ORIGINAL ARTICLE

PREDICTING THE OCCURRENCE OF PRIMARY OPEN-ANGLE GLAUCOMA DEPENDING ON THE GENETIC POLYMORPHISM ENDOTHELIAL NO SYNTHASE (*NOS3*) GENE

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ABSTRACT

The aim: To develop the model for predicting primary open – angle glaucoma (POAG) depending on the presence of the genetic polymorphism in the endothelial NO-synthase (*NOS3*) gene.

Materials and methods: The results of genotyping 153 patients (153 eyes) with POAG are included in this investigation. 47 patients were in the control group. Their age was 65,0±13,1 years, duration of disease – 4,9±5,3 years. The polymerase chain reaction was carried out in the patients' blood in the real time mode (Gene Amp® PCR System 7500 amplifier; USA) with the help of the TaqMan Mutation Detection Assays Life-Technology test system (USA). The program Statistica 10 (StatSoft, Inc., USA) was used for mathematical testing of the obtained results.

Results: The regression analysis confirmed the effect of rs1799983 and rs2070744 polymorphisms of the *NOS3* gene on the development of POAG. Calculating their specific gravity based on the degree of the impact on the probability of developing the disease showed that rs2070744 – 72.2% had the greater impact than rs1799983 – 38.5%. The regression model of POAG risk depending on the genotypes of the *NOS3* gene rs1799983 and rs2070744 polymorphisms was constructed with the satisfactory quality of mathematical prediction (-2log=202.59; χ^2=28.91; P<0.001). The value of probability of developing POAG exceeded the limit value (Cut-off=0.8), respectively, OR 4.39 (95% CI 1.00-19.30; P=0.048) and OR 14.15 (95% CI 1.88-106.28; P<0.001) in carriers of the rs1799983 and rs2070744 *GT-CC* and *TT-CC* haplotypes.

Conclusions: The results of the study proved the importance of risk genotypes (*TT* rs1799983 and *CC* rs 2070744) for the development of POAG in patients from the Ukrainian population. It has been shown that the significant increase in the risk of POAG exists for carriers of the *GT-CC* and *TT-CC* haplotypes.

KEY WORDS: primary open-angle glaucoma, *NOS3*, rs1799983 (*G894T*, Glu298Asp), rs2070744 (*T-786C*)

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INTRODUCTION

Primary open-angle glaucoma (POAG) is the most common type of glaucoma. It is characterized by asymptomatic development with the gradual decrease in peripheral vision [1]. The reason for this is the development of glaucoma optic neuropathy (GON) with damage to the optic nerve, inefficiency of the eye's drainage system with fluid accumulation and increased intraocular pressure [2].

Endothelial dysfunction plays the important role in the development of GON [3]. According to modern concepts, the vascular endothelium has the number of important functions both in normal and pathological conditions through the regulation of the vascular tone, microvascular permeability, angiogenesis, activity of the dorsal and proteolytic systems, mechanisms of cell adhesion and the development of inflammatory reactions [4, 5]. Among many regulatory factors, the leading role is played by nitric oxide (NO), its formation occurs with the involvement of the universal membrane enzyme – endothelial NO-synthase (eNOS; NOS3) [6].

The polymorphic condition of this gene can be the risk factor of the POAG development, according to the polymorphisms rs1799983 (*G894T,* Glu298Asp) and rs2070744 (*T-786C*) of the *NOS3* gene [7, 10].

The rs1799983 polymorphism (chr7:150999023, GRCh38.p12) is the result of the missense mutation *89T>A,G,* the replacement of aspartate with glutamine at position 298 (Glu298Asp) is the sequent, it leads to the decrease in eNOS activity [8-10].

The rs2070744 (*T-786C*) polymorphism (7q36.1; 7:150992991; GRCh38) is localized in the intron of the *NOS3* gene [10] and it is associated with the increased risk of POAG [10-12].

THE AIM

The aim of the work was to develop the model for predicting primary open – angle glaucoma (POAG) depending on the presence of the genetic polymorphism in the endothelial NO- synthase (*NOS3*) gene.

MATERIALS AND METHODS

The results of genotyping in 153 patients (153 eyes) with the established diagnosis of POAG have been included in this investigation. The control group included 47 patients without such diagnosis. There were 57 men (37.25%) and

Table I. Correspondence of categorical variables to indicator values which were used in regression analysis

Indicator	Variable name	Categorical value	Indicator value
rs1799983 NOS3 gene	1NOS3	GG	101
		GT	102
		TT	103
rs2070744 NOS3 gene	2NOS3	TT	101
		TC	102
		CC	103
Sex	Sex	male	101
		female	102

Table II. Independent variables included in the POAG probability prediction model and their statistical characteristics

Independent variables	β	±SE	95 % BI	Wald	p
Constant	-418,700	121,564	-(643,542-183,391)	8,73	0,003
1NOS3	1,568	0,647	0,300-2,837	5,87	0,015
2NOS3	2,527	0,680	1,195-3,859	13,82	<0,001
Sex	0,443	0,244	-0,661-0,866	3,31	0,069

Notes: β-regression coefficient; SE-standard error of the regression coefficient; 95% BI – 95% probable interval for the regression coefficient; Wald – Wald's statistics; P – significance of the discrepancy with the null hypothesis.

Table III. Classification of predicted and actual data by belonging to the categories of probability and risk assessment POAG

Cut-off	Actual data		Prediction (n)		Indicators (%)		
	Category	n	POAG+	POAG–	Sensitivity	Specificity	Accuracy
0,5	POAG +	153	140	13	91,5	6,38	71,5
	POAG –	47	44	3			
0,8	POAG +	153	111	42	72,5	70,2	72,0
	POAG –	47	14	33			

Note: Cut – off=0.5 is the standard cut-off point of P(POAG) values, calculated by Formula 2; Cut – off=0.8 is the optimal cut-off point.

96 women (62.75%) in the POAG group. There were 22 men (46.81%) and 25 women (53.19%) in the control group. According to the stages of POAG, patients were distributed as follows: stage I – 11.76%, stage II – 17.65%, stage III – 52.94% and stage IV – 17.65%.

According to the generally accepted protocol for examining patients with POAG [13], complaints and medical history were carefully collected from each patient, visometry, Hamphrey's perimetry, refractometry, pneumotonometry, biomicroscopy, gonioscopy, ophthalmoscopy, and optical coherence tomography (OCT) were performed.

Molecular genetic testing was performed in whole venous blood samples obtained from patients in the amount of three milliliters, in accordance with their permission and in compliance with the necessary biotic standards. The genotypes of the NOS3 gene rs1799983 (*G894T*, Glu298Asp) and rs2070744 (*T-786C*) polymorphisms were determined by real-time polymerase chain reaction in the automatic Gene Amp® PCR system 7500 amplifier ("Applied Biosystems", USA). At the first stage of the investigation, genomic DNA was isolated from whole venous blood using Purelink® Genomic DNA Kit For Purification of Genomic DNA ("INVITROGEN"; USA) reagents. Genetic analysis was performed using the unified test system TaqMan Mutation Detection Assays Life-Technology (USA).

Multi-factor logistic regression technology was used to construct the model for predicting the risk of the POAG development [14]. The analysis included data from 153 patients with POAG, united by the sign of the disease's presence and data from 47 patients in the control group. The POAG presence sign was used as the resulting variable regression. If the patient had POAG, the variable was assigned the indicator value of 1, and if it was absent – 0. The last value is taken as the reference value.

Stable indicators were used as factor signs in the construction of the model, they remained unchanged throughout the patient's life, and also they do not change with the progression of the disease. These signs included categorical indicators of gender and genotyping data for *NOS3* gene polymorphisms; they were converted into indicator values.

The selection of statistically significant independent variables of regression equations (predictors) was carried out in the step-by-step exclusion mode. Regression coefficients (β), their standard errors (SE), and the significance of their

Table IV. Distribution patients with POAG and the control group depending on the combination of genotypes of NOS3 gene polymorphisms rs1799983 and rs2070744

Haplotype(rs1799983-rs2070744)	$P_{(POAG)}$	POAG (n=153)	Control (n=47)	$P_{(EFC)}$	OR	95 % BI
GG-CC	0,487	0	7 (14,89%)	-	-	-
GG-CT	0,070	21 (13,72%)	10 (21,28%)	0,249	0,59	0,25-1,36
GG-TT	0,006	24 (15,69%)	8 (17,02%)	0,822	0,91	0,38-2,18
GT-CC	0,820	25 (16,34%)	2 (4,25%)	0,048	4,39	1,00-19,30
GT-CT	0,267	47 (30,72%)	15 (31,91%)	0,859	0,95	0,47-1,91
GT-TT	0,028	0	0	-	-	-
TT-CC	0,956	36 (23,53%)	1 (2,13%)	<0,001	14,15	1,88-106,28
TT-CT	0,636	0	4 (8,51%)	-		
TT-TT	0,122	0	0	-		

Note: P(POAG) is the probability of developing POAG calculated by Formula 2; P(EFC) is the statistical significance of genotype distribution comparisons using the exact Fisher's method.

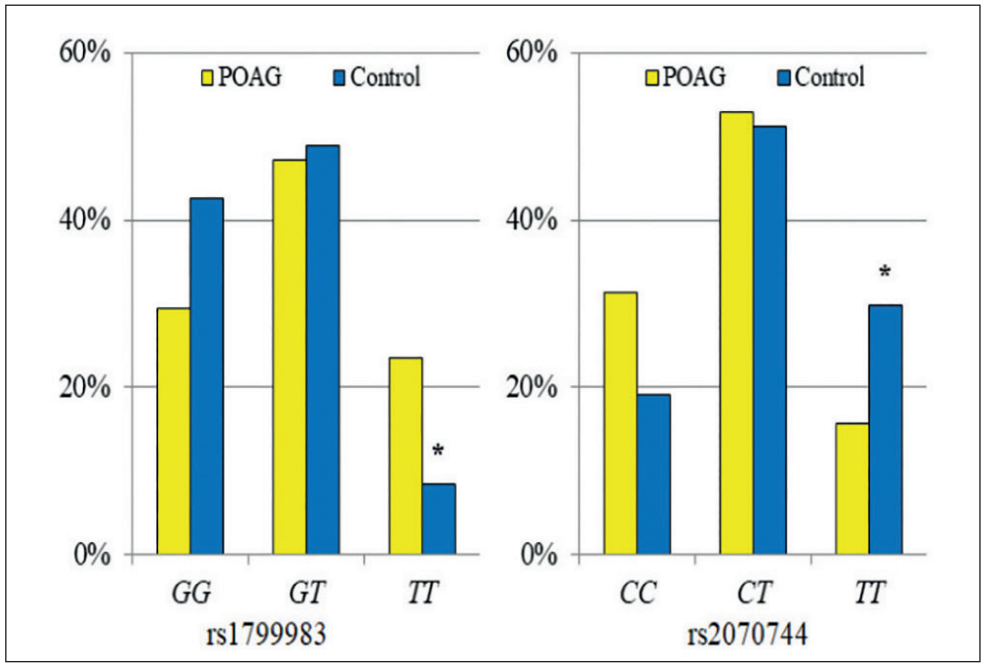

Fig. 1. Frequency of genotypes of rs1799983 and rs2070744 polymorphisms in patients with POAG and in the control group

differences from the null hypothesis were calculated using the Wald's statistics criterion (Wald) for logistic models.

The adequacy of logistics models was judged by receiver operating characteristic (ROC), and the area under the ROC – AUC (area under curve) curve was calculated. The model was considered adequate with the statistically significant difference AUC from 0.5. Wald's statistics, maximum likelihood coefficients (- 2log), xi-square (χ^2), and the Hosmer-Lemeshev's consent criterion were calculated for logistic models.

The percentage of influence of the selected regression indicators on the dependent variable was calculated using the formula:

$$d_k = \frac{\beta_k^2 * 100\%}{\sum_{i=1}^{n} \beta_i^2} \quad (1),$$

where d_k is the percentage of influence of this indicator; β_k – β is the coefficient of this independent variable of the regression equation; the denominator is the sum of the squares of the β-coefficients of all independent variables.

The degree of association of haplotypes with POAG was determined by calculating the odds ratio (OR) and the 95% certain interval (95% CI). In all cases of statistical estimation, the value of P<0.05 was considered probable.

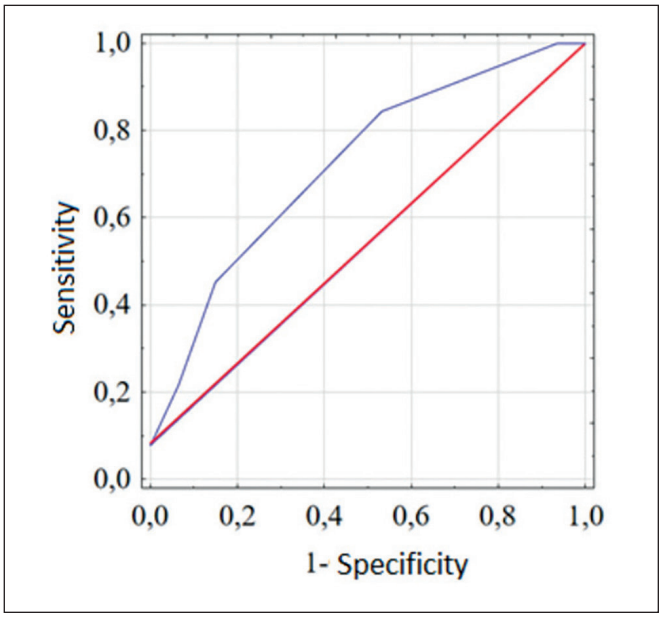

Fig. 2. ROC-diagram of the model for predicting the probability of POAG development

RESULTS AND DISCUSSION

The comparative analysis of the distribution of genotype frequency in patients with POAG and in the control group (fig. 1) showed that the certain difference was determined for both polymorphisms.

rs1799983 showed the decrease in the frequency of the hereditary homozygous *GG* genotype ((p_{Fet}=0,110)) and the increase in the frequency of the minor *TT* genotype (p_{Fet}=0,110) in patients with POAG compared to the control group. The Hardy-Weinberg's test for cases and controls showed random inheritance patterns in both POAG patients and the control group (χ^2=0,472; P=0,790 i χ^2=0,538; P=0,764, respectively).

rs2070744, the increase in the frequency of the hereditary homozygous *CC* genotype (p_{Fet} =0.139) was in the patients with POAG and the decrease in the frequency of the minor *TT* genotype (p_{Fet}=0,049) compared to the control group. The Hardy-Weinberg's test showed random inheritance in patients with POAG and in the control group (χ^2=1,119; P=0,571 i χ^2=0,051; P=0,975, respectively).

The statistical significance of independent variables included in the regression analysis is shown in Table II.

Based on the results of the analysis of Wald's statistics and interval characteristics of β-coefficients, the discrepancy between which and the null hypothesis was statistically significant, the following variables were selected as predictors for the regression model: the rs1799983 polymorphism of the *NOS3* gene (indicator value 1NOS3) and the rs2070744 polymorphism of the *NOS3* gene (indicator value 2NOS3). The following variables were selected as predictors for the regression model: rs1799983 polymorphism of the *NOS3* gene (indicator value 1NOS3) and rs2070744 polymorphism of the *NOS3* gene (indicator value 2NOS3). The following formula can be used as the mathematical expression of the developed model:

$$P(poag) = 1/(1 + e^{-(-418{,}7+1{,}568\times 1NOS3+2{,}527\times 2NOS3)}) \quad (2),$$

where: $P_{(POAG)}$ – probability of POAG developing;

1NOS3 – indicator value for the rs1799983 polymorphism of the *NOS3* gene:

GG = 101; *GT* = 102; *TT* = 103;

2NOS3 – indicator value for the rs2070744 polymorphism of the *NOS3* gene:

TT = 101; *TC* = 102; *CC* = 103.

Calculating the specific weight of predictors based on the degree of their influence on the dependent variable (Formula 1) allowed them to be arranged in the series in descending order as follows: the greatest influence was exerted by the genotype "2NOS3" (72.2%) and the smallest – by the genotype "1NOS3" (38.5%). At the same time, the value of the resulting regression variable was higher in patients with risk alleles in their genotypes: for rs2070744 of the *NOS3* allele *C* and for rs1799983 of the *NOS3* allele *T*.

Indicators that characterize the overall efficiency of the model based on the correspondence of the calculated data to the actual ones indicated the satisfactory quality of the mathematical forecast: -2log (Likelihood)=202.59; χ^2=28.91; P<0.001; Hosmer-Lemeshev's consent criterion: χ^2=12,17; p=0,144.

To evaluate the operational properties of the developed model, the ROC diagram was constructed and analyzed (fig. 2). In the developed mathematical model, the area under the ROC diagram was AUC=0.733±0.039 (95% CI 0.691-0.818; P<0.001), which satisfactorily characterized the quality of the predictive model.

Taking into account the satisfactory operational characteristics of the model as the whole, the attempt was made to find the optimal cut-off point for $P_{(POAG)}$values (Formula 2), the results of the forecast classification will be the best for this model. The search results are displayed on the frequency-probability diagram (fig. 3).

The continuous line shows the sensitivity diagram, the dotted line shows the specificity diagram, and the stipple line shows the accuracy diagram. Frequencies are plotted along the vertical axis, and probability values are plotted along the horizontal axis. The vertical line marked Cut-off=0.5 corresponds to the standard cut-off point, and the line marked Cut-off=0.8 corresponds to the optimal cut-off point.

The results of evaluating the discriminative model's ability are presented in the classification table (table III).

Analyzing the classification results at the standard point of separation of positive and negative prognostic results (Cut-off=0.5), it was found that the number of patients with the positive prognosis for the development of POAG coincided with the real condition in 91.5%. At the same time, the unmistakable negative prognosis for the absence of POAG was possible only in 6.38% of cases. This, even with the relatively high overall accuracy of the prognosis (71.5%; OR 0.73; 95% CI 0.20-2.69; P=0.641), cannot meet clinical requirements.

Analysis of the frequency-probability diagram allowed to determine the optimal threshold for cutting off positive and negative results of the prognosis Cut-off=0.8. At the

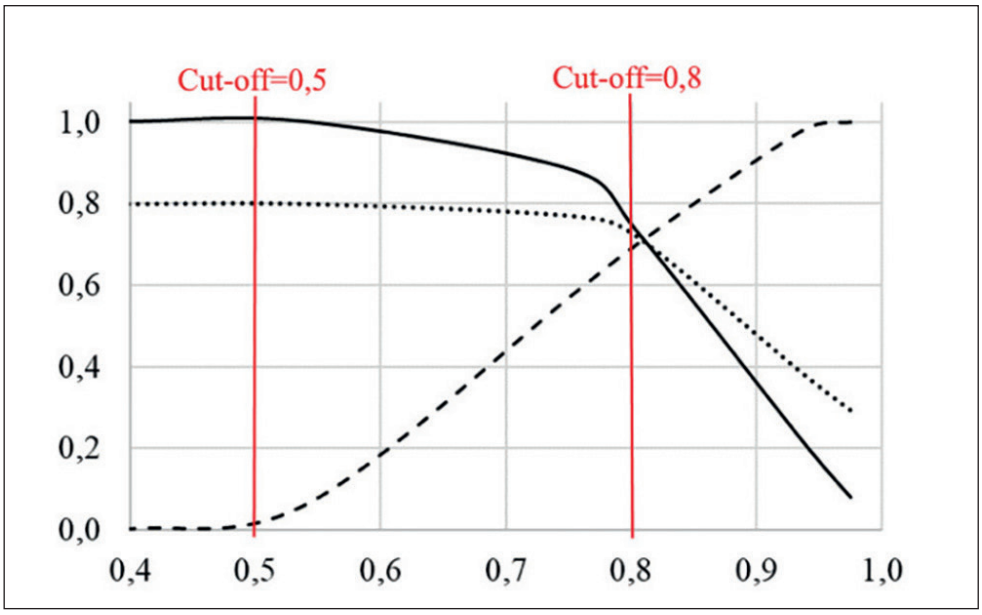

Fig. 3. Diagram of the dependence of sensitivity, specificity and accuracy on the probability of POAG development

same time, the detection of patients with the positive prognosis of POAG was less (72.5%) than the actual value, but the correct negative prognosis of POAG was increased to 70.2%. The overall accuracy of this model was 72.0% (OR 6.23; CI 3.03-12.78; P<0.001), which indicated satisfactory discriminative properties of the model.

Further, the distribution of patients with POAG was analyzed depending on the haplotype of the *NOS3* gene polymorphism rs1799983 and rs2070744 and the calculated probability of developing risk POAG (Formula 2) compared to the control group (table IV).

The actual distribution of patients with POAG and the control group confirmed the calculated data on the limit value of the developing POAG risk probability (Cut-off=0.8). In the *GT-CC* and *TT-CC* haplotypes the probability of developing POAG (Formula 2) exceeded the limit value (Cut-off=0.8). At the same time, the *GT-CC* haplotype increased the POAG risk developing with the value of OR 4.39 (95% CI 1.00-19.30; P=0.048). and the *TT-CC* haplotype increased the POAG risk with the value of OR 14.15 (95% CI 1.88-106.28; P<0.001) in the case-control study (90 patients with POAG and 127 controls).

The *T-786C* and Glu298Asp polymorphisms of the e*NOS* gene were shown to be associated with POAG [15]. Thus, the *T-786C* polymorphism was the risk factor among women (OR 2.28; 95% CI 1.11-4.70; P=0.024) and in patients over 52 years of age (OR 2.11; 95% CI 0.98-4.55; P=0.055).

CG T-786C and Glu298Asp haplotypes showed the borderline association with the POAG risk in the overall analysis (OR 1.76; 95% CI 0.98-3.14; P=0.055). among women (OR 2.02; 95% CI 0.98-4.16; P=0.052). and for the patients over 52 years of age (OR 3.48; 95% CI 1.54-7.84; P=0.002). We got the similar result – in the general analysis. the *GT-CC* haplotype (see table.4) had the positive association with POAG (OR 4.39; 95% CI 1.00-19.30; P=0.048).

It was observed also the association of the minor *TT* variant of the Glu298Asp *NOS*3 polymorphism with POAG in women in the investigation by J.H. Kang et al. (2011) [16]. In this regard, we separately analyzed the distribution of genotypes of this polymorphism by gender, but no statistical significance was found (P=0.195).

However, it was found that women had the association of allelic polymorphism with POAG: for the *G* allele OR = 0.50; CI 0.25-0.99; for the *T* allele OR = 2.00; CI 1.01-3.95 (P=0.043) when it was stratified by gender. This indicated the greater significance of the rs1799983 polymorphism for the POAG development in women compared to men. In our opinion, this observation should be confirmed in the examination of larger groups of patients.

By the way, there are investigations where the direct relationship between *NOS3* gene polymorphisms and POAG has not been established [9, 17, 18]. But the same time, it should be noted that the authors found certain associations with POAG phenotype: with low blood pressure [18] or with hemorrhages in the optic disc in normotensive glaucoma patients' groups [9].

According to these results, it is necessary to continue research in different populations and establish the link between *NOS*3 gene polymorphisms not only with the presence of the disease, but also with its manifestations and the degree of progression.

According to the case-control study (173 patients and 171 controls), the rs1799983 polymorphism was significantly associated with POAG especially in men (for the *T* allele: OR 1.77; 95% CI 1.07-2.94; P=0.025) [19]. At the same time, male-carriers of the *CT* haplotype rs2070744 and rs1799983 had the significantly increased risk of POAG (OR 2.60; 95% CI 1.16-5.82; P=0.016). In our study carriers of this haplotype had the significantly increased POAG risk developing (OR 14.15; 95% CI 1.88-106.28; P<0.001).

According to the meta-analysis the *TT* rs2070744 and *GG* rs1799983 genotypes are associated with the reduced POAG risk; it is more common in women [20]. Our investigation also showed the protective role of the *GG-TT* haplotype rs1799983 and rs2070744, which had the lowest probability of POAG developing ($P_{(POAG)}$=0.006; see table. 4).

Examining POAG patients (76 men and 84 women aged 41 to 75 years) showed the disease-related association for the *CC* genotype rs2070744 (OR 2.54; 95% CI 1.26-5.13; P=0.007), it was preserved for women when it was stratified by gender [11].

Unlike other investigations, the association of POAG with rs1799983 was not shown [11]. However, the lower total nitrate/nitrite (NOx) content was found in the blood plasma of POAG patients confirming the importance of endothelial dysfunction for the development of the disease [11].

The significance of the rs2070744 and rs1799983 polymorphisms of the *NOS*3 gene for POAG development was confirmed by meta-analysis in 2021. It covered the SID, MagIran, IranMedex, IranDoc, ScienceDirect, Embase, Scopus, PubMed, Web of Science and Google Scholar search engines with no time limit until May 2020 [10].

According to the results of 16 studies (1631 patients with POAG and 2405 controls for rs2070744 and 1456 patients and 2240 controls for rs1799983) the increased risk of POAG was found in carriers of *CC* genotypes rs2070744 (total OR 1.14) and *TT* rs1799983 (total OR 1.31). It fully coincides with the results of our investigation.

Thus, the results of the investigation are consistent with ones obtained for other patient populations and prove the importance of risk genotypes (*TT* rs1799983 and *CC* rs2070744) for the POAG development. In addition, we have shown that there is the significant increase in the POAG risk for carriers of the *GT-CC* haplotypes (OR 4.39) and *TT-CC* (OR 14.15).

CONCLUSIONS

1. Regression analysis confirmed the influence of polymorphisms rs1799983 and rs2070744 of the *NOS3* gene on the development of POAG in patients from the Ukrainian population. At the same time, rs2070744 had a greater influence on the probability of the disease developing.
2. The possibility of constructing an adequate regression model of POAG genetic risk proved the presence of rs1799983 and rs2070744 association with the disease (P<0.001).
3. Haplotypes rs1799983-rs2070744 *GT-CC* and *TT-CC* carriers had the highest probability of POAG developing, which may be the basis for the detection of genetic predisposition in the prehospital stage.

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