Relationship of eNOS gene variants to diseases that have in common an endothelial cell dysfunction

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Abstract

The endothelial cell (EC) dysfunction is a common characteristic of various pathologies that include atherosclerosis, hypertension, and Fabry's disease. Aware of the role of eNO and ACE in EC dysfunction, we questioned whether polymorphism of eNOS and/or ACE gene may be a common denominator in these pathologies. Patients with CHD (108), HT (109), Fabry's disease (37) and healthy subjects (control, 141) were genotyped for the eNOSG894T by RFLP-PCR technique and for eNOS4b/a, and ACEI/D polymorphisms by PCR amplification. The results of these studies were statistically evaluated. Compared to controls, the frequency of the eNOSG894T (T allele) was higher in CHD ($P=0.03$) and Fabry ($P=0.01$), while the eNOS4b/a (a allele) in CHD ($P=0.01$) and HT patients ($P=0.01$). The proportion of the ACEI/D was similar in all subjects. In CHD patients at "low risk" of atherogenic factors, the frequency of the T and a alleles of eNOS gene was high ($P=0.03$ and 0.02 , respectively). Carriers of the T allele of eNOSG894T were over-represented ($P=0.04$) in Fabry subgroup with renal failure. Compared to women, the eNOS894T alleles were more frequent ($P=0.03$) in men with CHD and HT, whereas ACE I/D in men ($P=0.03$) with HT. These findings suggest: (i) the frequency of eNOSG894T and/or eNOS4b/a is significantly associated with coronary dysfunction; (ii) eNOS4b/a confers a relatively high risk of hypertension in subjects with atherogenic risk factors; (iii) the frequency of eNOSG894T is high in Fabry hemizygotes with renal complications. Therefore, eNOS gene polymorphism represent a frequent risk factor for vascular abnormalities in CHD, HT and Fabry's disease, afflictions which have in common, the endothelial dysfunction.

Keywords: endothelium • coronary disease • hypertension • Fabry's disease •
eNOS • ACE • gene polymorphisms

Introduction

Atherosclerosis, hypertension, and Fabry's disease are distinct pathologies that have in common, the dysfunction of the endothelial cells (EC).

Atherosclerosis is a progressive disease that develops particularly in specific areas of the arterial bed (lesion-prone area). Based on current data, it is generally accepted that the initial stage in the formation of the atheromatous plaque involves a dysfunctional endothelium manifested by changes in permeability, expression of new adhesion molecules. With time, EC as well as monocyte-derived

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macrophages, smooth muscle cells (SMC) take up lipoproteins and turn into foam cells [1]. Atherosclerosis yields distinct clinical manifestations such as coronary heart disease (CHD).

Hypertension (HT), a polygenic and multifactorial disorder, induces in time, the structural and functional changes of arteries that thicken and become less compliant, enhancing greatly the risk of atherosclerosis. The underlying pathological defect in HT is due, at least in part, to the endothelial dysfunction [2].

Fabry's disease, is an X-linked inborn error of glycosphingolipid catabolism due to the genetic defects of the lysosomal hydrolase α -galactosidase A (α -gal A) resulting a lack or dramatic decrease of enzyme activity [3]. Accumulation of globotriaosylceramide (Gb3) in lysosomes occurs mainly in vascular EC and SMC. Transformation of EC into Gb3-loaded cells was shown recently by electron microscopy studies [4]. Expression of EC activation markers (i.e. ICAM-1) in Fabry's disease, suggests that Gb3 may lead to EC injury [5]. In contrast to atherosclerosis where lipid-loaded EC are restricted to the atherosclerotic plaque, in Fabry's disease they are located in all vasculature. In addition to the classical clinical manifestations (angiokeratomas of the skin, pain in the extremities, corneal and lenticular opacities) [6] the progressive Gb3 accumulation with age leads to systemic vasculopathy, cardiac disorders, kidney failure [4, 7, 8, 9]. Although the mechanisms of the formation of lipid loaded-EC in Fabry's disease and atherogenesis may be different, the EC dysfunction is a common feature. On this basis we hypothesize that the polymorphism of the eNOS or ACE genes may be a common feature and participate in the development of coronary disorders in patients with atherosclerosis and severe renal disease in Fabry patients.

The endothelial nitric oxide synthase (eNOS) catalyzes nitric oxide (NO) biosynthesis, the main regulator of the vessel wall [10]. A reduction in basal NO can initiate or contribute to the development of atherosclerosis [11]. It was reported that EC disturbances in essential HT is due to selective abnormalities of NO synthesis, possible a defect in phosphatidylinositol/ Ca^{2+} signalling pathway [12]. An increased nitrosylation of proteins in blood microvessels of Fabry patients suggests a chronic disturbance of NO pathways [13]. The most inves-

tigated eNOS gene polymorphisms are the missense Glu298Asp (G894T) [14] and the 27 bp tandem-repeat (4b/a) [15]. Thus far, there are controversial data whether the eNOS gene polymorphism represents a risk factor for CHD and HT (characterized by EC dysfunction) [14-19]. We reported that most patients with Fabry's disease carry the polymorphic forms (Glu298Asp and 4b/a) of eNOS gene [20].

Human angiotensin converting enzyme (ACE) has a significant function in cardiovascular homeostasis [21]. The polymorphism of ACE gene consists in the presence (I) or deletion (D) of a 287 bp within intron 16 [22]. Contradictory data on the association of ACEI/D variant with CHD and HT have been reported [23-25]. There are no data on the role of ACEI/D polymorphism on vascular disorders occurring in Fabry's disease.

The aim of this study was to analyze comparatively the polymorphism of eNOS and ACE genes with the CHD, HT, and Fabry's disease, disorders that have in common, the endothelial dysfunction. We report here that the eNOS gene polymorphism is associated with the coronary dysfunction in CHD, hypertension and renal disorders in Fabry's disease.

Materials and methods

Subjects

This study included on one hand Romanian subjects (108 CHD, 109 HT, and 102 healthy volunteers) and on the other subjects from France (37 with Fabry's disease and 39 healthy volunteers). All subjects gave the informed consent. The CHD and HT patients (30 to 60 years old) were clinically diagnosed and manifested changes on electrocardiography and echocardiography, as well as alterations in plasma troponin, lactic dehydrogenase, total creatine phosphokinase and cardiac isoenzyme - creatine phosphokinase. Only 15% of the total CHD patients had myocardial infarction. The CHD patients with hypertension were excluded from the study. The HT subjects had elevated systolic (>160 mm Hg) or diastolic (>95 mm Hg) blood pressure, or both, and did not presented coronary disorders. The blood pressure was measured supine. Fabry patients and their corresponding con-

Table 1 Characteristics of coronary heart disease (CHD) and hypertensive (HT) patients in comparison with control subjects.

Parameters	CHD	HT	Control
No. of subjects	108	109	102
Age	49.1±0.6	54.3±12.3	38.0±1.5
Men/women (P)	75/33 (0.13)	66/43 (0.80)	60/42
T-Cho, mg/dl	198.8±4.4	213.24±4.7	188.7±5.2
Tg, mg/dl	195.8±21.6	204.22±23.4	114.1±9.2
HDL-Cho, mg/dl	36.8±1.3	60.7±8.4	42.9±2.1
Glucose, mg/dl	98.4±2.8	106.3±5.1	91.3±2.1

Data are expressed as mean ± SEM. Significance (P) was calculated for patients vs. control subjects by ANOVA test, whereas the ratio men/women by χ^2 analysis. T-Cho, total cholesterol; Tg, triglyceride; HDL-Cho, cholesterol in high density lipoprotein.

trols were from Cochin Port-Royal Hospital, Paris, France. The patients were characterized by the lack or dramatic decrease of the α -gal A enzyme activity and the detection of its gene mutations as previously reported [20].

Biochemical determinations

Blood was collected after an overnight fast (at least 12 h). Serum total cholesterol (T-Cho), triglycerides (Tg), high-density lipoprotein-cholesterol (HDL-Cho) and glucose were determined by enzymatic methods (employing kits from SGM, Rome, Italy).

DNA polymorphism analysis

Genomic purified DNA extracted from peripheral blood leukocytes was used for genotyping Glu298Asp and 4b/a polymorphisms of eNOS gene (20) and ACEI/D variant [22]. About 15% of the samples were amplified again for checking the accuracy of the results.

Statistical analysis

The significance of biochemical determinations was evaluated by ANOVA test. Results are presented as mean ± SEM

or median (range). The genotypes (GG, GT, and TT of eNOSG894T, bb, ba, and aa of eNOS4b/a, and II, ID, and DD of ACEI/D) and the alleles (G and T of eNOSG894T, b and a of eNOS4b/a, and I and D of ACEI/D) were scored and the frequency was estimated. The proportions of the alleles and genotypes between patients and controls were compared with nonparametric tests (χ^2 ; odds ratio, OR; 95% confidence intervals, 95% CI). Statistical p-value (0.05 or less considered as significant) was calculated.

Results

Characterization of patients

The characteristics of the Romanian individuals are summarized in Table 1. The patients were slightly older than controls. The gender distribution was not significantly different among the Romanian subjects. The mean values of T-Cho, Tg, HDL-Cho, and glucose in CHD and HT were not significantly different from control.

From the 37 subjects clinically diagnosed with Fabry's disease, 28 patients (over 35 years old of age) were hemizygotes, presented classical symptoms of the disease and a dramatic decrease in α -gal A enzyme activity (less than 4 as compared with 41 nmol/h/mg protein, in control). They were carriers

Table 2 Frequency of endothelial nitric oxide synthase gene variants in patients with coronary heart disease (CHD), hypertension (HT), Fabry's disease and the corresponding control subjects.

	eNOS G894T				eNOS 4 b/a			
	Genotypes % (n)		Alleles % (n)		Genotypes % (n)		Alleles % (n)	
	GG	GT +TT	G	T	bb	ba +aa	b	a
CHD	20.4 (22)	79.6 (86)	48.1 (104)	51.9 (112)	61.2 (63)	38.8 (40)	79.6 (164)	20.4 (42)
HT	46.8 (37)	53.2 (42)	70.3 (111)	29.7 (47)	63.3 (69)	36.7 (40)	79.4 (173)	20.6 (45)
Control*	35.3 (36)	64.7 (66)	58.8 (120)	41.2 (84)	78.6 (66)	21.4 (18)	89.3 (150)	10.7 (18)
Fabry	34.6 (9)	65.4 (17)	51.9 (27)	48.1 (25)	58.3 (14)	41.7 (10)	79.2 (38)	20.8 (10)
Control †	53.4 (16)	46.6 (14)	75 (45)	25 (15)	76.9 (30)	23.1 (9)	87.2 (68)	12.8 (10)

Figures represent the percentage (%) and the number (n) of the subjects; eNOS, endothelial nitric oxide synthase; (*), controls for CHD and HT; in the case of Fabry patients and the corresponding control (†) all subjects were men.

of different mutations of the α -gal A gene (50% had missense mutation). About 74% out of Fabry hemizygotes presented renal failure (RF), out of which 4 subjects had kidney or/and heart transplant. The mean age of Fabry patients with RF was ~40 years old and of those without RF was ~50 years old. There are reports showing that in Fabry's disease the peak onset of proteinuria occurs in the fourth decade,

whereas the chronic renal insufficiency and end-stage renal disease take place in the fifth decade of life (9). The other 9 subjects were heterozygous females with a mutation of α -gal A gene, or suspected of Fabry's disease without having low activity of the α -gal A enzyme, they were not included in the study. The control from France was age-matched men without clinical vascular disorders.

Table 3 Frequency of angiotensin converting enzyme gene polymorphism in patients with coronary heart disease (CHD), hypertension (HT), Fabry's disease and the corresponding control subjects.

	ACE I/D			
	Genotypes % (n)		Alleles % (n)	
	I/I	ID + DD	I	D
CAD	15.4 (16)	84.6 (88)	38.0 (79)	62.0 (129)
HT	20.5 (16)	79.5 (62)	38.5 (60)	61.5 (96)
Control*	21.5 (20)	78.5 (73)	43.0 (80)	57.0 (106)
Fabry	22.2 (6)	77.8 (21)	46.3 (25)	53.7 (29)
Control †	16 (4)	84.0 (21)	36.0 (18)	64.0 (32)

Figures represent the percentage (%) and the number (n) of the subjects; ACE, angiotensin converting enzyme; (*), controls for CHD and HT; (†), controls for Fabry's disease.

Frequencies of Alleles and Genotypes

The results of the genotype and allele frequency were presented in Table 2 and 3. The eNOSG894T investigated in CHD patients revealed that the proportion of the T allele (51.9%) and the GT+TT genotypes (79.6%) was higher ($P=0.03$; $OR=1.54$; $95\%CI=1.31-1.77$, and $P=0.05$; $OR=2.13$; $CI=1.68-2.58$, respectively) than in controls (Table 2). In HT patients there was a similar distribution of T allele and GT+TT genotypes as in controls (Table 2).

In the case of Fabry patients, the frequency of the T allele (48%) and GT+TT genotypes (65%) was higher ($P=0.01$; $OR=2.78$; $95\%CI=1.69-3.87$, and $P=0.001$; $OR=2.16$; $95\%CI=1.13-3.19$, respectively) than in the control (Table 2); these data are in good agreement and extend our previous report on Fabry's disease [20].

As shown in Table 2, the eNOS4b/a examined in CHD patients revealed that the frequency of the a allele (20.4%) and the ba+aa genotypes (38.8%) was higher ($P=0.01$; $OR=2.13$; $95\%CI=1.72-2.54$, and $P=0.03$; $OR=2.32$; $95\%CI=1.60-3.04$, respectively) than in controls. Likewise, in HT, the proportion of the a allele (20.6%) and of the ba+aa genotypes (36.7%) was high ($P=0.01$; $OR=2.17$; $95\%CI=1.78-2.56$, and $P=0.02$; $OR=2.13$; $95\%CI=1.47-2.79$, Table 2). In contradistinction, there were no differences in the distribution of eNOS4b/a between Fabry patients and the corresponding control (Table 2). Studies of the ACEI/D revealed no significant differences either in the distribution of the D allele or ID+DD genotypes in the CHD, HT and Fabry's disease (Table 3).

Genetic Association Study

We further analyzed the relationship between eNOS or/and ACE gene polymorphism and the risk factors for atherogenesis or gender. In CHD, HT and the corresponding control, some of the subjects presented high concentrations of the T-Chol (>260 mg/dl), Tg (>140 mg/dl) and/or glucose (>110 mg/dl), considered as "high risk" factor. Consequently, the subjects in the three groups were divided in "high risk" and "low risk" individuals. The proportion of "low risk" subjects was 43 % in CHD, 49% in HT and 32% in control.

Fig. 1 Relative risk of coronary heart disease (CHD) and hypertension (HT) in correlation with the T allele of eNOSG894T (eNOS 894T), the a allele of eNOS4b/a (eNOS 4a) and the D allele of ACEI/D (ACE 16D) as a function of atherogenic "low risk" (LR) and "high risk" (HR) factors (hypercholesterolemia, hypertriglyceridemia and diabetes mellitus) and gender (G).

Interestingly, when compared the "low risk" subjects in CHD and control, there was a significant over-representation of the T and a alleles of the eNOS gene among the "low risk" CHD patients. The relative risk was 1.65 ($P=0.03$; $95\%CI=1.23-2.07$) for the T allele and 2.13 ($P=0.02$; $95\%CI=1.28-2.98$) for the a allele (Fig.1). These findings suggest that the frequency of mutant alleles of the eNOS gene is associated with the coro-

Fig. 2 Frequency of eNOS and ACE gene polymorphisms in Fabry patients with renal failure (RF) or without renal failure (RF-free). The T allele of eNOSG894T (eNOS 894T); the a allele of eNOS4b/a (eNOS 4a); the D allele of ACEI/D (ACE 16D). Note the high level of the distribution of eNOS894T alleles only among Fabry patients with RF.

nary dysfunction. In addition, a significant relationship of the a allele of eNOS4b/a was detected in the case of “high risk” CHD patients when compared to the control ($P=0.03$; $OR=2.14$; $95\%CI=1.15-3.13$).

In hypertensive individuals, the “low-risk” patients presented a similar distribution of the T and a alleles of eNOS gene variants as in control. The a allele of eNOS4b/a appeared to confer a 3.14 fold increased risk ($P<0.01$) of the occurrence of hypertension in “high risk” patients (Fig. 1).

Analysis of the distribution of eNOSG894T, eNOS4b/a and ACEI/D variants as a function of gender revealed that, there were more men than women carrying the T allele of eNOSG894T ($P=0.03$) in both CHD and HT cases. The relative risk was 1.60 ($95\%CI, 1.18-2.02$) in CHD and 2.13 ($95\%CI, 1.33-2.93$) in HT (Fig. 1). In the case of ACEI/D, only in HT, there were more men carrying the D allele ($P=0.03$; $OR=2.13$; $95\%CI=1.62-2.64$) than women (Fig. 1).

Comparison between Fabry patients with or without renal failure (RF) indicated that about 74% of the subjects had an associated renal dysfunction. The distribution of the T allele of eNOSG894T was higher ($P=0.04$) in Fabry patients with RF, as compared with those without RF. The OR was 4.64 ($P=0.04$; $95\%CI, 2.43-6.85$) suggesting an increased risk to develop renal disorders in Fabry hemizygotes carrying the T allele of eNOS gene.

No significant differences in the distribution of the a allele of eNOS4b/a and D allele of ACEI/D was detected among the Fabry patients with or without RF (Fig. 2).

Discussion

In the present study we examined the association between the frequency of eNOS and ACE gene polymorphisms and CHD, HT and Fabry’s disease.

The wild type G allele distribution of eNOSG894T varies greatly in normal Caucasian individuals. In our study, the frequency of this allele was 58.8% in Romanian control subjects. Interestingly, the proportion of the G allele among Fabry patients (from France) was comparable to that of Romanian controls, while in the controls from France this frequency was significantly low. There are controversial reports on the association of eNOS gene variants with CHD and HT in Caucasians [14-19]. Our data revealed that, as compared to control, both polymorphisms of the eNOS gene were significantly associated with CHD. While the “low risk” CHD patients carried significantly more T and a alleles of eNOS gene as compared with “low risk” controls, among the CHD patients the proportion of these alleles was comparable in both “low risk” and “high risk” subgroups. These data suggest that eNOS gene polymorphism might be an additional risk factor that contributes to the endothelial dysfunction in coronary disturbances.

In hypertension, the T allele frequency of eNOSG894T was not significantly different from that in controls, a result similar to that previously reported [17]. The a allele proportion of eNOS4b/a was significantly higher, a result that is in line with other report (19). The wild type b allele frequency of eNOS4b/a in controls was 78.6%; this figure is comparable to those reported [16] for Australia (72.3 %).

The data on ACEI/D polymorphism reported for CHD and HT are controversial [23-25]; our results are in line with those studies that found no correlation between this polymorphism and the respective diseases [23, 24]. Analysis of the eNOS and ACE gene polymorphisms as a function of gender indicated that among individuals with CHD or HT the

frequency of the T allele of eNOSG894T and the D allele of ACEI/D is significantly higher in men than in women; these results corroborate well with other reports [26, 27].

In the case of the Fabry's disease, we have found a significant association of eNOSG894T polymorphism with this affliction. The results obtained on a large number of Fabry hemizygotes confirm and extend our previous findings [20]. Unexpectedly, most of the Fabry hemizygotes with RF are carriers of the T allele of eNOSG894T. The association of this polymorphism with a faster renal decline was reported in men with autosomal dominant polycystic kidney disease where the eNOS gene polymorphism has a modifier effect [28]. In Fabry's disease the renal complications were related to the type of mutation of alpha-gal A gene and residual enzyme activity [9]. In our study, ~50% of Fabry hemizygotes with RF present missense mutation of the gene. We can safely assume that in Fabry's disease, a monogenic disease, the associated eNOSG894T polymorphism enhances the effect of the alpha-Gal A gene, and generates EC dysfunction resulting vascular disorders.

Since ACE polymorphism is particularly relevant for the renal disorders [24] we studied the ACEI/D variant, to find out whether there is an increase frequency of the D allele in Fabry patients with RF. Unexpectedly, no significant correlation was found. From all Fabry patients genotyped for the three polymorphisms about 63% carried two polymorphisms, particularly eNOSG894T and ACEI/D variants (data not shown).

Taken together, these data show that the ACEI/D gene polymorphism is associated with hypertension in man, only. The frequency of eNOS gene polymorphism is associated with CHD, HT and Fabry's disease, disorders that have in common the endothelial dysfunction. The eNOS gene polymorphism may act as an additional risk factor in the development of major vascular complications characteristic for these afflictions.

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References

1. **Simionescu N., Simionescu M.**, Alterations of the endothelium and subendothelium at the inception of hypercholesterolemic atherogenesis. In: *Functionality of the Endothelium in Health and Diseased States: a comprehensive review*, Editora del Gobierno del Estado de Veracruz, Llave, Mexic, 1984, pp. 269-284
2. **Panza J.A.**, Endothelial dysfunction in essential hypertension, *Clin. Cardiol.*, **11**:26-33, 1997
3. **Poenaru L.**, Les maladies lysosomales, *Rev. Prat.*, **44**: 645-655, 1994
4. **Nepomnyashchikh G.I., Soboleva M.K., Aidagulova S.V.**, Pathohistological and ultra-structural study of systemic vasculopathy (Fabry disease), *Bull. Exp. Biol. Med.*, **135**: 202-205, 2003
5. **DeGraba T., Azhar S., Dignat-George F. et al.**, Profile of endothelial and leukocyte activation in Fabry patients, *Ann. Neurol.*, **47**:229-233, 2000
6. **Desnick R.J., Brady R., Barranger J. et al.**, Fabry disease, an under-recognized multisystemic disorder: expert recommendations for diagnosis, management, and enzyme replacement therapy, *Ann. Intern. Med.*, **138**:338-346, 2003
7. **Meehan S.M., Junsanto T., Rydel J.J., Desnick R.J.**, Fabry disease: renal involvement limited to podocyte pathology and proteinuria in a septuagenarian cardiac variant. Pathologic and therapeutic implications, *Am. J. Kidney Dis.*, **43**:164-171, 2004
8. **Kimura K., Sato-Matsumura K.C., Nakamura H. et al.**, A novel A97P amino acid substitution in alpha-galactosidase A leads to a classical Fabry disease with cardiac manifestations, *Br. J. Dermatol.*, **147**:545-548, 2002
9. **Schiffmann R.**, Natural history of Fabry disease in males: preliminary observations, *J. Inherit. Metab. Dis.*, **24**:15-17, 2001
10. **Cosentino F., Luscher T.F.**, Nitric oxide and endothelial regulation of vascular tone, *Methods Neurosci.*, **31**: 215-228, 1996
11. **Oemas B.S., Tschudi M.R., Godoy N. et al.**, Reduced endothelial nitric oxide synthase expression and production in human atherosclerosis, *Circulation*, **97**: 2494-2498, 1998
12. **Cardillo C., Panza J.A.**, Impaired endothelial regulation of vascular tone in patients with systemic arterial hypertension, *Vasc. Med.*, **3**:138-144, 1998
13. **Moore D.F., Scott L.T., Gladwin M.T. et al.**, Regional cerebral hyperperfusion and nitric oxide pathway dysregulation in Fabry disease: reversal by enzyme replacement therapy, *Circulation*, **104**: 1506-1512, 2001
14. **Yoshimura M., Yasue H., Nakayama M. et al.**, A missense Glu298Asp variant in the endothelial nitric oxide synthase gene is associated with coronary spasm in the Japanese, *Human Genet.*, **103**: 65-69, 1998

15. **Wang X.L., Sim A.S., Badenhop R.F. et al.**, A smoking-dependent risk of coronary artery disease associated with a polymorphism of the eNOS gene, *Nature Med.*, **2**:41-45, 1996
16. **Granath B., Taylor R.R., van Bockxmeer F.M., et al.**, Lack of evidence for association between endothelial nitric oxide synthase gene polymorphisms and coronary artery disease in the Australian Caucasian population, *J. Cardiovasc. Risk*, **8**:235-241, 2001
17. **Karvonen J., Kauma H., Kervinen K. et al.**, Endothelial nitric oxide synthase gene Glu298Asp polymorphism and blood pressure, left ventricular mass and carotid artery atherosclerosis in a population-based cohort, *J. Intern. Med.*, **251**:102-110, 2002
18. **Shoji M., Tsutaya S., Saito R. et al.**, Positive association of endothelial nitric oxide synthase gene polymorphism with hypertension in northern Japan, *Life Sci.*, **66**:2557-2562, 2000
19. **Rodriguez-Esparragon F.J., Rodriguez-Perez J.C., Macias-Reyes A. et al.**, Peroxisome proliferator-activated receptor-gamma2-Pro12Ala and endothelial nitric oxide synthase-4a/b gene polymorphisms are associated with essential hypertension, *J. Hypertens.*, **1**:1649-1655, 2003
20. **Heltianu C., Costache G., Azibi K. et al.**, Endothelial nitric oxide synthase gene polymorphism in Fabry's disease, *Clin. Genet.*, **61**:423-429, 2002
21. **Igic R., Behnia R.**, Properties and distribution of Angiotensin I converting enzyme, *Curr. Pharm. Des.*, **9**:697-706, 2003
22. **Lindpaintner K., Pfeffer M.A., Kreutz R. et al.**, A prospective evaluation of an angiotensin-converting-enzyme gene polymorphism and the risk of ischemic heart disease, *N. Engl. J. Med.*, **332**:706-711, 1995
23. **Al-Fakhri N., Linhart R.E., Philipp M. et al.**, Endothelin-1 and vasopressin plasma levels are not associated with the insertion/deletion polymorphism of the human angiotensin I-converting enzyme gene in patients with coronary artery disease, *J. Hum. Hypertens.*, **17**:133-138, 2003
24. **Olivieri O., Grazioli S., Pizzolo F. et al.**, Different impact of deletion polymorphism of gene on the risk of renal and coronary artery disease, *J. Hypertens.*, **20**:37-43, 2002
25. **Schut A.F., Sayed-Tabatabaei F.A., Witteman J.C. et al.**, Smoking-dependent effects of the angiotensin-converting enzyme gene insertion/deletion polymorphism on blood pressure, *J. Hypertens.*, **22**:313-319, 2004
26. **Stankovic A., Zivkovic M., Alavantic D.**, Angiotensin I-converting enzyme gene polymorphism in a Serbian population: a gender-specific association with hypertension, *Scand. J. Clin. Lab. Invest.*, **62**: 469-475, 2002
27. **Orlowska-Baranowska E., Placha G., Gaciong Z. et al.**, Influence of insertion-deletion polymorphism of the angiotensin converting enzyme gene on left ventricular hypertrophy in patients with aortic stenosis—differences in men and women, *Pol. Arch. Med. Wewn.*, **108**: 855-866, 2002
28. **Persu A., Stoenoiu M.S., Messiaen T. et al.**, Modifier effect of ENOS in autosomal dominant polycystic kidney disease, *Hum. Mol. Genet.*, **11**:229-241, 2002