1. INTRODUCTION

An enzyme constitutively expressed mainly in endothelial cells, Endothelial Nitric Oxide Synthase (eNOS or NO3), is responsible for Nitric Oxide (NO) bioavailability at endothelial level. Alterations in endothelial-derived NO production occurs in various cardiovascular diseases (coronary artery disease, myocardial infarction, hypertension, pre-eclampsia, stroke, metabolic syndrome and diabetes [1–11]), associated with different polymorphisms in the eNOS gene – one of the most studied being represented by -786T/C (rs2070744) [1–11]. Even in relatively healthy people who are at low risk for cardiovascular disease, arterial stiffness increases with advancing age [12].

The interest in studying factors that modulate arterial stiffness is based on the relationship between arterial stiffness and pathogenesis of cardiovascular disease. A cluster entity characterized by hypertension, hyperglycemia, obesity, dyslipidemia, and insulin resistance is known as Metabolic Syndrome (MS). Atherosclerotic lesions in metabolic syndrome can be the result of endothelial dysfunction determined by alteration of nitric oxide production. NO has vasodilatory, antiproliferative and anti-inflammatory effects [13].

2. OBJECTIVES OF THE STUDY

In this study, we aimed to investigate the impact of eNOS T786C gene polymorphism on arterial stiffness, by measuring in patients with metabolic syndrome, arterial stiffness parameters - PWV and the augmentation index.
3. MATERIALS AND METHODS

3.1. Subjects

One hundred consecutive patients were included in the study, who were investigated in the service of Cardiology, 4th Medical Clinic, University of Cluj-Napoca. Based on International Diabetes Federation (IDF) criteria, 55% of patients were diagnosed with metabolic syndrome (obligatory presence abdominal obesity ≥ 94 cm in men and ≥ 80 cm in women), and another two criteria for blood pressure above 130/85 mmHg, low High-density Lipoprotein (HDL)-cholesterol, glycemia ≥ 100 mg/dl and triglycerides ≥ 150 mg/dl.

Anthropometric measurements were performed and included weight, height and waist circumference. Based on anthropometric measurements, the body mass index was calculated. Blood pressure measurements were performed at least twice, in a quiet room after lying down for 15 min (according to present guidelines) in order to diagnose hypertension.

WHO criteria were used for type 2 diabetes’ diagnosis. The levels of Triglycerides (TG), total cholesterol, Low-density Lipoproteins (LDL) and HDLs 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.

3.2. DNA Isolation

The T786C polymorphism located in the eNOS gene promoter (chr 7q36) was examined by polymerase chain reaction (PCR) amplification of genomic DNA and enzymatic digestion with the restriction endonuclease of the amplified fragment polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP technique).

For the identification of the above mentioned polymorphism, DNA analysis according to the modified method described by Negrao was performed. Amplification was performed in 25 μl reaction mixture, with the following reaction components: 20 ng genomic DNA, 0.2 mM dNTP, 0.2 μM forward and reverse primer [(the forward primer had the sequence: 5’-TGG AGA GTG CTG GTG TAC CCC A-3’; the reverse primer had the sequence: 5’-GCC TCC ACC CCC ACC CTG TC-3’) (Sigma Genosys, The Woodlands, TX 77380, USA), 1.5 mM Mg2+ and two units of Taq DNA polymerase (Sigma-Aldrich, St. Louis, MO 63103, USA)]. Identification of the T786C polymorphism was performed by enzymatic digestion of the fragment obtained by PCR amplification with the restriction endonuclease Mspl. The T786C polymorphism creates a restriction site for the Mspl enzyme.

3.2.1. Evaluation of arterial parameters

Brachial Augmentation Index (Aixb), Aortic Aix (AixAo), Pulse Wave Velocity (PWVao), central systolic pressure and aortic Pulse Pressure (PP) was performed with the TensioMed Arteriograph (Budapest, Hungary).

3.3. Statistics

Data were statistically processed using the statistical package SPSS 19.0 (IBM Corporation, Armonk, NY, USA) and Medcalc 10.3.0.0 version (MedCalc Software, Ostend, Belgium). The results are presented as mean ± standard deviation for quantitative variables with normal distribution (Kolmogorov test was used for testing the normality of data). Qualitative variables were presented as number (%). The difference between quantitative variables was assessed using the independent-sample t-test or Mann–Whitney test, and for qualitative variables, the χ²-test was used.

Data were presented as odds ratios and 95% confidence intervals. 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. Univariate analysis and logistic regression was used to identify independent predictive factors for metabolic syndrome. A p-value < 0.05 was considered statistically significant.

4. RESULTS

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).

Regarding T786C polymorphism, the distribution of the 100 subjects was the following: 13% were homozygous (CC), 30% were heterozygous (CT), and 57% of the subjects did not have the mutation (TT). No significant sex differences was found, with the only exception being the absence of the mutation (TT) which was more frequent in the female sex (66.7% vs. 38.2%, p-0.011). The homozygous state (CC) was more frequent in males (20.6% vs. 9.1%, p-NS) compared to females, without a statistically significant difference.

The relationship between the presence of metabolic syndrome and T786C polymorphism is presented in Table 1. Homozygous state (CC) was more frequently met in metabolic syndrome patients (CC - 16.4% vs. 8.9%). The TT status was more frequently present in metabolic syndrome patients (CC - 16.4% vs. 8.9%). The TT status was more frequently present in metabolic syndrome patients (CC - 16.4% vs. 8.9%).

The C allele was present in a proportion of 54.5% in subjects with metabolic syndrome and 28.9% in controls (p-0.01) – complete data are presented in Table 1.

Prevalence of hypertension and diabetes were increased in homozygous (CC) or heterozygous (CT) state vs. TT patients - 67.4% vs. 40.4%, p-0.0134, for hypertension and 32.6% vs. 10.5%, p-0.0130, for diabetes.

<table>
<thead>
<tr>
<th>Metabolic syndrome</th>
<th>Total</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>No (%)</td>
<td>4 (8.9)</td>
</tr>
<tr>
<td>T786C</td>
<td>CC</td>
<td>No (%)</td>
</tr>
<tr>
<td>mutation</td>
<td>CT</td>
<td>No (%)</td>
</tr>
<tr>
<td>CC or CT</td>
<td>No (%)</td>
<td>30 (54.6)</td>
</tr>
<tr>
<td>TT</td>
<td>No (%)</td>
<td>25 (45.5)</td>
</tr>
</tbody>
</table>

p between MS patients vs. those without MS, p < 0.05 was considered significantly statistic.
The presence of T786C gene polymorphism in homozygous (CC) or heterozygous (CT) state was found to be associated with an elevation of glycemia, total cholesterol, LDL-cholesterol, serum triglycerides and a decrease of HDL-cholesterol, with an increased abdominal circumference, but without reaching statistical significance (Table 2).

The eNOS T786C gene polymorphism was significantly associated with the presence of metabolic syndrome, subjects having homozygous state had higher risk of having MS compared to those without the polymorphism, OR - 2.790 (95% CI 0.76–10.13, *p*-0.1).

Sensitivity, specificity, positive predictive value, negative predictive value, +LR, –LR of allele presence (CC vs. TT) were calculated (taking into consideration as gold standard for diagnostic IDF criteria for metabolic syndrome definition).

The presence of the C allele was significantly associated with the presence of metabolic syndrome, OR - 2.86 (95% CI 1.2–6.6, *p*-0.014). All data are presented in Tables 1 and 3.

By univariate analysis, we investigated the role of the presence of T786C polymorphism in the development of metabolic syndrome. In univariate analysis, increased abdominal circumference, age, weight, elevated glicemia, serum triglycerides, HDL-cholesterol and PWV, were risk factors for the development of MS. The presence of the C allele of the T786C mutation was a risk factor for the development of metabolic syndrome (being significantly more frequently present in metabolic syndrome patients 54.5% vs. 28.9%)

| Table 2 | The relationship between the T786C mutation and biochemical parameters |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Mutation        | Mean            | Std. dev.       | Std. error mean | *p*             |
| Abdominal circumference (cm) |                   |                 |                 |                 |
| CC/CT           | 99.32           | 14.47           | 2.20            | 0.07            |
| TT              | 93.34           | 16.37           | 2.41            |                 |
| Glycemia (mg/dl) |                   |                 |                 |                 |
| CC/CT           | 105.06          | 28.64           | 4.36            | NS              |
| TT              | 97.76           | 28.07           | 4.09            |                 |
| Total cholesterol (mg/dl) |                   |                 |                 |                 |
| CC/CT           | 201.39          | 62.61           | 9.54            | NS              |
| TT              | 196.32          | 50.88           | 7.76            |                 |
| LDL-cholesterol (mg/dl) |                   |                 |                 |                 |
| CC/CT           | 127.60          | 49.10           | 7.96            | NS              |
| TT              | 119.67          | 41.14           | 7.38            |                 |
| HDL-cholesterol (mg/dl) |                   |                 |                 |                 |
| CC/CT           | 41.02           | 12.62           | 2.04            | NS              |
| TT              | 45.00           | 12.35           | 2.21            |                 |
| Triglycerides (mg/dl) |                   |                 |                 |                 |
| CC/CT           | 158.11          | 82.27           | 12.54           | NS              |
| TT              | 135.26          | 69.28           | 10.69           |                 |

5. DISCUSSION

The presence of the eNOS T786C polymorphism in homoygous or heterozygous state was associated with an increase in the prevalence of arterial hypertension (AHT) and diabetes. Like in this study, Fernandez showed that the T786C genotype was significantly more frequent in hypertensive patients with metabolic syndrome compared to those without metabolic syndrome (*p*-0.002), and concluded that the eNOS gene plays an important role in the pathogenesis of metabolic syndrome in hypertensive subjects [14].

González-Sánchez et al. [15] reported that the CC genotype was significantly more frequent in subjects with metabolic syndrome compared to those without metabolic syndrome (16.4% vs. 12.5%, *p*-0.010) and in subjects with low HDL cholesterol (16.1% vs. 12.7%, *p*-0.044). These data are in agreement with the data obtained in the current study.

The present study, the T786C gene polymorphism was significantly associated with the presence of MS. The eNOS C786T gene polymorphism was associated with the presence of metabolic syndrome in hypertensive subjects [14] and the eNOS haplotype, not the G894T polymorphism, was associated with the features of metabolic syndrome.

This inconsistency between studies regarding the association between the eNOS gene polymorphism and metabolic syndrome might be explained by genetic heterogeneity and by the difference between environmental factors that influence the phenotypic expression of the mutation [15]. Imamura et al. [16] demonstrated in a study that the C–786T allele is associated with increased blood pressure, which is significantly higher compared to subjects without the mutation. Previous studies provide clear evidence of an important physiological role of NO in the modulation of large artery properties [17–19].

Arterial stiffness varies in different arterial districts. In central arteries (such as the aorta) it is strongly influenced by age, elastin and collagen content. However, the tone of vascular smooth muscle through NO, influences the stiffness of the medium-sized muscular arteries.
Endothelium-derived NO (synthesized from L-arginine by eNOS [20–22]) has multiple physiological properties, modulating growth and migration of vascular smooth muscle cells and relaxing vascular smooth muscle [20,23]. Genetic factors, determining eNOS abnormalities, reduce bioavailability of NO [21,23].

In previous studies on mice, NOS3 knockout was associated with elevation in pulse pressure [21]. NOS3 has a role in the modulation of arterial properties, an association between NOS3 gene polymorphism and arterial function being found. In several studies, eNOS gene polymorphisms have been associated with arterial stiffness parameters [22,24].

Using ANOVA test, we found significant differences regarding PWV\textsubscript{Ao} between TT and CT and CC subjects. For Aix\textsubscript{b}, Aix\textsubscript{Ao} and aortic PP, homozgyous (CC) patients presenting higher values, but without statistical significance. Mayer et al. [22] demonstrated in a study that the homzygous and heterozygous state of T786C polymorphism is accompanied by significantly higher values of pulse wave velocity compared to mutation-free subjects (14.0 vs. 10.7 m/s, \( p < 0.002 \)); Mitchell showed that Glu298Asp polymorphism is correlated with pulse pressure and the reflected wave amplitude only in women [23]. After adjustment for multiple factors, the association between eNOS polymorphism and arterial stiffness was no longer maintained. Our group found that G894T polymorphism did not significantly influence the values of the arterial stiffness (PWV, Aix\textsubscript{b} and Aix\textsubscript{Ao}) [24]. The mutated T allele of rs3918226 polymorphism in the NOS3 gene was associated with parameters reflecting central arterial stiffness and wave reflection [25].

In women but not men, the genotype for the common NOS3 missense mutation (Glu298Asp, rs1799983) was related to central pulse pressure and forward wave amplitude [23]. In the current study, in univariate analysis, age, weight, increased abdominal circumference, elevated levels of glyceria and serum triglycerides, low HDL-cholesterol and high PWV\textsubscript{Ao} represented risk factors for the development of metabolic syndrome.

The presence of the C allele of the T786C mutation was a risk factor for the development of metabolic syndrome.

6. CONCLUSION

The eNOS T786C gene polymorphism in homozygous and heterozygous state was significantly associated with the presence of metabolic syndrome and with an increase in the prevalence of AHT and diabetes mellitus.

The T786C polymorphism influences arterial stiffness parameters, specifically PWV, which is the gold standard for arterial stiffness. Arterial stiffness is influenced by eNOS T786C gene polymorphisms, being a possible link between the increase cardiovascular risk and presence of metabolic syndrome in these patients.

CONFLICTS OF INTEREST

The authors declare they have no conflicts of interest.

AUTHORS’ CONTRIBUTION

AC, AV and ST contributed in concept and design of the study, data acquisition, analysis and interpretation of data, drafting and revising the article for intellectual content. OHO contributed in concept and design of the study, analysis and interpretation of data, drafting and revising the article for intellectual content. AF contributed in data acquisition, analysis and interpretation of data, drafting and revising the article. TA and VN contributed in analysis and interpretation of data, revising the article for intellectual content. LMP contributed in genetic analysis and revising the article for intellectual content. All authors read and approved the final manuscript.

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