

## ORIGINAL ARTICLE

# ASSOCIATIONS OF POLYMORPHISMS *NOS3-T-786C*, *MTHFR-C667T*, *P2RY12-T-744C*, (*GPIBA*) -*C482T* AND GENE INTERACTIONS IN MACROANGIOPATHIES IN PATIENTS WITH COMBINED HYPERTENSION AND TYPE DIABETES MELLITUS 2

DOI: 10.36740/WLek20220420115

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## ABSTRACT

**The aim:** To establish the role of allelic polymorphisms *NOS3-T-786C*, *MTHFR-C667T*, *P2RY12-T-744C*, (*GPIba*)-*C482T* in the development of vascular lesions in patients with hypertension and diabetes mellitus type 2.

**Materials and methods:** The study included 100 patients with hypertension and diabetes mellitus type 2 (main group) and 50 patients without type 2 diabetes (control group). Patients underwent echocardiography, color duplex scanning of extracranial, brachiocephalic and femoral vessels. The distribution of allelic polymorphisms was investigated by isolation DNA from leukocytes and polymerase chain reaction (PCR).

**Results:** The risk of vascular damages increases 2-fold when carrying all 4 risk alleles in monozygotic genotypes of polymorphic loci in patients with hypertension with concomitant type 2 diabetes ( $p < 0,05$ ). In gene-gene interaction, the values of contributions and directions of interaction between alleles of polymorphic loci are established ( $p < 0,05$ ). Genes create a paired hierarchy of interaction according to their functional activity; the largest contribution to the probable vascular damage depends on the allelic polymorphism *NOS3-786CT* ( $p < 0,05$ ), the lowest - on the allelic polymorphism *P2RY12-744CC* (*H2H2*). The genetic polymorphism of the *MTHFR* gene is independent of the influence of other studied polymorphisms ( $p < 0,05$ ); the genes *P2RY12-744CT* and *GPIba 482CT* act synergistically with the gene *NOS3-786CT*, being in a weak negative interaction with each other.

**Conclusions:** Phenotypic manifestations of endothelial dysfunction may be modified by allelic polymorphism of genes associated with endothelial and platelet functions with the risk of vascular complications.

**KEY WORDS:** arterial hypertension, diabetes mellitus type 2, angiopathy, candidate genes

Wiad Lek. 2022;75(4 p2):1002-1008

## INTRODUCTION

Multifactorial endothelial damage is reflected by the presence of a significant number of biochemical markers, including a decrease in the synthesis of nitrogen monoxide and its metabolites [1]. Therefore, the gene that are responsible for the production of endothelial nitrogen monoxide synthase (*NOS3*) is considered a candidate gene for the development of hypertension (AH) and type 2 diabetes mellitus (DM) complications. [2]. Given the role of nitrogen monoxide in the involvement of vascular damage in the combined course of hypertension and type 2 diabetes, the study of the association of allelic polymorphism of the NO-synthase gene is important for predicting the severity of polyopathy and the effectiveness of antihypertensive therapy.

Hyperhomocysteinemia is a proven risk factor for thrombosis [3]. A slight excess of homocysteine levels is much more common and is associated with mutations and polymorphisms in the methylenetetrahydrofolate reductase gene. (*MTHFR*) [4]. Carrying the *MTHFR 677T* allele under certain conditions may cause hyperhomocysteinemia and increase the risk of arterial thrombosis [5].

According to Aga Q.A.A. et al. [6], the contribution of genetic factors to the variability of platelet reactivity is about 30%. Functional evaluation of the effect of polymorphic variants of the *GPIba* gene on the rate of thrombosis was performed only in some small studies [7]. Other studies have shown that the occurrence of *H2*-haplotype is associated with increased platelet ADP dependent aggregation with different concentrations of inducers, which may be due to increased platelet ADP receptor expression [8]. The association of the haplotype of *H2* presence with peripheral vascular thrombosis and the development of peripheral arterial damage in the absence of other influencing factors (smoking, weight gain, hyperlipidemia, etc.) was also determined [9].

## THE AIM

The aim of the study was to establish the role of allelic polymorphisms *NOS3 - T-786C*, *MTHFR - C667T*, *P2RY12 - T-744C*, (*GPIba*) - *C482T* in the development of vascular lesions of vessels with medium caliber in patients with hypertension and diabetes mellitus type 2.

**Table I.** Distribution of gene polymorphisms and their correspondence to Hardy-Weinberg equilibrium

Gene	Poly-morphism	Genotype	Distribution of genotypes in the control group		p1 for $\chi^2$ , p2 Fisher's exact	Distribution of genotypes in the main group		p1 for $\chi^2$ , p2 Fisher's exact
			N	%		N	%	
<i>NOS3</i>	<i>T-786C</i>	<b>-786CC</b>	4	8	p1=0,25 p2=0,35	15	15	p1<0,01 p2<0,01
		-786CT	26	52		72	72	
		-786TT	20	40		13	13	
<i>MTHFR</i>	<i>C667T</i>	667CC	21	42	p1=0,18 p2=0,26	19	19	p1<0,01 p2<0,01
		667CT	25	50		69	69	
		<b>667TT</b>	4	8		12	12	
<i>P2RY12</i>	<i>T-744C (H1/H2)</i>	-744TT (H1H1)	25	50	p1=0,51 p2=0,73	37	37	p1=0,15 p2=0,21
		-744TC (H1H2)	22	44		53	53	
		<b>-744CC (H2H2)</b>	3	6		10	10	
<i>GPIba</i>	<i>C482T</i>	482CC	16	32	p1=0,06 p2=0,08	4	4	p1<0,01 p2<0,01
		482CT	30	60		86	86	
		482TT	4	8		10	10	

Note: Genotypes that are potential risk factors for vascular lesions in hypertension and type 2 diabetes are highlighted in bold

## MATERIALS AND METHODS

The study included 100 patients with hypertension and diabetes mellitus type 2 (main group) and 50 patients with hypertension without type 2 diabetes (control group). Verification of the diagnosis of hypertension was performed using the criteria recommended by the European Society of Hypertension and Cardiology, verification of diabetes was carried out on the basis of WHO criteria. All representatives belonged to the general population of Ukrainians in Chernivtsi region (Northern Bukovyna).

The distribution of alleles of polymorphic regions was investigated by isolation of genomic DNA from peripheral blood leukocytes and subsequent amplification by polymerase chain reaction (PCR) on the amplifier "Amplify-4L". All patients underwent echocardiography, color duplex scanning of extracranial vessels, duplex scanning of brachiocephalic and femoral vessels on the EnVisor HD device (Philips, USA). Attention was paid to the presence of stenotic lesions of large vessels, assessment of intraluminal diameter of vessels and thickening of the complex of intima-media walls of large vessels for verification of macroangiopathies.

For statistical analysis of the obtained results we used a program packages Statistica for Windows 8.0 (Stat Soft inc., USA), WinPEPI 11.43 (School of Public Health and Community Medicine, Hebrew University, Israel), MDR 3.0.2 (Perelman School of Medicine of the University of Pennsylvania, USA), GMDR 0.7 (Department of Psychiatry and Neurobehavioral Sciences University of Virginia, USA). Values are presented in the frequencies (percentage of observations to the total number of subjects). DeFinetti online programs (<https://ihg.gsf.de/cgi-bin/hw/hwa1.pl>) (Institute of Human

Genetics, Helmholtz Center, Munich, Germany) were used to study the associations of genetic polymorphisms. Comparison of the frequency of alleles and genotypes was performed using the criterion  $\chi^2$  of maximum likelihood (ML  $\chi^2$ ) and modification of the exact Fisher's test (mid-p). At level of  $p < 0,05$  discrepancies were considered statistically significant.

## RESULTS

At the initial stage of the study, the distribution of genotypes of polymorphisms of genes of 4 loci, respectively, according to the Hardy-Weinberg equilibrium in the main and control groups were calculated. The results of the compliance analysis are presented in table I.

In the control group there was no statistical deviation from Hardy-Weinberg equilibrium. In contrast, the genotype distribution of the main group polymorphisms for the *NOS3*, *MTHFR* and *GPIba* genes was characterized by a probable deviation from Hardy-Weinberg equilibrium, and no statistically significant deviation from equilibrium was found for the *P2RY12* gene polymorphism.

A monocus analysis of the associations of alleles and genotypes of the corresponding polymorphisms with the risk of vascular damage of the elastic type in patients with hypertension and concomitant type 2 diabetes showed the presence of changes between the main and control groups. Tests of associations of allelic polymorphism T-786C of the *NOS3* gene are presented in table II.

The variability of the -786C allele was associated with an increased risk (2.55-fold increase) (Table II) of large vessel damage in the examined patients compared with the control. The main group also differed in the level of

**Table II.** Association of T-786C alleles of the NOS3 gene in patients with macrovascular lesions in hypertension and diabetes mellitus compared with control

Simple frequency of alleles	The degree of heterozygosity	The degree of homozygosity	Allelic positivity	Linear trend (Armitage's test)
<b>Allele risk 2 (T)</b>				
[1]<->[2]	[11]<->[12]	[11]<->[22]	[11]<->[12+22]	General OR
OR=0,49 95%CI=0,30-0,81 $\chi^2=7,77$ p=0,005	OR=0,74 95%CI=0,23-2,43 $\chi^2=0,25$ p=0,62	OR=0,17 95%CI=0,05-0,64 $\chi^2=7,59$ p=0,006	OR=0,49 95%CI=0,16-1,57 $\chi^2=1,48$ p=0,22	OR=0,38 $\chi^2=11,4$ p<0,001
<b>Allele risk 1 (C)</b>				
[2]<->[1]	[22]<->[12]	[22]<->[11]	[11+12]<->[22]	General OR
OR=2,02 95%CI=1,23-3,33 $\chi^2=7,77$ p=0,005	OR=4,27 95%CI=1,86-9,77 $\chi^2=12,58$ p=0,003	OR=5,77 95%CI=1,56-21,28 $\chi^2=7,59$ p=0,006	OR=4,46 95%CI=1,98-10,05 $\chi^2=14,16$ p<0,001	OR=2,55 $\chi^2=11,4$ p<0,001

**Table III.** Association of C667T alleles of the MTHFR gene in patients with macrovascular lesions in hypertension and diabetes mellitus compared with control

Simple frequency of alleles	The degree of heterozygosity	The degree of homozygosity	Allelic positivity	Linear trend (Armitage's test)
<b>Allele risk 2 (T)</b>				
[1]<->[2]	[11]<->[12]	[11]<->[22]	[11]<->[12+22]	General OR
OR=1,77 95%CI=1,18-2,65 $\chi^2=7,61$ p=0,006	OR=3,05 95%CI=1,59-5,6 $\chi^2=11,60$ p=0,006	OR=3,32 95%CI=1,17-9,44 $\chi^2=5,32$ p=0,02	OR=3,09 95%CI=1,63-5,84 $\chi^2=12,48$ p<0,001	OR=1,98 $\chi^2=10,04$ p=0,002
<b>Allele risk 1 (C)</b>				
[2]<->[1]	[22]<->[12]	[22]<->[11]	[11+12]<->[22]	General OR
OR=0,57 95%CI=0,38-0,85 $\chi^2=7,61$ p=0,006	OR=0,92 95%CI=0,35-2,42 $\chi^2=0,53$ p=0,86	OR=0,30 95%CI=0,11-0,86 $\chi^2=5,32$ p=0,02	OR=0,64 95%CI=0,25-1,63 $\chi^2=0,89$ p=0,35	OR=0,49 $\chi^2=10,04$ p=0,002

**Table IV.** Association of T-744C (H1 / H2) alleles of the P2RY12 gene in patients with macrovascular lesions in hypertension and diabetes mellitus compared with controls

Simple frequency of alleles	The degree of heterozygosity	The degree of homozygosity	Allelic positivity	Linear trend (Armitage's test)
<b>Allele risk 2 (T) (H2)</b>				
[1]<->[2]	[11]<->[12]	[11]<->[22]	[11]<->[12+22]	General OR
OR=1,48 95%CI=0,88-2,49 $\chi^2=2,16$ p=0,15	OR=1,62 95%CI=0,80-3,31 $\chi^2=0,25$ p=0,62	OR=2,52 95%CI=0,56-9,01 $\chi^2=1,37$ p=0,24	OR=1,70 95%CI=0,86-3,39 $\chi^2=2,32$ p=0,13	OR=1,53 $\chi^2=2,45$ p=0,12
<b>Allele risk 1 (T)(H1)</b>				
[2]<->[1]	[22]<->[12]	[22]<->[11]	[11+12]<->[22]	General OR
OR=0,68 95%CI=0,40-1,14 $\chi^2=2,16$ p=0,15	OR=0,72 95%CI=0,18-2,88 $\chi^2=0,21$ p=0,64	OR=0,44 95%CI=0,11-1,78 $\chi^2=1,37$ p=0,24	OR=0,57 95%CI=0,15-2,19 $\chi^2=0,67$ p=0,41	OR=0,65 $\chi^2=2,45$ p=0,12

heterozygosity (OR = 4.27; 95% CI = 1,86 – 9,77; p<0,05) from the control, which explained the deviation of the frequency of genotypes from Hardy-Weinberg equilibrium.

Analysis of the associations of alleles of the C667T polymorphic locus of the MTHFR gene showed a constant

difference in the “risk” allele of T in patients with hypertension with concomitant type 2 diabetes. Adjusted for the linear trend, the odds ratio (OR) is 1,98 (p = 0,002), table III. The model is also characterized by an increased degree of heterozygosity and the presence of allelic positivity in

**Table V.** Association of C482T alleles of GPIba gene in patients with macrovascular lesions in hypertension and diabetes mellitus type 2 compared with control

Simple frequency of alleles	The degree of heterozygosity	The degree of homozygosity	Allelic positivity	Linear trend (Armitage's test)
<b>Allele risk 2 (T)</b>				
[1]<->[2]	[11]<->[12]	[11]<->[22]	[11]<->[12+22]	General OR
OR=1,84 95%CI=1,13-3,00 $\chi^2=6,01$ p=0,014	OR=11,46 95%CI=3,55-37,01 $\chi^2=22,34$ p<0,001	OR=10,00 95%CI=2,03-49,28 $\chi^2=8,99$ p=0,003	OR=11,29 95%CI=3,53-36,15 $\chi^2=22,62$ p<0,001	OR=3,29 $\chi^2=13,33$ p<0,001
<b>Allele risk 1 (C)</b>				
[2]<->[1]	[22]<->[12]	[22]<->[11]	[11+12]<->[22]	General OR
OR=0,54 95%CI=0,33-0,89 $\chi^2=6,01$ p=0,014	OR=1,15 95%CI=0,34-3,93 $\chi^2=0,05$ p=0,83	OR=0,10 95%CI=0,02-0,49 $\chi^2=8,99$ p=0,003	OR=0,78 95%CI=0,23-2,63 $\chi^2=0,16$ p=0,69	OR=0,29 $\chi^2=13,33$ p<0,001

**Table VI.** GRS for two-locus, three-locus, and four-locus models of vascular complications in patients with hypertension and diabetes type 2

Genes with polymorphic loci	OR and 95% CI	$\chi^2$ , p
<i>MTHFR</i>	1,62 (0,50 – 5,32)	0,56; 0,46
<i>MTHFR</i> + <i>NOS3</i>	1,94 (0,80 – 4,60)	2,25; 0,13
<i>MTHFR</i> + <i>P2RY12</i>	1,73 (0,70 – 4,67)	1,36; 0,24
<i>MTHFR</i> + <i>GPIba</i>	1,48 (0,61 – 3,80)	0,75; 0,39
<i>NOS3</i> + <i>P2RY12</i>	2,05 (0,83 – 5,46)	2,40; 0,12
<i>NOS3</i> + <i>GPIba</i>	1,75 (0,73 – 4,45)	1,57; 0,21
<i>P2RY12</i> + <i>GPIba</i>	1,54 (0,61 – 4,17)	0,81; 0,37
<i>MTHFR</i> + <i>NOS3</i> + <i>P2RY12</i>	2,08 (0,96 – 4,71)	3,45; 0,063
<i>MTHFR</i> + <i>NOS3</i> + <i>GPIba</i>	1,87 (0,87 – 4,11)	2,56; 0,11
<i>NOS3</i> + <i>P2RY12</i> + <i>GPIba</i>	1,91 (0,88 – 4,32)	2,65; 0,4
<i>MTHFR</i> + <i>P2RY12</i> + <i>GPIba</i>	1,67 (0,76 – 3,79)	1,63; 0,20
<i>MTHFR</i> + <i>NOS3</i> + <i>P2RY12</i> + <i>GPIba</i>	2,07 (1,05 – 4,32)	3,96; 0,046

**Table VII.** Models of gene-gene interactions in patients with vascular lesions of large caliber in the combined course of hypertension and diabetes mellitus type 2

Combinations of polymorphisms in the model	Training balance accuracy	Testing balance accuracy	Test the significance of the model (sign (p) test)	Reproducibility of the model (CV consistency)
<i>MTHFR</i> + <i>NOS3</i> + <i>P2RY12</i> + <i>GPIba</i>	0,82	0,76	9(0,011)	10/10
<i>NOS3</i> + <i>P2RY12</i> + <i>GPIba</i>	0,78	0,77	10(0,001)	10/10

the main group (respectively 3,3 and 4,8 times at  $p<0,001$ ).

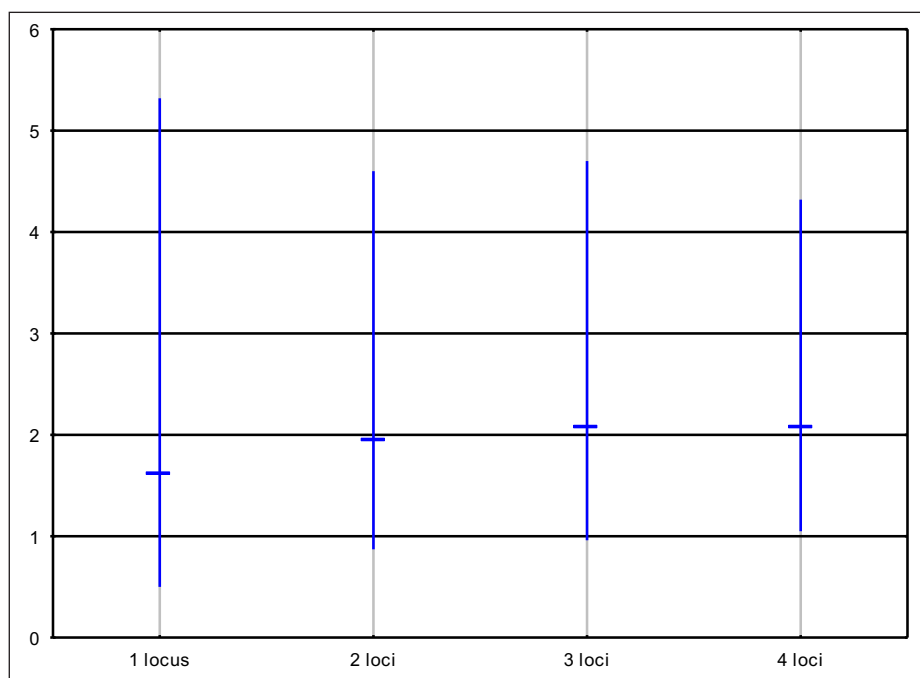
The ratio of alleles of the ADP receptor gene (*T-744C*, *P2RY12*) in patients was quite uneven: in  $63,5\% \pm 3,15\%$  of cases the presence of *-744T* allele, in  $36,5\% \pm 3,15\%$  - *-744C*-allele ( $p<0,05$ ). The distribution of genotypes in the main group (H1/H1; H1/H2; H2/H2) of carriers of this polymorphism was determined as follows: H1/H1 homozygotes accounted for 37,0% of cases, homozygotes with the presence of the "risk" allele - H2/H2 - 10,0 %, heterozygotes H1/H2 - 53,0% ( $p<0,05$ ), table IV.

The frequency distribution of alleles of the platelet glycoprotein receptor gene (*C482T*, *GPIba*) was more uniform than the alleles of the *P2RY12* receptor. Thus, the *482C* allele was found in 90% of patients (its frequency in the group -  $47,0 \pm 1,8\%$ ), and the *482T* allele - in 96% of pa-

tients (total frequency in the group -  $5,0 \pm 1,8\%$ ). However, the analysis of associations of the *C482T* polymorphic locus of the *GPIba* gene revealed a probable excess of the T allele frequency in patients with hypertension with concomitant type 2 diabetes (OR = 3,29 in the trend test, increasing the degree of heterozygosity for the allele " risk ", increase in the degree of allelic positivity ( $p<0,001$ )), table V.

Thus, based on data analysis, it can be assumed that allelic polymorphisms of the *NOS3*, *MTHFR*, and *GPIba* genes can be considered as genetic markers of markers associated with vascular damage in the general cohort of patients with combined hypertension and type 2 diabetes.

Our monolocus analysis of allelic polymorphisms of four genes revealed a significant difference ( $p<0,05$ ) in reducing the incidence of "protective" alleles and genotypes in pa-



**Fig. 1.** Changes in the GRS index depending on the frequency of interaction of polymorphic loci of genes responsible for endothelial dysfunction.

Note: GRS for the models with different number of loci (from 1 to 4). The estimation of risk of macrovascular damage in patients with hypertension and diabetes mellitus type 2 (OR (95% CI)).

tients with hypertension and diabetes with macrovascular lesions, but monolocus analysis of the frequency of “risk” alleles and genotypes found only a tendency to increase their frequency in patients of the main group ( $p > 0,05$ ).

To validate gene interactions, analysis was performed using the Genetic Risk Score (GRS) interlocus conjugation model. According to this model, in the main and control group for the *GPIb $\alpha$*  gene the frequency of “risk” alleles was 53% and 38%, for the *P2RY12* gene - 36% and 28%, for the *NOS3* gene - 51% and 34%, for the *MTHFR* gene - 46% and 33%, with a total median frequency of 48,5% for the main group and 33,5% for the control group. On average, there were 1,33 alleles per person in the control group, and 1.86 alleles in the main group, which was in 1,42 folds greater ( $t = 3.07$ ;  $p = 0,022$ ;  $p = 0,04$  by median Mood test). Thus, in contrast to the single-locus model of allelic polymorphism, carriers of “risky” allelic polymorphisms simultaneously for all 4 genes significantly ( $p < 0,05$ ) increases the likelihood of macrovascular complications, which depends on multilocus gene interaction. GRS values for complications dependent on genotype interactions were calculated from models of two, three and four-gene interactions. Indicators of the ratio of chances (OR) with confidence intervals and the corresponding frequencies of risk genotypes are given in table VI.

Thus, the overall risk of macrovascular complications in patients with hypertension and diabetes mellitus 2 is a measure of the effect of a combination of polymorphisms of the four so-called “risky” alleles, of course, without considering the possible influence of additional polymorphisms or mutations in patients’ genotypes.

Changes in the GRS index depending on the type of multiple model (including loci of models *MTHFR* (1 locus), *MTHFR* + *NOS3* (2 loci), *MTHFR* + *NOS3* + *P2RY12* (3 loci), *MTHFR* + *NOS3* + *P2RY12* + *GPIb $\alpha$*  (4 loci)) are illustrated in fig. 1.

Given that the regions of the studied polymorphisms are located on different chromosomes, it is possible that there is an epistatic or complementary interaction of the genes included in our analysis with each other. To evaluate these types of interactions, namely, how mononucleotide polymorphisms affect each other’s function, we modeled the interaction of nucleotide polymorphisms by the method of multifactorial dimensional reduction (MDR). According to the analysis of this method in the sample of sick and healthy people, the optimal models of combinations of gene interactions were determined, with the establishment of their accuracy and reproducibility. Forced Search Algorithm was used to analyze the interaction models, which assessed the nature of genotype combinations in the direction of the development of macrovascular complications in patients with hypertension and type II diabetes. As a result, we selected 2 models with the highest degree of reproducibility (10/10 - 100%) - for three loci of polymorphism and for four loci of polymorphism, table VII.

Thus, the risk of developing macrovascular complications in patients with hypertension and diabetes mellitus type 2 associated with allelic polymorphism depends not only on the type of monolocus polymorphism, but has a more complex nature of dependence due to intergenic multidirectional interactions.

## DISCUSSION

Analysis of current literature data on the association of different polymorphic markers of the eNOS gene and hypertension shows contradictions. For example, in a Canadian study, an association of the polymorphic allele -786T>C with the risk of developing hypertension was found, but in Japanese studies, the existence of this association was not confirmed [10]. The polymorphic variant of the *Glu298Asp* gene in the eNOS gene has been shown to be associated with the risk



of acute myocardial infarction [11], coronary artery disease (CAD) [12], AH [13]. The minisatellite polymorphism eNOS 4a / 4b has been studied in detail. In the Chinese population, allele 4a is defined as an independent risk factor for atherosclerosis and diseases that are accompanied by impaired formation of nitrogen monoxide, including hypertension [14]. However, in another study, on the contrary, the protective role of the 4a allele in the development of stroke in patients with hypertension [15]. Genotypes eNOS-786C / C and allele eNOS-922G in case-control studies were more common in patients with hypertension compared with the control group [16].

An association with arterial thrombosis depending of the C667T polymorphism of the MTHFR gene has been found in Chinese populations [17], Turkish population [18], Polish population [19], in Italians [20].

## CONCLUSIONS

1. The risk of vascular damages increases 2-fold when carrying all 4 risk alleles in monozygotic genotypes of polymorphic loci in patients with hypertension with concomitant type 2 diabetes ( $p < 0,05$ ).
2. In gene-gene interaction, the values of contributions and directions of interaction between alleles of polymorphic loci are established ( $p < 0,05$ ). Genes create a paired hierarchy of interaction according to their functional activity; the largest contribution to the probable vascular damage depends on the allelic polymorphism NOS3-786CT ( $p < 0,05$ ), the lowest - on the allelic polymorphism P2RY12-744CC (H2H2).
3. The genetic polymorphism of the MTHFR gene is independent of the influence of other studied polymorphisms ( $p < 0,05$ ); the genes P2RY12-744CT and GPIBa 482CT act synergistically with the gene NOS3-786CT, being in a weak negative interaction with each other.

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*This work is a fragment of the research work «Clinical, pathogenetic and pharmacotherapeutic features of internal diseases», registration number 0119U101344 (2019-2023).*

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**Conflict of interest:**

*The Authors declare no conflict of interest.*

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**Received:** 18.10.2021

**Accepted:** 30.03.2022

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**A** - Work concept and design, **B** - Data collection and analysis, **C** - Responsibility for statistical analysis,

**D** - Writing the article, **E** - Critical review, **F** - Final approval of the article