The Relationship Between eNOS (*G894T*) Gene Polymorphism and Arterial Stiffness in Patients with Metabolic Syndrome

ANGELA COZMA[1,2#], ADELA SITAR TAUT[1,2#], OLGA ORASAN[1,2#], LUCIA MARIA PROCOPCIUC[3], ANCA DANIELA FARCAS[1,4*], ADINA STAN[5,6], VASILE NEGREAN[1,2], DOREL SAMPELEAN[1,2], DANA POP[1,7], DUMITRU ZDRENGHEA[1,7], ADRIANA FODOR[8,9]

[1] Iuliu Hatieganu University of Medicine and Pharmacy, Internal Medicine Department, 8 Victor Babes Str., 400012, Cluj Napoca, Romania

[2] 4th Medical Clinic, 16-18-20 Republicii Str., 400015, Cluj Napoca, Romania

[3] Iuliu Hatieganu University of Medicine and Pharmacy, Molecular Sciences Department, 68 Pasteur Str., 400349, Cluj Napoca, Romania

[4] Emergency Clinical County Hospital, Cardiology Department, 3-5 Clinicilor Str, 400006, Cluj Napoca, Romania

[5] Iuliu Hatieganu University of Medicine and Pharmacy, Neuroscience Department, 43 Victor Babes Str, 400012, Cluj Napoca, Romania

[6] Emergency Clinical County Hospital, Neurology Department, 43 Victor Babes Str, 400012, Cluj Napoca, Romania

[7] Cardiology Department, Clinical Rehabilitation Hospital, 46-50 Viilor Str, 400347, Cluj Napoca, Romania

[8] Iuliu Hatieganu University of Medicine and Pharmacy, Medical Specialites Department, 2-4 Clinicilor Str., 400006, Cluj Napoca, Romania

[9] Clinical Center of Diabetes, Nutrition and Metabolic diseases, 2-4 Clinicilor Str., 400006, Cluj Napoca, Romania

Metabolic syndrome (MS) is a clustering entity characterized by obesity, hypertension, hyperglycemia, dyslipidemia, and insulin resistance. Atherosclerotic lesions may be a complication of MS, arising from endothelial dysfunction and induced by decreased nitric oxide (NO) production. NO is synthesized by nitric oxide synthase (eNOS), encoded by the NOS3 gene, and displays anti-inflammatory, vasodilatory, and antiproliferative effects.We aimed to investigate the relationship between the G894T polymorphism of the eNOS gene and metabolic syndrome (including its components) and the association of this polymorphism with arterial function, assessed by determining pulse wave velocity and the augmentation index.The study included 100 consecutive patients, 55% with metabolic syndrome (based on IDF criteria -study group), 45% without MS (control group). Arterial stiffness was measured using TensioMed™ Arteriograph. The presence of the homozygous (TT) or heterozygous (GT) state was associated, compared to subjects without the mutation (GG), with an increased prevalence of arterial hypertension, diabetes mellitus, with an increase of abdominal circumference, an increase of triglycerides, without significantly influencing the level of HDL. No significant differences were found between patients with G894T polymorphism compared to those without the mutation regarding the arterial stiffness. The eNOS gene polymorphism: 894G>T was significantly associated with the presence of MS; the polymorphism in homozygous and heterozygous state was associated with an increased risk of metabolic syndrome. G894T polymorphism did not significantly influence the values of the studied arterial parameters (pulse wave velocity, aortic and brachial augmentation index)

Keywords: G894T polymorphism, arterial stiffness, metabolic syndrome

Metabolic syndrome (MS) is a clustering entity characterized by obesity, hypertension, hyperglycemia, dyslipidemia, and insulin resistance. Atherosclerotic lesions may be a complication of MS [1], arising from endothelial dysfunction and induced by decreased nitric oxide (NO) production. NO is synthesized by nitric oxide synthase (eNOS), encoded by the NOS3 gene, and displays anti-inflammatory, vasodilatory, and antiproliferative effects [2].

The eNOS gene is located on chromosome 7q35-36, including 26 exons and 25 introns, with a weight of 21kb. eNOS gene polymorphisms contribute to endothelial dysfunction and diminish nitric oxide production [3]. The most studied eNOS gene polymorphisms *(T786C, G894T* and *4a4b*) have been associated with various diseases, including: ischemic cardiopathy [4], myocardial infarction [5], risk of intrastent stenosis [6], coronary spasm, arterial hypertension [7], end-stage chronic kidney disease, and diabetic nephropathy [8].

G894T polymorphism has also been associated with hypertension [9], with the risk of intracerebral aneurysm [10] and with ischemic cerebrovascular accident [11-13]. Even in preeclampsia, an exacerbated inflammatory status associated with endothelial dysfunction and neovascularization [14], G894T polymorphism was related with the risk of hypertension [15]. Animal studies have suggested that mice without nitric oxide synthase 3 have a similar phenotype to that of metabolic syndrome [16].

The relationship between eNOS gene polymorphism and MS has been studied in a number of articles, but the results are controversial. However, there are few studies that report a possible association between eNOS gene polymorphism and metabolic syndrome [17,18]. These studies have shown a positive association between eNOS polymorphism and MS [19] while an Italian study evidenced an association with DM and insulin resistance [20]. Moreover, a positive association between *G894T* polymorphism and MS was revealed in a study carried out by Chinese and Japanese authors [21-23]. eNOS gene polymorphism was also associated with a component of MS: non-alcoholic fatty liver [24].

The relationship between eNOS and endothelial dysfunction measured non-invasively was evidenced by Kumar et al. in healthy subjects [25]. It is also known that nitric oxide can influence arterial stiffness by vascular tone regulation. The presence of the eNOS gene mutation may influence arterial stiffness and can be a connection

** email: ancafarcas@yahoo.com; Phone: 0744780873* *#Authors with equal contribution*

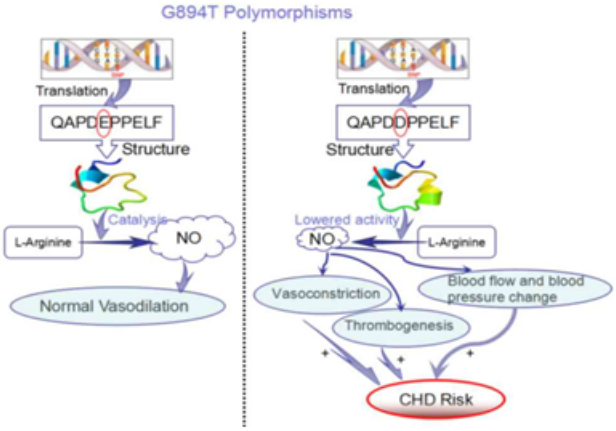

Fig.1. The relationship between G894T polymorphism and the development of CHD [26]

pathway between metabolic syndrome and the increased cardiovascular risk of these patients (fig. 1).

The identification of endothelial dysfunction by determining genetic polymorphisms has an extremely important role and can provide details about the interaction between genetic factors and environmental factors, as well as their role in the development of atherosclerosis [27].

Furthermore, in recent studies, eNOS polymorphism has been associated with arterial stiffness [28], evaluated by the measurement of pulse wave velocity [29,30].

In contrast, there are few studies that have investigated the eNOS-arterial stiffness relationship, and to our knowledge, no study has investigated this relationship with MS.

In this study, we aimed to investigate the relationship between the G894T polymorphism of the eNOS gene and metabolic syndrome (including its components), on the one hand, and the association of this polymorphism with arterial function, assessed by determining pulse wave velocity and the augmentation index, on the other hand.

Experimental part

Subjects

The study included 100 consecutive patients, who were investigated in the service of Cardiology, Medical Clinic IV, University of Cluj-Napoca. Of the 100 subjects, 55% were diagnosed with metabolic syndrome (based on IDF criteria – study group), 45% without MS (control group).

IDF criteria for the diagnosis of metabolic syndrome involve the obligatory presence of abdominal obesity (abdominal circumference ≥ 94 cm in men and ≥80 cm in women) and another two of the following abnormalities: BP > 130/85 mmHg, serum triglycerides ≥150 mg/dL, HDL cholesterol < 40 mg/dL in men and < 50 mg/dL in women, and serum glucose ≥100 mg/dL.

Anthropometric measurements were carried out and included weight, height and waist circumference. Based on anthropometric measurements, the body mass index (BMI) was calculated. Hypertension was diagnosed based on blood pressure measurements performed at least twice during two or three different appointments, in a quiet room after lying down for 15 min. Type 2 diabetes or impaired fasting glucose was diagnosed using WHO criteria. The levels of triglycerides (TG), total cholesterol, low-density lipoproteins and high-density lipoproteins were estimated according to standard protocols. The study protocol was approved by the local Ethics Committee, and all subjects provided an oral and written informed consent. The study was conducted in compliance with the Declaration of Helsinki.

DNA extraction and genotype analysis

DNA extraction was performed using a Zymo Research extraction kit. DNA concentration and purity were determined by measuring optical density at 260 nm and 280 nm. All samples having a DO260/DO280 ratio < 1.8 were considered pure. *Glu298Asp (G894T)* polymorphism located in exon 7 of the eNOS gene (chr 7q36) was examined by PCR amplification of genomic DNA and enzymatic digestion with the restriction endonuclease of the amplified fragment (PCR-RFLP technique).

For the identification of the above mentioned polymorphism, DNA analysis according to the modified method described by Lustberg was carried out. Amplification was performed in 25 mL reaction mixture, under the following amplification conditions: 20 ng genomic DNA, 0.2 mM dNTP, 0.2µM forward primer and reverse primer (the forward primer had the sequence: 5'-TCCCTGAGGAGGGCATGAGGC-3'; the reverse primer had the sequence: 5'- TGAGGGTCACACAGGTTCCT-3'), 1.5 mM Mg^{2+} and 2 units of Taq DNA polymerase.

Identification of the Glu287Asp polymorphism was performed by enzymatic digestion of the PCR-amplified fragment (457 bp) with the restriction endonuclease BanII. The Glu287Asp polymorphism creates a restriction site for the BanII enzyme. The normal allele with guanine at position 894 (G894) forms by enzymatic digestion an undigested fragment of 457 bp, while the mutated allele with thymine at position 894 (T894) forms by enzymatic digestion 2 fragments of 320 and 137 bp.

Evaluation of arterial stiffness

Evaluation of arterial parameters: pulse wave velocity -PWVAo -, brachial augmentation index -Aixb-, aortic augmentation index -AixAo-, pulse pressure -PP-was performed with the TensioMed™ Arteriograph.

Statistical analysis

Data were statistically processed using the statistical package SPSS 19.0 (Demo Version). The results were presented as mean ± standard deviation for quantitative variables with normal distribution (assess by using Kolmogorov test). Quantitative data was presented as number and percentages. The difference between quantitative variables was assessed using the independent-sample T test, and the difference between qualitative variables was evaluated by the χ^2 test. Data were presented as odds ratios and 95% confidence intervals.

ANOVA test was used in order to identify differences between categories.

The sensitivity, specificity, positive and negative predictive values, positive likelihood ratio and negative likelihood ratio of a certain mutation/allele in the development of metabolic syndrome were calculated.

Logistic regression was used to identify independent prediction factors. A p value < 0.05 was considered statistically significant.

Good practice principles in scientific research were respected.

Results and discussions

The mean age of the patients included in the study was 56.91±14.39 years, sex distribution being: 66 women and 34 men. There was no significant sex difference regarding the prevalence of MS (53% in women vs 58.8% in men, p=NS) (table1).

The presence of *G894T* polymorphism among patients with MS compared to the control group was the following: 47.3% vs. 20% had G894T in heterozygous state (GT) and

20% vs. 4.4% in homozygous state (TT), while 32.7% vs. 75.6% did not have the G894T polymorphism (GG). Genotype distribution was statistically significantly different between the group with MS and the control group (for GG p=0.0001, for GT p=0.008, TT=0.04).

The T (Asp) allele was present in a proportion of 67.3% in patients with MS and 24.4% in controls (p < 0.0001).

The presence of the homozygous (TT) or heterozygous (GT) state was associated, compared to subjects without the mutation (GG), with an increased prevalence of arterial hypertension (AHT) (64.6% vs 40.4%, p=0.013), diabetes mellitus (DM) (27.1% vs 13.5%, p=0.03), with an increase of abdominal circumference (99.82±14.19 cm vs 92.56±16.44 cm, p=0.02), an increase of triglycerides (169.04±88.9 mg/dL vs 122.97±51.8 mg/dL, p=0.005), without significantly influencing the level of HDL (42.52±13.11 mg/dL vs 43.25±11.9 mg/dL, p=NS). Data are shown in table 2.

The eNOs G894T gene polymorphism was significantly associated with the presence of metabolic syndrome: subjects with the polymorphism present in heterozygous state (GT) had a more than 5 times higher risk of developing MS compared to those without the polymorphism (GG), OR=5.457 (95%CI 1.92-15.87, p<0.001), while subjects with the G894T polymorphism in homozygous state (TT) had a more than 10 times higher risk compared to those without the polymorphism (GG), OR=10.38 (95%CI 1.83-76.61, p=0.002), and the presence of the T allele was associated with a 6 times higher risk of having MS, OR=6.354 (95%CI 2.41-17.05, p<0.001).

By univariate analysis, we investigated the role of the presence of G894T polymorphism in the development of metabolic syndrome, as well as its identification as an independent risk factor for metabolic syndrome (by multivariate analysis).

As shown in table 3, in univariate analysis, age, weight, increased abdominal circumference, increased glicemia and serum triglyceride levels, HDL-cholesterol and pulse wave velocity represent risk factors for the development of metabolic syndrome.

The presence of the TT homozygous state of G894T and the presence of the T allele of the G894T mutation represent risk factors for the development of metabolic syndrome.

Parameter	Study group MS + 55 patients	Control group MS-45 patients	P-value
Gender – W/M – No (%)	35 (63.63)/ 20 (36.36)	31 (68.88)/ 14 (31.11)	NS
Age – mean±SD	61.87±9.46	50.10±17.11	0.0002
BMI (kg/m^2) – mean±SD	30.97±3.89	24.47±4.47	< 0.0001
Weight (kg) – mean±SD	86.25±14.36	68.28±15.58	< 0.0001
Waist circumference (cm) – mean±SD	103.96±10.91	83.73±14.17	< 0.0001
Glicemia (mg/dl) – mean±SD	108.16±31.37	90.4±18.81	0.0012
LDL (mg/dl) – mean±SD	124.10±44.54	123.78±51.08	NS
HDL (mg/dl) – mean±SD	40.74±9.97	50.92±18.01	0.05
TG (mg/dl) – mean±SD	167.50±80.5	108.9±51.05	0.0001
TAS (mmHg) – mean±SD	142.40±23.52	133±23.67	NS
TAD (mmHg) – mean±SD	85.27±19.84	75.38±12.94	0.0044
Hypertension – No (%)	43 (78.18)	9(20)	< 0.0001
Diabetes – No (%)	20 (36.36)	0 (0)	0.0001
Smoking – No (%)	7 (12.72)	6(13.33)	NS
Cardiovascular disease – No (%)	20(36.36)	10 (22.2)	NS
PWVAo (m/sec)	10.52±1.68	9.05±1.83	0.0029
AixAo	38.79±16.77	32.26±18.63	NS
Aixb	5.4±28.88	-6.69±33.47	NS
PP (mmHg)	54.61±14.61	52.15±12.26	NS

Results expressed as mean values ± SD. Abbreviations: BMI: body mass index, LDL: low density lipoproteins, HDL: high density lipoproteins, TG: triglicerides, PWVAo: Pulse wave velocity, Aixb: brachial augmentation index, AixAo: the aortic augmentation index , PP: pulse pressure

Table 1
BASELINE CHARACTERISTICS OF THE STUDY GROUP AND THE CONTROL GROUP

	Mutation	Mean	Std. Dev.	Std. Err. Mean	P
Waist circumference (cm)	GG	92.56	16.44	2.47	0.02
	TT/GT	99.82	14.19	2.11	
Glicemia (mg/dl)	GG	98.68	27.92	4.20	0.03
	TT/GT	103.71	28.99	4.27	
Cholesterol total (mg/dl)	GG	189.76	52.95	8.17	NS
	TT/GT	207.54	59.49	8.96	
LDL – cholesterol (mg/dl)	GG	114.33	44.47	8.55	NS
	TT/GT	130.28	45.65	7.04	
HDL – cholesterol (mg/dl)	GG	43.25	11.90	2.29	NS
	TT/GT	42.52	13.11	2.02	
Triglyceride (mg/dl)	GG	122.97	51.80	8.09	0.005
	TT/GT	169.04	88.90	13.40	

Table 2
THE RELATIONSHIP BETWEEN THE *G894T* MUTATION AND BIOCHEMICAL PARAMETERS

	MS	Mean	Std. Dev.	Std. Err. Mean	p
Age	Yes	61.87	9.47	1.28	p<0.05
	No	50.10	17.11	2.71	
Weight (Kg)	Yes	86.25	14.36	1.94	p<0.05
	No	68.29	15.58	2.46	
Waist circumference (cm)	Yes	103.96	10.92	1.47	p<0.05
	No	83.74	14.18	2.43	
Glicemia (mg/dl)	Yes	108.16	31.37	4.23	p<0.05
	No	90.40	18.82	3.18	
Cholesterol total	Yes	197.96	57.53	7.76	NS
	No	200.45	56.31	10.11	
LDL - cholesterol (mg/dl)	Yes	124.11	44.54	6.01	NS
	No	123.79	51.09	13.65	
HDL - cholesterol (mg/dl)	Yes	40.75	9.97	1.34	p<0.05
	No	50.93	18.02	4.82	
Triglycerides (mg/dl)	Yes	167.51	80.51	10.86	p<0.05
	No	108.90	51.06	9.32	
Aixb	Yes	5.41	28.88	4.04	NS
	No	-6.69	33.48	7.68	
AixAo	Yes	38.80	16.78	2.33	NS
	No	32.26	18.63	4.27	
PWVAo (m/sec)	Yes	10.52	1.69	0.24	p<0.05
	No	9.05	1.83	0.43	
PP (mmHg)	Yes	54.62	14.61	2.03	NS
	No	52.16	12.26	2.81	

Table 3
QUANTITATIVE RISK FACTORS FOR THE DEVELOPMENT OF METABOLIC SYNDROME

PWVAo: Pulse wave velocity, Aixb: brachial augmentation index, AixAo: the aortic augmentation index , PP: pulse pressure

(894G>T in homozygous state: 20% vs. 4.4%; p=0.04 and presence of the T allele: 67.3% vs 24.4%; p<0.0001).

In multivariate analysis, using the backward logistic regression method (enter variable if p<0.05, remove variable if p>0.1), the following of the previously mentioned factors are considered to be independent variables: glicemia, HDL-cholesterol (a high value is a protective factor) and the presence of the T allele (its absence is a protective factor).

The model does not include age, weight, abdominal circumference, serum triglycerides, PWVAo, the presence of diabetes mellitus and AHT.

Coefficients, standard errors and odds ratios for the presented variables are illustrated in table 4.

Relationship between *G894T* polymorphism and arterial parameters: pulse wave velocity, aortic and brachial augmentation index

As shown in table 5, the ANOVA test evidenced no significant differences in the values of pulse wave velocity (PWVAo), the brachial augmentation index (Aixb), the aortic augmentation index (AixAo), pulse pressure (PP) of subjects without the G894T polymorphism (GG) compared to those having the polymorphism in heterozygous state (GT) and compared to those having the polymorphism in homozygous state (TT) (PWVAo p = 0.5, Aixb p = 0.58, AixAo p = 0.37, PP p = 0.64). However, homozygous patients (TT) had higher values of the brachial augmentation index (Aixb) and pulse pressure (PP) compared to heterozygous subjects and mutation-free subjects.

The relationship between 894G>T polymorphism and metabolic syndrome

In this study, the presence of the eNOS *894G>T* mutation in homozygous (TT) or heterozygous (GT) state was associated with an increase in the prevalence of AHT, DM with an increase in the abdominal circumference, an increase of triglycerides, without significantly influencing the level of HDL. In another study, Ukkola et al. [31] showed that the TT genotype of the eNOS gene is associated with high LDL-cholesterol and low HDL-cholesterol, compared to GG and GT genotypes. Besides, low HDL- cholesterol levels increased pulse wave velocity and hence increased arterial stiffness in treated and untreated hypertensive patients [32]. The mechanism by which *G894T* polymorphism is associated with the lipid profile remains unknown, although it has been demonstrated that mice without eNOS have high cholesterol levels [16]. A significant number of studies have led to the consideration of the idea of association between eNOS polymorphism and one or two risk factors belonging to metabolic syndrome, such as arterial hypertension, type 2 diabetes mellitus [33] and dyslipidemia. Some of these studies have shown a positive association between eNOS poly-

Variable	Coefficient	Std. error	*P*
Glicemia	0.1229	0.03	0.001442
HDL-cholesterol	-0.2070	0.06	0.001688
PRESENCE of the T allele = NO	-2.1422	1.08	0.04788
Constant	1.1457		

Table 4
COEFFICIENTS AND STANDARD ERRORS

		Mean	Std. Dev.	Std. Err.	95% Confidence Interval for Mean		Min.	Max.
					Lower Bound	Upper Bound		
PWVAo	GG	10.10	1.98	.37	9.32	10.87	5.80	14.10
	GT	10.36	1.79	.33	9.67	11.04	7.00	14.60
	TT	9.60	1.53	.46	8.56	10.63	8.10	12.60
Aixb	GG	-1.80	30.34	5.53	-13.13	9.52	68.30	54.60
	GT	3.65	29.79	5.53	-7.67	14.98	59.60	55.60
	TT	8.80	33.67	10.15	-13.81	31.43	59.60	52.90
AixAo	GG	34.22	17.95	3.27	27.51	40.92	5.00	65.30
	GT	40.5207	16.26	3.01	34.3354	46.7060	7.50	72.10
	TT	35.7250	18.66	5.38	23.8672	47.5828	6.00	55.40
PP	GG	52.2000	12.71	2.32	47.4530	56.9470	35.00	77.00
	GT	54.8276	15.38	2.85	48.9752	60.6800	39.00	100.00
	TT	56.2500	14.09	4.06	47.2967	65.2033	41.00	91.00

Table 5
ARTERIAL PARAMETERS DEPENDING ON THE *894G>T* MUTATION

PWVAo: Pulse wave velocity, Aixb: brachial augmentation index, AixAo: the aortic augmentation index, PP: pulse pressure

morphism and these factors, suggesting that NOS3 *894G>T* plays a role in the pathogenesis of metabolic syndrome. For example, a Brazilian study on 1577 subjects showed that NOS3 *894G>T* polymorphism is associated with arterial hypertension risk only in subjects with total cholesterol levels higher than 209 mg/dL. The authors concluded that eNOS polymorphism influences blood pressure depending on the lipid level [34].

Periaswamy reported the same results in a recent study (35) on 438 hypertensive subjects and 444 healthy controls, finding a positive correlation between the NOS3 *894G>T* polymorphism and hypertension in women. In another study, Jia et al.[36] suggested that certain eNOS polymorphisms are an important risk factor for essential arterial hypertension, in Chinese subjects. Recently, no correlation was found between eNOS gene polymorphism and hypertension [35].

A prospective study carried out by Tsao et al. [37] evidenced that NOS3 *894G>T* polymorphism is predictive of glycemic status at 5 years of follow-up, in 256 Chinese subjects with altered basal glycemia.

The T allele is a predictive factor for glycemic status compared to subjects without the mutation (GG subjects). In the current study, the *894G>T* polymorphism does not significantly influence glycemic values.

Previous studies have shown an association between the eNOS *894G>T* gene polymorphism and metabolic syndrome. In the current study, the homozygous state (TT) was significantly associated with metabolic syndrome.

The eNOs *894G>T* gene polymorphism was significantly associated with the presence of MS, which is in accordance with the data presented by other authors [38]. Ianas et al.[39] reported a risk of metabolic syndrome with an OR=2.09 for GT genotype, and a risk of 3.08 for TT genotype.

A number of studies have associated eNOS gene polymorphisms with arterial stiffness parameters [40]. In the current study, *G894T* polymorphism does not show significant differences between the values of pulse wave velocity (PWVAo), the aortic augmentation index (AixAo), the brachial augmentation index (Aixb), pulse pressure (PP) in subjects with GG vs GT vs TT genotype. However, homozygous patients (TT) had higher values of Aixb and PP.

Mayer et al.[41] demonstrated in a study that the homozygous and heterozygous status of *T786C* polymorphism is accompanied by significantly higher values of pulse wave velocity compared to mutation-free subjects; the same results were reported for *G894T* polymorphism, in smokers. In contrast, in the group of non-smokers, eNOS gene polymorphisms were not associated with a significantly higher pulse wave velocity. Mitchell showed that *Glu298Asp* polymorphism is correlated with pulse pressure and the reflected wave amplitude only in women [42]. After adjustment for multiple factors, the association between eNOS polymorphism and arterial stiffness is no longer maintained. In this study, in univariate analysis, age, weight, increased abdominal circumference, increased glycemia and serum triglycerides, decreased HDL-cholesterol and increased PWVAo are risk factors for the development of metabolic syndrome.

Among qualitative variables, the presence of the TT homozygous state of the *894G>T* mutation, the presence of the T allele of the *894G>T* mutation represent risk factors for the development of metabolic syndrome. In multivariate analysis, using the backward logistic regression method, among the previously mentioned factors, the following are considered independent variables for the development of metabolic syndrome: glycemia, HDL-cholesterol (a high value is a protective factor) and the presence of the T allele (its absence is a protective factor) of the *894G>T* mutation.

Conclusions

The eNOS gene polymorphism: *894G>T* was significantly associated with the presence of MS; the polymorphism in homozygous and heterozygous state was associated with an increased risk of metabolic syndrome. Patients in whom the T allele was identified were more susceptible to the development of MS features, as well as to the development of MS as an entity.

The presence of *894G>T* polymorphism in homozygous (TT) or heterozygous (GT) state was associated with an increased prevalence of AHT and diabetes mellitus.

G894T polymorphism did not significantly influence the values of the studied arterial parameters (pulse wave velocity, aortic and brachial augmentation index).

In univariate analysis, the following were identified as risk factors for the development of MS: the presence of the TT homozygous state of the *894G>T* mutation, the presence of the T allele of the *894G>T* mutation. Of these, the presence of the T allele of the *894G>T* mutation is an independent predictive factor for the development of metabolic syndrome (multivariate analysis).

In current practice, the determination of eNOS gene polymorphisms might allow the identification of a group of subjects at high risk for metabolic syndrome, diabetes mellitus and arterial hypertension.

The presence of eNOS gene polymorphisms influences arterial stiffness and can be a connection pathway between metabolic syndrome and the increased cardiovascular risk of these patients.

The *894G>T* polymorphism is sensitive in the identification of patients at high risk for the development of metabolic syndrome.

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