood pressure readings of 140/90 mmHg and higher, and/or if subjects were receiving antihypertensive therapy.

All patients were subjected to ophthalmological examination, which included corrected visual acuity, funduscopic examination and photography, and examination by slit-lamp microscopy with and without preset lens. DR was determined and graded by an ophthalmologist, and was defined as at least one microaneurysm, haemorrhage or exudate in either eye. Fluorescein angiography was performed on some patients to confirm the funduscopic findings.

eNOS genotyping

eNOS Glu298Asp and T-786C genotype analysis was performed by PCR-RFLP analysis, using MboI and MspI digestion, respectively. For Glu298Asp, PCR amplification of exon 7 with the primers 5′-CAT GAG GCT CAG CCC CAG AAC-3′ (sense) and 5′-AGT CAA TCC CTT TGG TGC TCA C-3′ (antisense), was followed by MboI restriction. Digested products were separated on 7% SDS-PAGE gels; the Glu allele was visualized as a 206-bp band, while the Asp allele was visualized as 119 and 87 bp fragments. The T-786C polymorphism was assessed by PCR-RFLP analysis of the 236 bp PCR product (Msp I digestion) using the following primers: (sense) 5′-CACCCAGGCCCACCCCAACT-3′, and (antisense): 5′-GCCGCAGGTCGACAGAGAGACT-3′; restricted fragments were separated by electrophoresis on 20% SDS-PAGE. The 4a4b polymorphism was detected by PCR using the following primers: (sense) 5′-CTATGGTAGTGCCTTGGCTGGAGG-3′, and (antisense) 5′-ACCGCCCAGGGAACTCCGCT-3′. PCR products comprised a 420-bp band corresponding to the five 27-bp repeats (b allele), and a 393-bp band corresponding to the four 27-bp repeats (a allele).

Statistical analysis

Statistical analysis was performed using SPSS version 13·0 software (SPSS Inc., Chicago, IL). Data were expressed as mean ± SD (continuous variables), or as percentages of the total (categorical variables). Pearson's χ^2-test or Fisher's exact test was used to assess intergroup significance, and Student's *t*-test was used to determine differences in means. Allele frequencies were calculated by the gene-counting method, and each polymorphism was tested for Hardy–Weinberg equilibrium using χ^2 goodness-of-fit test, using HPlus 2·5 software (http://qge.fhcrc.org). Differences in the allele and genotype frequencies of the eNOS gene variants were tested by Pearson's χ^2-test and Fisher's exact test.

eNOS haplotype estimation was carried out by the expectation maximization method using HPlus 2·5, where the sum of probability estimates for all possible haplotypes equals 1·0. Where haplotype assignment was uncertain (heterozygous carriers), the haplotype assignment probability estimate was used to determine the individual's contribution to that haplotype. eNOS haplotypes were coded as per the allele at each locus (1 for wild-type allele, 2 for mutant allele). The first number refers to Glu298Asp (1 for Glu298, 2 for Asp298), the second to 4b4a (1 for 4b, 2 for 4a), and the third number refers to T-786C (1 for -786T; 2 for -786C). Univariate and multivariate regression analysis was determined using HPlus 2·5 and HAPStat haplotype analysis software (http://bios.unc.edu); results were expressed as *P*-value, odds ratio (OR) and 95% confidence intervals (CI). Statistical significance was set at $P < 0{\cdot}05$.

Results

Study subjects

The characteristics of DR and DWR patients are shown in Table 1. There were 489 DWR patients with no evidence of retinopathy, and 383 patients with confirmed DR. The two groups were matched for gender, age, BMI, age at disease onset, and duration of diabetes,

© 2007 The Authors
Journal compilation © 2007 Blackwell Publishing Ltd,

Table 2. eNOS G894T, 4b4a and T-786C allele and genotype frequencies

	Allele/genotype	DWR group (489)	DR group (383)	*P*
G894T	G	0·64 ± 0·01†	0·61 ± 0·02	0·456
	T	0·36 ± 0·01	0·39 ± 0·02	0·172
	G/G	194 (39·8)‡	137 (35·9)	0·260
	G/T	231 (47·4)	190 (49·7)	0·544
	T/T	62 (12·7)	55 (14·4)	0·539
4b4a	4b	0·76 ± 0·01	0·81 ± 0·01	0·201
	4a	0·24 ± 0·01	0·19 ± 0·01	0·011
	4b/4b	276 (56·4)	251 (65·5)	0·008
	4b/4a	180 (36·8)	115 (30·0)	0·042
	4a/4a	33 (6·7)	17 (4·4)	0·190
T-786C	T	0·75 ± 0·01	0·70 ± 0·02	0·024
	C	0·25 ± 0·01	0·30 ± 0·02	0·054
	T/T	278 (56·9)	192 (50·1)	0·072
	T/C	182 (37·2)	155 (40·5)	0·431
	C/C	29 (5·9)	36 (9·4)	0·069

†Allele frequencies ± SD. ‡Number of individuals (per cent).

together with systolic and diastolic blood pressure. Fasting glucose, HbA1c, HDL and LDL levels were comparable between both patient groups, while higher total cholesterol ($P < 0·001$) and triglyceride ($P < 0·001$) levels were seen in DR than in DWR patients. Whereas initial management of diabetes was comparable between DR and DWR groups ($P = 0·069$), consisting primarily of oral hypoglycaemics (64·9 *vs.* 63·9%) and diet (20·8 *vs.* 20·2%), a significantly higher proportion of DR patients required later (> 5 years postinitial diagnosis) supplementation with insulin (46·8 *vs.* 20·7%; $P < 0·001$).

Genotype analysis

The genotype frequency distributions of the three polymorphisms did not deviate from Hardy–Weinberg equilibrium among participants ($P = 0·39$ for Glu298Asp, $P = 0·43$ for 4b4a, $P = 0·52$ for T-786C). There was a lower frequency of the 4a allele ($P = 0·011$) and heterozygous 4b/4a ($P = 0·042$) in the DR than in the DWR group (Table 2). The allele and genotype distribution of the Glu298Asp and T-786C SNPs was comparable between both groups of T2DM patients (Table 2).

Table 3. eNOS haplotype distribution in patients and controls

Haplotype†	DWR group (489)	DR group (383)	*P*‡	OR (95% CI)
111	0·356 ± 0·018	0·326 ± 0·015	0·393	0·88 (0·66–1·16)
211	0·220 ± 0·025	0·238 ± 0·025	0·589	1·11 (0·81–1·52)
121	0·119 ± 0·028	0·096 ± 0·028	0·303	0·78 (0·50–1·21)
112	0·104 ± 0·026	0·165 ± 0·031	0·009	1·71 (1·16–2·53)
212	0·073 ± 0·029	0·078 ± 0·019	0·852	1·08 (0·66–1·79)
221	0·060 ± 0·027	0·039 ± 0·021	0·272	0·67 (0·37–1·27)
122	0·057 ± 0·025	0·018 ± 0·120	0·004	0·27 (0·12–0·68)
222	0·011 ± 0·150	0·040 ± 0·022	0·009	3·64 (1·37–8·75)

†Haplotype (G894T-4b/a-T-786C) frequency determined by the maximum likelihood method. ‡Fisher's exact test.

Haplotype distribution

The three-locus eNOS haplotype analysis, stratified by study subjects, is shown in Table 3. Of the eight major eNOS haplotypes identified, select eNOS haplotypes were positively or negatively associated with DR. These comprised the 112 ($P = 0·009$) and 222 ($P = 0·009$) haplotypes, which were higher, and the 122 haplotype ($P = 0·004$), which was lower among DR than DWR patients, thus conferring a disease susceptibility and protective nature to these haplotypes, respectively.

Regression analysis

The association between eNOS polymorphic variants and DR was examined first at univariate, and then at multivariate levels. Taking the 111 haplotype as reference, univariate analysis identified the 122 haplotype to be negatively associated ($P = 0·003$; OR = 0·46; 95% CI = 0·28–0·77), and the 222 haplotype to be positively associated ($P = 0·012$; OR = 2·79; 95% CI = 1·26–6·19) with DR (Table 4).

Table 4. Analysis of eNOS haplotypes in diabetic retinopathy

	Unadjusted			Multivariate*		
Haplotype†	Z-score	*P*	OR (95% CI)	Z-score	*P*	aOR‡ (95% CI)
111			1·00			1·00
211	0·29	0·769	1·05 (0·76–1·45)	1·39	0·164	1·20 (0·93–1·56)
112	1·31	0·190	1·25 (0·89–1·76)	2·20	0·027	1·34 (1·03–1·73)
121	−0·57	0·569	0·91 (0·67–1·24)	−0·69	0·492	0·91 (0·69–1·20)
212	0·84	0·398	1·15 (0·83–1·59)	1·25	0·211	1·18 (0·91–1·55)
221	−0·21	0·836	0·95 (0·59–1·54)	−1·50	0·134	0·70 (0·44–1·12)
122	−2·95	0·003	0·46 (0·28–0·77)	−2·44	0·015	0·51 (0·30–0·88)
222	2·52	0·012	2·79 (1·26–6·19)	1·97	0·048	2·55 (1·01–6·44)

*Adjusted for age, gender, age of disease onset, HbA1c, hypertension, total cholesterol concentrations, and medications used (antihypertensive and lipid-lowering). †G894T-4a/b-T-786C haplotype. ‡aOR, adjusted odds ratio.

© 2007 The Authors
Journal compilation © 2007 Blackwell Publishing Ltd,

Multivariate analysis confirmed the association between the 122 and 222 haplotypes and DR, and in addition identified the 112 haplotype ($P = 0{\cdot}027$; OR = 1·34; 95% CI = 1·03–1·73) to be associated with DR after adjustment for the covariates age, gender, age of disease onset, HbA1c, hypertension, and total cholesterol concentrations (Table 4).

Discussion

A few studies have investigated the association between the eNOS polymorphisms and DR, but with inconsistent results. We found an association between genetic variation in the eNOS gene and DR in Tunisian T2DM patients, which was confirmed by haplotype analysis, with protective (haplotype 122) and susceptible (112 and 222) haplotypes identified, hence indicating an important role of the NO pathway in DR pathogenesis. DR and DWR patients were matched according to DR risk factors, including duration of diabetes (and age of onset), and HbA1c, thereby ruling out the possibility that patients were more susceptible to DR because of longer exposure to hyperglycaemia and poor glycaemic control.[2,3] Both DR and DWR patients had elevated total cholesterol, with higher total cholesterol seen in the DR group ($P < 0{\cdot}001$), as seen elsewhere.[2]

The eNOS gene polymorphic variants, in particular the 4b/4a polymorphism (intron-4), were significantly associated with DR. This is in disagreement with the study by Awata, which showed that the 4a allele, which was in linkage disequilibrium with the -786C allele, was significantly associated with DR,[12] and by others who failed to demonstrate any association between the 4b/4a polymorphism and DR.[14–16] Our results are in agreement with two smaller European studies, which similarly found that the 4b allele, and hence the 4b4b genotype, were associated with severe DR.[13,17] The most likely explanation for these apparently conflicting results is differences in ethnicity,[14–16] sample size,[14,15] type of diabetes[17] and the failure to control for confounding factors (in particular gender, HbA1c level, and obesity, and duration of diabetes) by some of these studies, which may have masked potential effects of eNOS gene variants on DR.

The association between eNOS gene variants and DR was confirmed by haplotype analysis at the univariate and multivariate levels, and specific eNOS haplotypes were positively (112 and 222) or negatively (122) associated with DR (multivariate analysis). Similar studies examining the relationship between eNOS haplotypes and DR are scarce. Sandrim reported that haplotype 112 was present at low, while haplotype 212 was present at high frequencies among hypertensive T2DM patients.[18] On the other hand, de Syllos suggested that haplotype 112 was protective of T2DM, as it was present at low frequencies among T2DM patients.[14] While not addressing DR, Franks demonstrated the select association between specific eNOS haplotypes and T2DM and associated conditions (energy metabolism).[19] The differences in eNOS haplotype distribution among Tunisian Arabs with other populations may be explained by ethnic variation in the eNOS haplotype distribution, highlighted by the high prevalence of the 111 haplotype among Asians (77%) compared to other ethnic groups (46%).[20]

eNOS polymorphic variants may influence NO production by different mechanisms. The Glu298Asp acts by controlling eNOS intracellular distribution, and interacts with proteins involved in its degradative processing,[21] while the T-786C SNP reduces by 50% eNOS gene promoter activity, leading to lower eNOS mRNA accumulation and NO production.[22] The exact functional role of the 4b4a polymorphism in DR pathogenesis, which results from deleting one of five nucleotide repeats in intron 4 of eNOS gene, remains to be seen, although it appears to be distinct from that exerted by the T-786C or Glu298Asp variants. The 4a variant may act by modulating eNOS transcriptional and/or post-transcriptional rates by binding as enhancer/repressor to nuclear proteins, thus modulating eNOS gene transcription efficiency, as has been suggested.[23] Impaired eNOS expression in the retina may contribute to the development of retinopathy, including breakdown of the endothelium-maintained BRB, altered vascular tone, and stimulation of leucocyte adhesion to the endothelium.[24]

In conclusion, eNOS gene polymorphism appears to be an independent risk factor for the development of retinopathy in T2DM patients, which was predictive even after controlling for potential confounders, including HbA1c and disease duration. A limitation of this study was that it was limited to Tunisian Arabs, thus necessitating follow-up studies in T2DM patients with DR from different ethnic groups. In spite of these shortcomings, the association between the eNOS gene variants and DN susceptibility will strengthen our understanding of the link between NO production, T2DM and DR pathogenesis.

References

1 Ciulla, T.A., Amador, A.G. & Zinman, B. (2003) Diabetic retinopathy and diabetic macular edema: pathophysiology, screening, and novel therapies. *Diabetes Care*, **26**, 2653–2664.

2 Cundiff, D.K. & Nigg, C.R. (2005) Diet and diabetic retinopathy: insights from the Diabetes Control and Complications Trial (DCCT). *Medscape General Medicine*, **7**, 3.

3 Henricsson, M., Nilsson, A., Janzon, L. & Groop, L. (1997) The effect of glycaemic control and the introduction of insulin therapy on retinopathy in non-insulin-dependent diabetes mellitus. *Diabetic Medicine*, **14**, 123–131.

4 Williams, M.D. & Nadler, J.L. (2007) Inflammatory mechanisms of diabetic complications. *Current Diabetes Reports*, **7**, 242–248.

5 Joussen, A.M., Smyth, N. & Niessen, C. (2007) Pathophysiology of diabetic macular edema. *Developmental Ophthalmology*, **39**, 1–12.

6 Christoffersen, N. & Larsen, M. (2004) Unilateral diabetic macular oedema secondary to central retinal vein congestion. *Acta Ophthalmologica Scandinavica*, **82**, 591–595.

7 Toda, N. & Nakanishi-Toda, M. (2007) Nitric oxide: ocular blood flow, glaucoma, and diabetic retinopathy. *Progress in Retinal and Eye Research*, **26**, 205–238.

8 Wang, Y. & Marsden, P.A. (1995) Nitric oxide synthases: biochemical and molecular regulation. *Current Opinion in Nephrology and Hypertension*, **4**, 12–22.

9 Moncada, S. & Higgs, E.A. (2006) The discovery of nitric oxide and its role in vascular biology. *British Journal of Pharmacology*, **147**, S193–S201.

10 Liu, Y., Burdon, K.P., Langefeld, C.D., Beck, S.R., Wagenknecht, L.E., Rich, S.S., Bowden, D.W. & Freedman, B.I. (2005) T-786C polymorphism of the endothelial nitric oxide synthase gene is associated with albuminuria in the diabetes heart study. *Journal of the American Society of Nephrology*, **16**, 1085–1090.

© 2007 The Authors
Journal compilation © 2007 Blackwell Publishing Ltd,

11 Zhang, C., Lopez-Ridaura, R., Hunter, D.J., Rifai, N. & Hu, F.B. (2006) Common variants of the endothelial nitric oxide synthase gene and the risk of coronary heart disease among U.S. diabetic men. *Diabetes*, **55**, 2140–2147.

12 Awata, T., Neda, T., Iizuka, H., Kurihara, S., Ohkubo, T., Takata, N., Osaki, M., Watanabe, M., Nakashima, Y., Sawa, T., Inukai, K., Inoue, I., Shibuya, M., Mori, K., Yoneya, S. & Katayama, S. (2004) Endothelial nitric oxide synthase gene is associated with diabetic macular edema in type 2 diabetes. *Diabetes Care*, **27**, 2184–2190.

13 Taverna, M.J., Elgrably, F., Selmi, H., Selam, J.L. & Slama, G. (2005) The T-786C and C774T endothelial nitric oxide synthase gene polymorphisms independently affect the onset pattern of severe diabetic retinopathy. *Nitric Oxide*, **13**, 88–92.

14 de Syllos, R.W., Sandrim, V.C., Lisboa, H.R., Tres, G.S. & Tanus-Santos, J.E. (2006) Endothelial nitric oxide synthase genotype and haplotype are not associated with diabetic retinopathy in type 2 diabetes patients. *Nitric Oxide*, **15**, 417–422.

15 Suganthalakshmi, B., Anand, R., Kim, R., Mahalakshmi, R., Karthikprakash, S., Namperumalsamy, P. & Sundaresan, P. (2006) Association of VEGF and eNOS gene polymorphisms in type 2 diabetic retinopathy. *Molecular Vision*, **12**, 336–341.

16 Neugebauer, S., Baba, T. & Watanabe, T. (2000) Association of the nitric oxide synthase gene polymorphism with an increased risk for progression to diabetic nephropathy in type 2 diabetes. *Diabetes*, **49**, 500–503.

17 Frost, D., Chitu, J., Meyer, M., Beischer, W. & Pfohl, M. (2003) Endothelial nitric oxide synthase (ecNOS) 4 a/b gene polymorphism and carotid artery intima-media thickness in type-1 diabetic patients. *Experimental and Clinical Endocrinology and Diabetes*, **111**, 12–15.

18 Sandrim, V.C., de Syllos, R.W., Lisboa, H.R., Tres, G.S. & Tanus-Santos, J.E. (2006) Endothelial nitric oxide synthase haplotypes affect the susceptibility to hypertension in patients with type 2 diabetes mellitus. *Atherosclerosis*, **189**, 241–246.

19 Franks, P.W., Luan, J., Barroso, I., Brage, S., Gonzalez Sanchez, J.L., Ekelund, U., Rios, M.S., Schafer, A.J., O'Rahilly, S. & Wareham, N.J. (2005) Variation in the eNOS gene modifies the association between total energy expenditure and glucose intolerance. *Diabetes*, **54**, 2795–2801.

20 Tanus-Santos, J.E., Desai, M. & Flockhart, D.A. (2001) Effects of ethnicity on the distribution of clinically relevant endothelial nitric oxide variants. *Pharmacogenetics*, **11**, 719–725.

21 Brouet, A., Sonveaux, P., Dessy, C., Balligand, J.L. & Feron, O. (2001) Hsp90 ensures the transition from the early Ca^{2+}-dependent to the late phosphorylation-dependent activation of the endothelial nitric-oxide synthase in vascular endothelial growth factor-exposed endothelial cells. *Journal of Biological Chemistry*, **276**, 32663–32669.

22 Nakayama, M., Yasue, H., Yoshimura, M., Shimasaki, Y., Kugiyama, K., Ogawa, H., Motoyama, T., Saito, Y., Ogawa, Y., Miyamoto, Y. & Nakao, K. (1999) T-786→C mutation in the 5′-flanking region of the endothelial nitric oxide synthase gene is associated with coronary spasm. *Circulation*, **99**, 2864–2870.

23 Wang, J., Dudley, D. & Wang, X.L. (2002) Haplotype-specific effects on endothelial NO synthase promoter efficiency: modifiable by cigarette smoking. *Arteriosclerosis, Thrombosis, and Vascular Biology*, **22**, e1–e4.

24 Fairchild, T.A., Fulton, D., Fontana, J.T., Gratton, J.P., McCabe, T.J. & Sessa, W.C. (2001) Acidic hydrolysis as a mechanism for the cleavage of the Glu^{298}→Asp variant of human endothelial nitric-oxide synthase. *Journal of Biological Chemistry*, **276**, 26674–26679.

© 2007 The Authors
Journal compilation © 2007 Blackwell Publishing Ltd,