Original Article

Association of the *eNOS* 4a/b and -786T/C Polymormphisms with Coronary Artery Disease, Obesity and Diabetes Mellitus

(coronary artery disease / nitric oxide synthase / polymorphism / obesity)

V. KINCL[1], A. VAŠKŮ[2], J. MELUZÍN[1], R. PANOVSKÝ[1], J. SEMÉNKA[1], L. GROCH[1]

[1]1st Department of Internal Medicine and Cardioangiology, St. Anne's University Hospital,
[2] Department of Pathophysiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic

Abstract: The aim was to assess the relationship between *eNOS* 4a/b and -786T/C polymorphisms with coronary artery disease (CAD), obesity and diabetes mellitus. Total number of 1313 patients underwent coronary angiography, 939 had significant CAD (stenosis of ≥ 1 coronary artery ≥ 50%), 222 had smooth coronary arteries. Patients with insignificant atherosclerosis were excluded, the study finally comprised 1161 patients. The analysis of *eNOS* 4a/b and -786T/C polymorphisms was performed by polymerase chain reaction. No significant interaction was found between -786T/C polymorphism and solitary CAD or CAD with diabetes and obesity. For 4a/b polymorphism, genotypes aa+ab were almost three times more frequent in diabetic patients without CAD versus patients without CAD and without diabetes – OR 2.79; P = 0.009, Pcorr = 0.03. In 4a/b polymorphism and CAD with obesity and diabetes: bb genotype was significantly more frequent: in patients with CAD, diabetes and obesity in comparison with obese diabetic patients without CAD (OR = 3.63, Pcorr = 0.05); in non-diabetic non-obese patients with CAD, versus diabetic and obese patients without CAD (OR = 3.38, Pcorr = 0.05); in obese non-diabetic patients without CAD vs. obese diabetic patients without CAD (OR = 5.91, Pcorr = 0.01); in patients without CAD, obesity and diabetes vs. obese diabetic patients without CAD (OR = 3.59, Pcorr = 0.05). The eNOS 4a/b polymorphism has significant association with diabetes mellitus in CAD-negative patients, and with CAD in combination with obesity and diabetes mellitus. No association between 4a/b or -786T/C polymorphism and solitary CAD was found.

Introduction

The cardiovascular diseases, especially the coronary artery disease (CAD), represent the most frequent mortality cause in developed countries. There are many risk factors of CAD, but the genetic background that underlies the susceptibility, occurrence and severity of CAD is still poorly elucidated. Endothelial dysfunction is one of the conditions that determine the occurrence of atherosclerosis (Hingorani, 2001).

Nitric oxide (NO), which is a powerful endothelial relaxing factor, is produced by NO synthase (NOS). This enzyme has three isoforms – inducible (iNOS), neuronal (nNOS) and the most important endothelial (eNOS). NO has more effects than endothelial relaxation including limitation of leukocyte adhesion (Lefer, 1997), platelet-vessel wall interaction (Radomski et al., 1987), smooth muscle cell proliferation and migration (Garg et al., 1989), and scavenging superoxide radicals (Loscalzo et al., 1995). There are some single nucleotide polymorphisms (SNP) in the *eNOS* gene, which may influence the activity of eNOS and NO production.

One of them, the T-786C polymorphism, has thymidine replaced by cytosine at nucleotide -786 in the 5′-flanking region of the *eNOS* gene (Nakayama et al., 1999). This variant causes reduction of *eNOS* gene promoter activity and has been reported as a risk factor for coronary spasm in the Japanese population (Nakayama et al., 1999). The second one, 27-base pair (bp) repeat polymorphism is located in intron 4 of the *eNOS* gene (4a4b polymorphism) and has been associated with changes in plasma NO levels (Wang et al., 1997). Some studies (Wang et al., 1996) showed influence in prediction of smoking-dependent risk of CAD, but other studies have not described association between this polymorphism and CAD in German (Gardemann et al., 2002) and Taiwanese (Hwang et al., 2003) population. Patients with diabetes mellitus have decreased NO gen-

Received January 20, 2009. Accepted June 30, 2009.

This study was supported in part by grant of the Ministry of Education of the Czech Republic No. MSM 0021622402.

Corresponding author: Vladimír Kincl, 1st Department of Internal Medicine and Cardioangiology, St. Anne's University Hospital, Masaryk University, Pekařská 53, 656 91 Brno, Czech Republic. Phone: (+ 420) 543 182 233; e-mail: vladimir.kincl@fnusa.cz

Abbreviations: bp – base pair, CAD – coronary artery disease, eNOS – endothelial nitric oxide, iNOS – inducible nitric oxide, nNOS – neuronal nitric oxide, NO – nitric oxide, NOS – nitric oxide synthase, PCR – polymerase chain reaction, SNP – single nucleotide polymorphisms.

eration and increased degradation of the NO (Bucala et al., 1991) and also have more frequent vascular complications that could be facilitated by impaired NO production (Pieper, 1999). In our study we investigated the association of these two polymorphisms with CAD, diabetes mellitus, and obesity.

Material and Methods

Patients

The study comprised 1313 patients referred to the 1st Department of Internal Medicine/Cardioangiology for coronary angiography. During the short-term hospitalization the patients underwent full cardiologic investigation (history, physical examination, electrocardiography, biochemical and haematological screening and coronary angiography). All patients gave informed consent to examinations and genetic analysis of blood samples and the study was approved by the institutional ethics committee. Patients with renal or hepatic failure, anaemia, endocrine or neurological diseases or malignancy were excluded. We also excluded patients with insignificant atherosclerosis, unclear diagnosis or suspected spastic angina pectoris. Finally we analysed 1161 patients. Clinical characteristics of the patients' cohort are presented in Table 1.

Coronary angiography

Coronary angiography was performed using the standard technique by experienced investigators. The coronary lesions were visually analysed in multiple projections. Based on morphology of coronary arteries, two patient groups were defined: l, patients with significant coronary atherosclerosis (at least one coronary artery with luminal diameter narrowing of 50 % or more, CAD group) and 2, patients with normal smooth coronary arteries.

Definitions of cardiovascular risk factors

Clinical risk factors for CAD were defined subsequently: hypercholesterolaemia as current treatment with hypolipidaemic drugs or diet or total plasma cholesterol > 5.0 mmol/l on hospital admission, diabetes mellitus as current treatment with insulin or oral antidiabetic drugs or diet or repeated fasting glucose > 7.0 mmol/l during hospitalization, hypertension as current treatment with antihypertensive drugs or repeat resting blood pressure > 140/90 mmHg during hospitalization, obesity as body mass index higher than 30 kg/m^2.

Table 1. Demographic and clinical characteristics of the patient cohor

Number of patients / men	1161 / 813
Age, mean (y)	64.2
CAD/ smooth coronary arteries (n)	939 / 222
Hypertension (n/%)	906 / 78 %
Hyperlipoproteinaemia (n/%)	712 / 61.3 %
Diabetes mellitus (n/%)	348 / 30 %
Obesity (n/%)	345 / 29.7 %
Total cholesterol (mean ± SD)	4.53 ± 1.1 mmol/l
Body mass index (mean ± SD)	28.7 ± 4.2

Genetic analysis

The DNA was extracted from peripheral blood leukocytes using proteinase K and precipitated by isopropanol and chloroform. Isolated DNA samples were tested for *eNOS 3* intron 4a/b polymorphism and *eNOS* -796T/C polymorphism by polymerase chain reaction (PCR). The PCR of 4a/b polymorphism was performed in DNA thermocycler using following primers: 5′- ACCTCAG-CCCAGTAGTG-3′sense,5′-GCAAGTGTCAGATAGGATT-3′ antisense, and Taq polymerase. The PCR products had 573 bp for aa, 604 bp for bb and 573+604 bp for ab alleles. These fragments were separated and analysed by electrophoresis in 1.5% agarose gel with ethidium bromide.

For -786T/C polymorphism, primers 5′-TGGAGAGTGCTGGTGTACCCCA-3′ sense, 5′-GCCTCCACCCCCACCCTGTC-3′ antisense and Taq polymerase were used. The PCR products of 180 bp were incubated with *Msp*I restriction endonuclease at 37 °C for 5 h. The final fragments had 140+40 bp for TT, 90+50+40 for CC and 140+90+50+40 for TC alleles and were resolved by electrophoresis in 4% agarose gel with ethidium bromide.

Statistical analysis

The interactions between genotypes and coronary artery disease or other comorbidities were identified by multivariation analysis – Wilk's test, General Linear Models; significant asociations between genotypes or alleles and diseases were verified by χ^2 and Fisher exact test, program package Statistica (version 8.0, StatSoft, Tulsa, OK).

Results

The distribution of genotypes of the whole cohort of patients was CC = 13 %, CT = 50 %, TT = 37 %; T allele frequency was 62.5 %, C allele frequency 37.5 % for -786T/C polymorphism. For intron 4a/b polymorphism the following distribution was found: aa = 3.8 %, ab = 28.8 %, bb = 67.4 %. The frequency of alleles was 81.8 % for b and 18.2 % for a. Both polymorphisms genotypes were in Hardy-Weinberg equilibrium.

The multivariate analysis did not identify any significant interaction between -786T/C polymorphism and solitary CAD or CAD in combination with diabetes, hyperlipoproteinaemia or obesity. For the 4a/b polymorphism, no significant interaction was found between 4a/b polymorphism and solitary CAD (Table 2) or CAD in combination with obesity (Table 3). On the other hand, significant interactions of CAD were found in combination with diabetes. Genotypes aa+ab were almost three times more frequent in diabetic patients without CAD in comparison with patients without CAD and without diabetes – OR 2.79; 95% CI 1.27–6.07, P =

Table 2. Association between the 4a/b polymorphism and CAD, counts of patients according to genotypes; P for genotypes = 0.964, P for alleles = 0.987

Summary Frequency Table – 4 a/b polymorphism and CAD				
CAD	**4a/b – ab**	**4a/b – bb**	**4a/b – aa**	**Row – Totals**
1	272	632	35	939
2	63	150	9	222
All	335	782	44	1161

CAD 1 = CAD-positive; CAD 2 = CAD-negative controls

0.009, Pcorr = 0.03 (Table 4). Association between the 4a/b polymorphism and CAD in combination with obesity and diabetes mellitus was found as well. The bb genotype was significantly more frequent: in patients with CAD, diabetes and obesity in comparison with obese diabetic patients without CAD (OR = 3.63, 95% CI 1.23–10.67, Pcorr = 0.05); in non-diabetic non-obese patients with CAD, versus diabetic and obese patients without CAD (OR = 3.38, 95% CI 1.21–9.46, Pcorr = 0.05); in obese non-diabetic patients without CAD in comparison with obese diabetic pacients without CAD (OR = 5.91 (1.76–19.88), Pcorr = 0.01); in patients without CAD, obesity and diabetes versus obese diabetic patients without CAD (OR = 3.59 (1.23–10.50), Pcorr = 0.05) (Table 5).

Discussion

The association between T-786C and coronary artery disease was investigated in many reports, but the results remain controversial (Colombo et al., 2003; Casas et. al., 2004,; Fatini et al., 2004; Rossi et al., 2006). Fatini et al. (2004) found that the *eNOS* 4a4a genotype was an independent predisposing factor for acute coronary syndromes (ACS) (OR 2.5, 95% CI 1.1-5.4, P = 0.02) but there was no association between the *eNOS* -786CC and 894TT genotypes. The presence of the -786CC genotype intensified the predisposition to ACS in 4a4a homozygotes.

Meta-analysis of 26 studies involving 23,028 subjects (Casas et al., 2004) showed significantly increased risk of CAD in 4a4a homozygotes but not in homozygous carriers of the -786C allele. Rossi et al. (2006) found more cardiovascular deaths in -786TT than in -786 TC + CC carriers in the follow-up study of more than one thousand patients. These authors suppose that this genetic variant is associated with higher NO bioactivity, which means increased production of reactive oxygen and nitrogen forms. This results in activation of matrix metalloproteinases and following predisposition to rupture of the atherosclerotic plaque. In our study, multivariate analysis did not confirm any significant association between the -786T/C polymorphism and solitary CAD or CAD in combination with obesity and diabetes mellitus.

The influence of the intron 4a/b polymorphism on plasmatic NO level was presented in several reports (Tsukada et al., 1998, Wang et al., 2000), but this has not been confirmed by other studies (Wang et al., 1997; Yoon et al., 2000; Jeeroburkhan et al., 2001). This polymorphism is located in the intron of the gene, so it is

Table 3. The 4a/b polymorphism and CAD and obesity, counts of patients according to genotypes – no significant interaction was found

Summary Frequency Table – 4a/b polymorphism and CAD and obesity						
Group	**CAD**	**Obesity**	**4a/b – ab**	**4a/b – bb**	**4a/b – aa**	**Row – Totals**
1	1	1	83	190	6	279
2	1	2	189	442	29	660
	Total		272	632	35	939
3	2	1	20	45	1	66
4	2	2	43	105	8	156
	Total		63	150	9	222
	Column Total		335	782	44	1161

CAD 1 = CAD-positive; CAD 2 = CAD-negative; Obesity 1 = obese, Obesity 2 = non-obese

Table 4.The 4a/b polymorphism and CAD with diabetes mellitus, counts of patients according to genotypes

Summary Frequency Table – 4a/b polymorphism and CAD and diabetes mellitus						
Group	**CAD**	**Diabetes**	**4a/b – ab**	**4a/b – bb**	**4a/b – aa**	**Row – Totals**
1	1	1	92	215	11	318
2	1	2	180	417	24	621
	Total		272	632	35	939
3*	2	1	15	14	1	30
4*	2	2	48	136	8	192
	Total		63	150	9	222
	Column Total		335	782	44	1161

*Significant differences between groups 3 a 4 for ab+aa: 16/14 vs. 56/136, OR = 2.79, 95% CI 1.27–6.07, P = 0.009, Pcorr = 0.03
CAD 1 = CAD-positive; CAD 2 = CAD-negative; Diabetes 1 = diabetic patients; Diabetes 2 = non-diabetic patients

Table 5. The 4a/b polymorphism and CAD with obesity and diabetes mellitus, counts of patients according to genotypes

Summary Frequency Table – 4a/b polymorphism and CAD with obesity and diabetes mellitus							
Group	**CAD**	**Diabetes**	**Obesity**	**4a/b – ab**	**4a/b – bb**	**4a/b – aa**	**Row – Totals**
1	1	1	1	39	87	1	127
2	1	1	2	53	128	10	191
	Total			92	215	11	318
3	1	2	1	44	103	5	152
4	1	2	2	136	314	19	469
	Total			180	417	24	621
5	2	1	1	10	6	0	16
6	2	1	2	5	8	1	14
	Total			15	14	1	30
7	2	2	1	10	39	1	50
8	2	2	2	38	97	7	142
	Total			48	136	8	192
	Column Total			335	782	44	1161

Significant differences:
Group 1 vs. 5 for bb: 87/40 vs. 6/10, OR = 3.63, 95% CI 1.23–10.67, Pcorr = 0.05
Group 4 vs. 5 for bb: 314/155 vs. 6/10, OR = 3.38, 95% CI 1.21–9.46, Pcorr = 0.05
Group 5 vs. 7 for bb: 6/10 vs. 39/11, OR = 5.91 (1.76–19.88), Pcorr = 0.01
Group 5 vs. 8 for bb: 6/10 vs. 97/45, OR = 3.59 (1.23–10.50), Pcorr = 0.05
CAD 1 = CAD-positive; CAD 2 = CAD-negative; Diabetes 1 = diabetic patients; Diabetes 2 = non-diabetic patients; Obesity 1 = obese; Obesity 2 = non-obese patients

unlikely to influence the protein structure, but it may play a role as a marker in linkage disequilibrium with other variants of the *eNOS* gene (Wang et al., 1997; Yoshimura et al., 2000; Jeeroburkhan et al., 2001).

Alvarez et al. (2001) found the CC variant of the T-786C polymorphism to increase risk of early CAD in a Caucasian population, but not the 4a/b polymorphism. In contrast, as mentioned above, Fatini et al. (2004) documented that susceptibility to acute coronary syndromes is markedly influenced by the haplotype-specific pattern 4a4a/786CC.

Association between intron 4a/b polymorphism and diabetes mellitus was described by Galanakis et al. (2008). These authors found a significant relationship of the presence of the a allele and diabetes mellitus with no difference between type I and II diabetes. This conforms with our results, which show significantly higher frequency of the aa and ab genotype in diabetic patients without CAD in comparison with patients without CAD and without diabetes. The relation between diabetes and *eNOS* polymorphisms has not yet been elucidated. Laboratory studies by microarray analysis of insulin-producing cells treated by cytokines found that about 50 % of the genes influenced by cytokines were NO-dependent (Kutlu et al., 2003).

The relation of the 4a/b polymorphism and obesity was not investigated, either. Hoffmann et al (2005) did not find significant association between *eNOS* 4a/b and Glu298Asp polymorphisms and either obesity or fasting glucose and blood pressure. This finding is also concordant with our work; obesity either in CAD patients or in controls did not show significant association with the 4a/b polymorphism. The limitation of our study was especially selected population, so that it does not represent common population but patients refferred for coronary angiography, and also a low number of patients in some subgroups.

In conclusion, our study demonstrates that the *eNOS* 4a/b polymorphism has significant association with diabetes mellitus in CAD-negative patients, and with coronary artery disease in combination with obesity and diabetes mellitus. No association between the 4a/b polymorphism or -786T/C polymorphism and solitary CAD was found. However, more detailed genetic analyses are necessary to elucidate the associations of genetic abnormalities and CAD and its risk factors.

References

Alvarez, R., Gonzales, P., Batala, A., Reguero, J. R., Iglesias-Cubero, G., Hevia, S., Cortina, A., Merino, E., Gonzalez, I., Alvarez, V., Coto, E. (2001) Association between the NOS3 (-786T/C) and the ACE (I/D) DNA genotypes and early coronary artery disease. *Nitric Oxide* **5**, 43-48.

Bucala, R., Ritchlin, C., Winchester, R., Cerami, A. (1991) Constitutive production of inflammatory and mitogenic cytokines by rheumatoid synovial fibroblasts. *J. Exp. Med.* **173**, 569-574.

Casas, J. P., Bautista, L. E., Humpries, S. E., Hingorani, A. D. (2004) Endothelial nitric oxide synthase genotype and ischemic heart disease. *Circulation* **109**, 1359-1365.

Colombo, M. G., Paradossi, U., Andreassi, M. G., Botto, N., Manfredi, S., Masetti, S., Biagini, A., Clerico, A. (2003) Endothelial nitric oxide synthase gene polymorphisms and risk of coronary artery disease. *Clin. Chem.* **49**, 389-395.

Fatini, C., Sofi, F., Sticchi, E., Gensini, F., Gori, A. M., Fedi, S., Lapini, I., Rostagno, C., Comeglio, M., Brogi, D., Gensini, G., Abbate, R. (2004) Influence of endothelial nitric oxide synthase gene polymorphisms (G894T, 4a4b, T-786C) and hyperhomocysteinemia on the predisposition to acute coronary syndromes. *Am. Heart J.* **147**, 516-521.

Galanakis, E., Kofteridis, D., Stratigi, K., Petraki, E., Vazgiourakis, V., Fragouli, E., Mamoulakis, D., Boumpas, D. T., Goulielmos, G. N. (2008) Intron 4 a/b polymorphism of the endothelial nitric oxide synthase gene is associated with both type 1 and type 2 diabetes in a genetically homogeneous population. *Hum. Immunol*. **69**, 279-283.

Gardemann, A., Lohre, J., Cayci, S., Katz, N., Tillmanns, H., Haberbosch, W. (2002) The T allele of the missense Glu298Asp endothelial nitric oxide gene polymorphism is associated with coronary heart disease in younger individuals with high atherosclerosis risk profile. *Atherosclerosis* **160**, 167-175.

Garg, U. C., Hassid, A. (1989) Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophospate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J. Clin. Invest.* **83**, 1774-1777.

Hingorani, A. D. (2001) Polymorphisms in endothelial nitric oxide synthase and atherogenesis: John French Lecture. *Atherosclerosis* **154**, 521-527

Hoffmann, I. S., Tavares-Mordwinkin, R., Castejon, A. M., Alfieri, A. B., Cubeddu, L. X. (2005) Endothelial nitric oxide synthase polymorphism, nitric oxide production, salt sensitivity and cardiovascular risk factors in Hispanics. *J. Hum. Hypertens.* **19**, 233-240.

Hwang, J. J., Tsai, C. T., Yeh, H. M., Chiang, F. T., Hsu, K. L., Tseng, C. D., Liau, C. S., Tseng, Y. Z., Lai, L. P. (2002) The 27-bp tandem repeat polymorphism in intron 4 of the endothelial nitric oxide synthase gene is not associated with coronary artery disease in a hospital-based Taiwanese population. *Cardiology* **97**, 67-72.

Jeerooburkhan, N., Jones, L. C., Bujac, S., Cooper, J. A., Miller, G. J., Vallance, P., Humphries, S. E., Hingorani, A. D. (2001) Genetic and environmental determinants of plasma nitrogen oxides and risk of ischemic heart disease. *Hypertension* **38**, 1054-1061.

Kutlu, B., Cardozo, A. K., Darville, M. I., Kruhoffer, M., Magnusson, N., Orntoft, T., Eizirik, D. L. (2003) Discovery of gene networks regulating cytokine-induced dysfunction and apoptosis in insulin-producing INS-1 cells. *Diabetes* **52**, 2701-2719.

Lefer, A. M. (1997) Nitric oxide: nature's naturally occurring leukocyte inhibitor. *Circulation* **95**, 553-554.

Loscalzo, J., Welch, G. (1995) Nitric oxide and its role in the cardiovascular system. *Prog. Cardiovasc. Dis.* **38**, 87-104.

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

Pieper, G. M. (1999) Enhanced, unaltered and impaired nitric oxide-mediated endothelium-dependent relaxation in experimental diabetes mellitus: Importance of disease duration. *Diabetologia* **42**, 204-213.

Radomski, M. W., Palmer, R. M. J., Moncada, S. (1987) Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. *Lancet* **2**, 1057-1058.

Rossi, G. P., Maiolino, G., Zanchetta, M., Sticchi, D., Pedon, L., Cesari, M., Montemurro, D., De Toni, R., Zavattiero, S., Pessina, A. C. (2006) The T-786C endothelial nitric oxide synthase genotype predicts cardiovascular mortality in high risk patients. *J. Am. Coll. Cardiol*. **48**, 1166-1174.

Tsukada, T., Yokoyama, K., Arai, T., Takemoto, F., Hara, S., Yamada, A., Kawaguchi, Y., Hosoya, T., Igari, J. (1998) Evidence of association of the eNOS gene polymorphism with plasma NO metabolite levels in humans. *Biochem. Biophys. Res. Comun.* **245**,190-193.

Wang, X. L., Sim, A. S., Badenhop, R. F., McCredie, R. M, Wilcken, D. E. L. (1996) A smoking-dependent risk of coronary artery disease associated with a polymorphism of the endothelial nitric oxide synthase gene. *Nat. Med.* **2**, 41-45.

Wang, X. L., Mahaney, M. C., Sim, A. S., Wang, J., Blangero, J., Almasy, L., Badenhop, R. B., Wilcken, D. E. L. (1997) Genetic contribution of the endothelial constitutive nitric oxide synthase gene to plasma nitric oxide levels. *Arterioscler. Thromb. Vasc. Biol.* **17**, 3147-3153.

Wang, X. L., Sim, A. S., Wang, M. X., Murrell, G. A. C., Trudinger, B., Wang, J. (2000) Genotype dependent and cigarette specific effects on endothelial nitric oxide synthase gene expression and enzyme activity. *FEBS Lett.* **471**, 45-50.

Yoon, Y., Song, J., Hong, S. H, Kim, J. Q. (2000) Plasma nitric oxide concentrations and nitric oxide synthase gene polymorphisms in coronary artery disease. *Clin. Chem.* **46**,1626-1630.

Yoshimura, M., Yasue, H., Nakayama, M., Shimasaki, Y., Ogawa, H., Kugiyama, K., Saito, Y., Miyamoto, Y., Ogawa, Y., Kaneshige, T., Hiramatsu, H., Yoshioka, T., Kamitani, S., Teraoka, H., Nakao, K. (2000) Genetic risk factors for coronary artery spasm: signigicance of endothelial nitric oxide synthase gene T-786C and missense Glu298Asp variants. *J. Invest. Med.* **48**, 367-374.