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The role of *NOS3* (rs2070744) and *GNB3* (rs5443) genes' polymorphisms in endothelial dysfunction pathway and carotid intima-media thickness in hypertensive patients

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Abstract. The mechanisms orchestrating the balance between nitric oxide and endothelium-derived contracting factors, and genetic predisposition to endothelial dysfunction in hypertensive patients remain to be determined. One-hundred hypertensive patients participated in the case-control study to clarify the risk of endothelial dysfunction and carotid "intima media" thickness (IMT) changes depending on *NOS3* (rs2070744) and *GNB3* (rs5443) genes' polymorphisms. It is found that presence of *NOS3* gene's *C*-allele significantly elevates the risk of atherosclerotic plaques on carotid arteries (OR95%CI: 1.24–11.20; $p = 0.019$) and the probability of low *NOS3* gene expression (OR95%CI: 17.72–520.0; $p < 0.001$). Homozygous carriage of *C*-allele of *GNB3* gene is protective and corresponds to the lowest chances of the carotid IMT increase, atherosclerotic plaques formation and sVCAM-1 elevation (OR = 0.10–0.34; OR95%CI: 0.03–0.95; $p \leq 0.035$–0.001). *Vice versa*, *T*-allele of *GNB3* gene significantly augments the risk of the carotid IMT increase (OR95%CI: 1.09–7.74; $p = 0.027$) including development of atherosclerotic plaques, associating *GNB3* (rs5443) with cardiovascular pathology.

Key words: Endothelial dysfunction — Hypertension risk — *NOS3* (rs2070744), *GNB3* (rs5443) genes' polymorphism

Abbreviations: BP, blood pressure; CCA, common carotid artery; CV, cardiovascular; EAH, essential arterial hypertension; FMD BA, flow-mediated dilation of the brachial artery; *GNB3* (rs5443), genetic variation of guanine nucleotide-binding protein beta-3; HMOD, hypertensive-mediated organ damage; ICA, internal carotid artery; IMT, "intima media" thickness; *NOS3* (rs2070744), genetic variation of endothelial nitric oxide synthase; NO_2–/NO_3–, total monoxide nitrogen metabolites; sVCAM-1, soluble vascular cell adhesion molecule; SBP/DBP, systolic/diastolic blood pressure.

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Introduction

Arterial hypertension remains a growing worldwide epidemic and an important challenge for a healthcare in most countries (Williams et al. 2018; Visseren et al. 2021). A fundamental role in the pathogenesis of essential arterial hypertension (EAH) is attributed to endothelial dysfunction associated with impaired production of monoxide nitrogen (NO). A healthy inert endothelium permanently releases potent vasodilators or constrictors in response to blood flow change to sustain a proper vascular resistance (Brandes 2014). On the other hand, the attenuated endothelium-dependent vasodilation may result with endothelial dysfunction followed by activation of endothelial inflammation, peripheral vasoconstriction and blood pressure (BP) elevation as well. *Vice versa*, normalization of endothelial function does not inevitably affect BP. In some studies, endothelial dysfunction correlates with vascular stiffness not only in terms of systemic vascular aging, but it relates to pulse pressure and pulse wave velocity (Duprez et al. 2013). Endothelium also mediates processes of vascular remodelling and inflammation *via* such inflammatory cytokines as TNF-α, IL-β, IL-6, etc. (De Ciuceis et al. 2005). Endothelial dysfunction induced by TNF-α is partially modulated by insufficient endothelial nitric oxide synthase (eNOS) mRNA activity *via* miR-155. While miR-155 is induced by TNF-α (Sun et al. 2012), chronically hyperactivated Renin-angiotensin-aldosterone system (RAAS) with depressed signal transducer and activator of transcription 3 (STAT3) system induce vascular dysfunction through the signalling pathway from the endothelium-derived contracting factors (EDCF), like angiotensin II (AGTII), endotelin-1, thromboxane A2 and especially cyclooxygenase-derived prostanoids and superoxide anions as well, to Nox induction and oxidative stress (Johnson et al. 2013; Virdis et al. 2013; Incalza et al. 2018; Bruder-Nascimento et al. 2020; Jiang et al. 2020). EDCF potentiate inflammation and increase formation of vascular reactive oxygen species (ROS), with oxygen free radicals release, activates Nox NADPH oxidases, adhesion molecules release and promotes generation of hydrogen peroxide (H_2O_2) sufficient to attenuate endothelial NO release (Takac et al. 2012; Virdis et al. 2013; Carrizzo et al. 2020). Soluble vascular cell adhesion molecule-1 (sVCAM-1) promotes inflammatory cells adhesion to the vascular endothelium and facilitates its migration through the endothelium membrane (Dörr et al. 2012; Tchalla et al. 2015, 2018). Some evidence prove that these processes might be at least partially genetically determined by genes regulating RAAS or NO activity or code the enzymes' expression or synthesis, or associate with hypertrophy and remodeling of vascular smooth muscles (Sydorchuk et al. 2013, 2015, 2020a; Ji et al. 2017; Pimphen Charoen et al. 2019; Smyth et al. 2019; Dzhuryak et al. 2020; Tegegne et al. 2020; Repchuk et al. 2021; Semianiv et al. 2021). However, the mechanisms that orchestrate the balance between NO and EDCF, genetic predisposition to endothelial dysfunction in hypertensive patients still remain to be determined. Moreover, the pathogenetic linkages transforming the protective endothelium to a source of inflammatory, adhesive, pro-apoptosis mediators and vasoconstrictor, diminishing the NO-dependent vasodilation, requires further research, too.

In this regard, the objective of our study was to clarify the risk of endothelial dysfunction and carotid "intima media" thickness (IMT) changes depending on guanine nucleotide-binding protein beta-3 (*GNB3*, rs5443) and endothelial nitric oxide synthase (*NOS3*, rs2070744) genes polymorphisms in EAH patients.

Material and Methods

Compliance with bioethics

The Study fully adhered to European Convention on Human Rights and Biomedicine, GCP, GLP principles, EUC directive #609 and other EU and international legislations on bioethics. The Study Protocol was approved by the Ethics' Committee of the Bukovinian State Medical University (Protocol No. 2 from 10.10.2019), the written consent was obtained from all patients. The research is defined as prospective, case-control study.

Inclusion/exclusion criteria

The study included EAH patients with hypertensive-mediated organ damage (HMOD) estimated according to European Societies of Hypertension and Cardiology recommendations (ESH/ESC 2018): target-organs damage – 2nd stage (asymptomatic EAH), from the 1st through to the 3rd grade of BP elevation; moderate-high cardiovascular (CV) risk; aged from 45 to 65 years.

We excluded patients with EAH stage 3 (established CV disease, chronic kidney diseases (CKD) – with estimated glomerular filtration rate (eGFR) less than 30 ml/min/1.73 m^2); secondary arterial hypertension; chronic heart failure higher than II functional class (NYHA III–IV); diabetes mellitus type 1 (DM1), sub- and decompensated DM2 (with diabetic target-organ damage); malignant or uncontrolled arterial hypertension; sub- and decompensated liver diseases (triple growth over the normal level of aspartate aminotransferase, alanine aminotransferase); bronchial asthma, chronic obstructive pulmonary disease of III–IV stage with C or D risk value (GOLD 2019); exacerbated infectious diseases or during unstable remission; psychological disorders; malignancies of any location; administration of oral corticosteroids or contraceptives; pregnancy or lactation.

After screening of matching inclusion and exclusion criteria, 100 patients were selected for further examination (75% women, 25% men, mean age 59.87 ± 7.98 years). The genetic examination was performed in 72 patients. The control group included 48 practically healthy individuals who were not relatives of the patients and without reliable differences in gender distribution (62.5% females, 37.5% males) and mean age (49.13 ± 6.28 years) with the study group. All enrolled subjects signed a consent form to participate in the study.

Essential arterial hypertension assessment

Hypertension was defined as office systolic BP (SBP) values ≥140 mmHg and/or diastolic BP (DBP) values ≥90 mmHg at least for three measurements during a month, according to European Societies of Hypertension and Cardiology (ESH/ESC) recommendations (Bryan et al. 2018; Visseren et al. 2021).

All enrolled patients underwent a complex of examinations: general clinical examinations, complete blood count, creatinine, glucose, total cholesterol (TC) level, triglycerides (TG) and low/high density level cholesterol (LDL-C, HDL-C), Atherogenicity Index (AI = (TC – HDL-C)/HDL-C, Unit), body mass index (BMI, kg/m^2) for evaluation of overweight and abdominal obesity (AO), Waist-to-Hip ratio (WHR), office measurement of SBP, DBP, heart rate (HR), GFR calculation (according to CKD Epidemiology Collaboration (CKD-EPI) equation with creatinine level), ECG in 12 leads, EchoCG, consultations of ophthalmologist and neurologist according to European recommendations (ESC 2018, 2021).

Markers of endothelial function

The serum level of soluble vascular cell adhesion molecule (sVCAM-1; CD 106) was determined in enzyme linked-immuno-sorbent assay (ELISA) according to the Manufacturer's Guidelines with highly sensitive sVCAM-1 ELISA KIT® (Diaclon SAS, France) on a "StatFax 303" equipment (USA). The sVCAM-1 assay has a sensitivity of 0.6 ng/ml.

The monoxide nitrogen metabolites (NO_{2-}/NO_{3-}) concentration was evaluated in serum, stabilized with EDTA (1 mg/ml), by colorimetric method with Total NO/ NO_{2-}/ NO_{3-}Assay Kit (RDS, UK) on a 550 nm spectrophotometer (TS8210, China). Detection principle: after recovery of nitrate to nitrite by nitrate-reductase enzyme nitrite reacts with chromogenic agent producing light red azo-compound and the content of nitrite can be calculated by measuring the OD value at 550 nm.

Transcriptional activity of NOS3 gene in the peripheral blood was validated by pathway-specific PCR array with Maxima SYBR Green/ROX qPCR MasterMix (2X) set (ThermoScientific™, USA). Total RNA was isolated from blood leucocytes using NucleoZOL (Macherey-Nagel, Germany) according to the Manufacturer's guideline. The quality and value of the isolated total RNA was determined using NanoDrop spectrophotometer (Thermo Scientific™, USA). The purified RNA underwent reverse transcription (RT) with a cDNA conversion RT² Strand Kit (OT-1, Syntol™, RF). RNA samples within the range of –2.0–2.2 were selected. Amplification was performed on samples in triplicate. Samples were assigned to both groups: control and study. Quantity detection of mRNA performed by calculating the relative normalized amount of cDNA of the *NOS3* gene when the control group data were taken as "1" and the data of the study/test group were determined relative to the control group. Relative cDNA values were normalized and based on ΔΔCt method with the reference gene *GAPDH*. The data for analysis was uploaded to the GeneGlobe portal and computed fold change/expression using delta-delta Ct method (ΔΔCt) as: ΔCt study/test group (ΔCt *NOS3* gene) – delta Ct control group (ΔCt average of *GAPDH* reference gene) with following calculation of fold change using $2^{-\Delta\Delta Ct}$ equation.

Statistical analysis of PCR array data: RT2 Profiler PCR Array Data Analysis software does not perform any statistical analysis beyond the calculation of *p*-values using a Student's *t*-test (two-tail distribution and equal variances between the two samples) based on the triplicate $2^{-\Delta\Delta Ct}$ values of *NOS3* gene between patients and the control group. The Microarray Quality Control indicated that sample numbers and *p*-value calculation was sufficient to demonstrate reproducible results across microarrays and PCR arrays, including the RT2 Profiler PCR arrays.

Carotid intima-media thickness and endothelium-dependent flow-mediated dilation of the brachial artery ultrasound assessment

Endothelial-dependent flow-mediated dilation of the brachial artery (FMD BA) measurement according to the FMD assessment Guidelines (Corretti et al. 2002; Thijssen et al. 2019; Maruhashi et al. 2020, Holder et al. 2021) using ultrasonographic complex "ACCUVIX A30" (Samsung Medison, South Korea) with duplex scanning of brachial arteries (BA), high-frequency vascular transducer, color and spectral Doppler and an internal ECG monitor. A blood pressure cuff was applied to the forearm and inflated to a pressure that was 50 mmHg above the baseline SBP for 5 min. From 30 s before to 2 min after cuff deflation, the BA diameter was recorded on ultrasound. Cuff deflation induces a reactive hyperaemia – a brief high-flow through the brachial artery to accommodate the dilated resistance vessels. An increase of internal BA diameter was expressed in the percentage of the baseline BA diameter. The diameter increase less than 10% was determined as endothelial dysfunction or FMD insufficiency.

Carotid *intima-media* thickness (C-IMT) and carotid plaque, including mean-maximal or composite IMT measures of common carotid artery (CCA) and internal carotid artery (ICA) from both sides in a region free of plaque was assessed on ultrasound complex "ACCUVIX A30" (Samsung Medison, South Korea) in B-mode regime with high-frequency vascular transducer, color and spectral Doppler and an internal ECG monitor according to the Mannheim CIMT 2012 Consensus Report and ESC 2021 Recommendation (Den Ruijter et al. 2012; Touboul et al. 2012; Ravani et al. 2015; Kabłak-Ziembicka and Przewłocki 2021; Visseren et al. 2021). An upper limit of 0.9 mm has been used as a cut off value that denotes an increased IMT. Plaque was defined as the presence of a focal wall thickening that is ≥50% greater than the surrounding vessel wall, or as a focal region with an IMT measurement ≥1.5 mm that protrudes into the lumen (ESC 2021).

Genotyping of the endothelial nitric oxide synthase (NOS3, rs2070744) and guanine nucleotide-binding protein beta-3 (GNB3, rs5443) gene polymorphisms; DNA isolation, amplification and genotyping

Venous blood was collected in a sterile vacutainer, stabilized by K2-EDTA. DNA was isolated from the whole venous blood lymphocytes' nuclei and purified according to GeneJET Genomic DNA Purification Kit Manufacturer's Guidance (Thermo Fisher Scientific, USA). DNA fragments of analyzed genes amplified by qRT-PCR with specific for each gene TaqMan probes and genotyping with TaqMan Genotyping Master Mix on CFX96 Touch™ RT-PCR Detection System (Bio-Rad Laboratories, Inc., USA). The genotyping protocol was described in our previous publications (Dzhuryak et al. 2020; Sydorchuk et al. 2020a, 2020b; Repchuk et al. 2021; Semianiv et al. 2021). Alleles' discrimination of *NOS3* (rs2070744) and *GNB3* (rs*5443*) gene polymorphisms was analyzed by licensed CFX96 RT-PCR Detection System Software (Microsoft, USA).

Statistical analysis

Statistical analysis performed using StatSoft Statistica v.7.0 software (StatSoft Inc., USA). We used Pearson's criterion (χ^2) for the genotype distribution comparison. Analysis of qualitative data (categorical variables), risk of pathology severity was assessed by a binary logistic regression model using relative risk (RelR); risk ratio (RR) was estimated by odds ratio (OR) with 95% confidence interval (95% CI) using a chi-square test (χ^2) (df = 1). Differences were regarded as significant at $p < 0.05$ values.

Results

Endothelial dysfunction parameters, carotid IMT and atherosclerotic plaques depending on EAH severity (after BP value)

Table 1. Frequency of endothelial dysfunction biomarkers, carotid "intima-media" thickness (IMT) and signs of atherosclerosis depending on the hypertension severity

		Patients in total *n* (%)	Hypertensive patients *n* (%) SBP/DBP (mmHg)		c^2	*p*
			<160/<100	≥160/≥100		
FMD BA	N	5 (6.94)	5 (11.90)	0		0.061
	↓(<10.0 %)	67 (93.06)	37 (88.09)	30 (100.0)		
Carotid IMT	N	29 (40.28)	22 (52.38)	7 (23.33)	6.14	0.013
	↑(>0.9 mm)	43 (59.72)	20 (47.62)	23 (76.67)		
Atherosclerotic plaques on the CCA	Unilateral	52 (72.22)	26 (61.90)	26 (86.67)	5.35	0.021
	Bilateral	46 (63.89)	22 (52.38)	24 (80.0)	5.79	0.016
NO_{2-}/NO_{3-}	N	5 (6.94)	4 (9.52)	1 (3.33)		>0.05
	↓(<25.0 μmol/l)	67 (93.06)	38 (90.48)	29 (96.67)		
sVCAM-1	N	20 (27.78)	10 (23.81)	10 (33.33)	1.12	>0.05
	↑(>1050 ng/ml)	52 (72.22)	32 (76.19)	20 (66.67)		
Transcriptional activity of *NOS3* gene after mRNA	≥0.5 U	21 (29.17)	16 (38.09)	5 (16.67)	3.89	0.049
	<0.5 U	51 (70.83)	26 (61.91)	25 (83.33)		
Number of patients		72	42	30		

FMD BA, flow-mediated dilation of the brachial artery; IMT CCA, ICA, *intima-media* thickness of common carotid artery, internal carotid artery; SBP, DBP, systolic, diastolic blood pressure; NO_{2-}/NO_{3-}, total nitrite/total nitrate; sVCAM-1/CD 106, soluble vascular cell adhesion molecule; *p*, significance of differences between groups with various BP values. N, number of patients with normal values; ↓ number of patients with lowered values; ↑ number of patients with increased values.

Table 2. Risk of severe arterial hypertension course (SBP≥160/DBP≥100 mmHg) depending on endothelial dysfunction biomarkers, carotid "intima-media" thickness, signs of atherosclerosis and *NOS3* gene transcriptional activity

		RR	RR 95%CI	OR	OR 95%CI	*p*
↓ FMD BA (<10.0%)		1.10	0.96–1.25	3.92	0.43–35.42	>0.05
↑ Carotid IMT (>0.9 mm)		1.61	1.11–2.34	3.61	1.28–10.23	0.012
Atherosclerotic plaques in the CCA	Unilateral	1.40	1.06–1.84	4.0	1.18–13.59	0.018
	Bilateral	1.53	1.09–2.14	3.64	1.23–10.71	0.014
↓ Total NO metabolites (NO_2–/NO_3–), (<25 μmol/l)		1.06	0.95–1.20	3.05	0.32–28.79	>0.05
↑ sVCAM-1 (>1050 ng/ml)		0.88	0.65–1.19	0.63	0.22–1.77	>0.05
↓ *NOS3* gene expression after mRNA (<0.5 U)		1.35	1.01–1.79	3.08	1.0–9.66	0.042

For abbreviations, see Table 1.

are presented in Table 1. Endothelial dysfunction after FMD BA and total NO metabolites decrease were found in 93% of patients ($n = 67$) and did not depend on the BP value. Whereas, an increased CCA IMT and number of atherosclerotic plaques with uni- and/or bilateral location prevailed among patients with more severe EAH course (SBP≥160/DPB≥100 mmHg) by 29.05% ($c^2 = 6.14$; $p = 0.013$), 24.77% ($c^2 = 5.35$; $p = 0.021$) and 27.62% ($c^2 = 5.79$; $p = 0.016$), respectively, high sVCAM-1 values (above reference >1050 ng/ml) were found in 72.22% patients ($n = 52$), but it did not depend on BP level ($p > 0.05$). The frequency of subjects with lowered *NOS3* gene transcriptional activity after the mRNA level (<0.5 U) prevailed among patients suffering from EAH with more severe course of the disease by 21.42% ($c^2 = 3.89$; $p = 0.049$).

The risk of more severe EAH course (SBP≥160/DPB≥100 mmHg) is associated with an increase of carotid IMT (>0.9 mm) more than 3.5 times (OR 95%CI: 1.28–10.23; $p = 0.012$), unilateral occurrence of atherosclerotic plaques in CCA – 4 times as much (OR 95%CI: 1.18–13.59; $p = 0.018$), and on bilateral – over 3.5 times (OR 95%CI: 1.23–10.71; $p = 0.014$); and decreased *NOS3* gene expression after the level of mRNA (<0.5 U) – 3 times as much (OR 95%CI: 1.0–9.66; $p = 0.042$), respectively (Table 2).

Insomuch as the total risk of severe EAH course (after BP values) did not depend on distinguished endothelial dysfunction biomarkers, the cohort of patients was divided according to endothelial dysfunction severity considering FMD BA deviations, total NO metabolites decrease and sVCAM-1 concentration elevation (Table 3). Mild endothelial dysfunction grade (1st degree) was regarded by reduced FMD BA below the upper quartile (percentile) of the patient's group (10.0–8.0%) and total NO metabolites (25–21 μmol/l) as well, with normal sVCAM-1 level (<1050 ng/ml); Moderate endothelial dysfunction grade (2nd degree) – decrease of the examined variables within the interquartile range (Q2): FMD BA – 7.9–7.0%, NO_2/NO_3 – 20.9–18.0 μmol/l, with increased sVCAM-1 value of 1050–1390 ng/ml; Severe endothelial dysfunction grade (3rd degree) was extrapolated by FMD BA and total NO metabolites decrease below the lower quartile (Q1): FMD BA < 7.0%, NO_2/NO_3 < 18.0 μmol/l, and an increase of sVCAM-1 >1390 ng/ml.

Moderate and severe endothelial dysfunction grades (2nd, 3rd degrees) increase the risk of severe EAH course more than 3 and 5.5 times (OR 95%CI: 1.13–9.34; $p = 0.025$ and OR 95%CI: 1.96–14.45; $p < 0.001$), respectively (Table 3).

The endothelial dysfunction markers rate analysis, carotid IMT and signs of atherosclerosis depending on *NOS3* (rs2070744) gene's polymorphism proved that in the *C*-allele carriers of NOS3 gene more often the total NO metabolites

Table 3. Risk of severe arterial hypertension course (SBP/DBP ≥160/≥100 mmHg) depending on endothelial dysfunction severity grade

Endothelial dysfunction	FMD BA (%)	Total NO metabolites (μmol/l)	sVCAM-1 (ng/ml)	RR	RR 95%CI	OR	OR 95%CI	*p*
Mild	10.0–8.0	25–21	<1050	0.70	0.27–1.84	0.64	0.19–2.11	>0.05
Moderate	7.9–7.0	20.9–18.0l	1050–1390	2.27	1.08–4.80	3.25	1.13–9.34	0.025
Severe	<7.0	<18	>1390	2.20	1.36–3.55	5.50	1.96–14.45	<0.001

For abbreviations, see Table 1.

Table 4. Frequency of endothelial dysfunction, carotid "intima-media" thickness increase and signs of atherosclerosis depending on the polymorphic variants of *NOS3* (rs2070744) gene

		Genotypes of *NOS3* gene *n* (%)		c^2	*p*
		TT-	*TC*-, *CC*-		
FMD BA	N	3 (14.29)	2 (3.92)	<1.0	>0.05
	↓(<10.0%)	18 (85.71)	49 (96.08)		
IMT CCA	N	9 (42.86)	20 (39.22)	<1.0	>0.05
	↑ (>0.9 mm)	12 (57.14)	31 (60.78)		
Atherosclerotic plaques on the CCA	Unilateral	11 (52.38)	41 (80.39)	5.82	0.016
	Bilateral	9 (42.86)	37 (72.55)	5.68	0.017
Total NO metabolites (NO_{2-}/NO_{3-})	N	4 (19.05)	1 (1.96)	–	0.023
	↓ (<25 μmol/l)	17 (80.95)	50 (98.04)		
sVCAM-1	N	10 (47.62)	10 (19.61)	5.82	0.016
	↑ (>1050 ng/ml)	11 (52.38)	41 (80.39)		
NOS3 gene expression after mRNA	≥0.5 U	18 (85.71)	3 (5.88)	45.89	<0.001
	<0.5 U	3 (14.29)	48 (94.12)		
n		21	51		

p, significance of differences between groups. For abbreviations, see Table 1.

decrease and sVCAM-1 elevation was observed by 17.09% ($p = 0.023$) and 28.01% ($c^2 = 5.82$; $p = 0.016$), respectively; with lack of such dependence after FMD BA (Table 4). Atherosclerotic plaques were found more frequently in *C*-allele carriers as well: with unilateral location by 28.01% ($c^2 = 5.82$; $p = 0.016$), bilateral by 29.69% ($c^2 = 5.68$; $p = 0.017$), respectively. Moreover, individuals with decreased *NOS3* gene expression by the mRNA level were found more often in the *C*-allele carriers of *NOS3* (rs2070744) gene, than among those with *TT*-genotype by 79.87% ($c^2 = 45.89$; $p < 0.001$).

C-allele of *NOS3* gene (786*T*>*C*) increases the risk of CCA atherosclerotic uni- and bilateral plaques by over 3.5 times (OR = 3.73; OR 95%CI: 1.24–11.20; $p = 0.019$ and OR = 3.52; OR 95%CI: 1.22–10.18; $p = 0.018$), endothelial dysfunction with decreased total NO metabolites (<25 μmol/l) and elevated sVCAM-1 value (>1050 ng/ml) almost 12 and 4 times (OR = 11.77; OR 95%CI: 1.23–112.7; $p = 0.023$ and

Table 5. Risk of endothelial dysfunction, carotid "*intima–media*" thickness increase and atherosclerotic plaques depending on polymorphic variants of *NOS3* (rs2070744) gene

		RR	RR 95%CI	OR	OR 95%CI	*p*
C–allele of *NOS3* (786*T*>*C*, rs2070744) gene						
↓ FMD BA (<10.0 %)		1.12	0.93–1.35	4.08	0.63–26.47	>0.05
↑ IMT CCA (>0.9 mm)		1.06	0.69–1.64	1.16	0.41–3.26	>0.05
Atherosclerotic plaques in CCA	Unilateral	1.53	1.0–2.36	3.73	1.24–11.20	0.019
	Bilateral	1.69	1.0–2.85	3.52	1.22–10.18	0.018
↓ Total NO metabolites (<25 μmol/l)		1.21	0.98–1.50	11.77	1.23–112.7	0.023
↑ sVCAM-1 (>1050 ng/ml)		1.53	1.0–2.36	3.73	1.24–11.20	0.019
↓ *NOS3* gene expression after mRNA (<0.5 U)		6.59	2.31–18.82	69.0	17.72–520	<0.001
TT–genotype of *NOS3* (786*T*>*C*, rs2070744) gene						
↓ FMD BA (<10.0 %)		0.89	0.74–1.07	0.24	0.04–1.59	>0.05
↑ IMT CCA (>0.9 mm)		0.94	0.61–1.45	0.86	0.31–2.41	>0.05
Atherosclerotic plaques on the CCA	Unilateral	0.65	0.42–1.0	0.27	0.09–0.81	0.016
	Bilateral	0.59	0.35–0.99	0.28	0.10–0.82	0.017
↓ Total NO metabolites (<25 μmol/l)		0.83	0.67–1.02	0.09	0.01–0.81	0.023
↑ sVCAM-1 (>1050 ng/ml)		0.65	0.42–1.0	0.27	0.09–0.81	0.016
↓ *NOS3* gene expression after mRNA (<0.5 U)		0.15	0.05–0.43	0.01	0.002–0.06	<0.001

For abbreviations, see Table 1.

OR = 3.73; OR 95%CI: 1.24–11.20; $p = 0.019$), respectively (Table 5). The likelihood of low *NOS3* gene expression by the mRNA level (<0.5 U) increases 69 times (OR 95%CI: 17.72–520.0; $p < 0.001$) in *C*-allele carriers of *NOS3* gene. On the contrary, *TT*-genotype shows protective properties concerning atherosclerosis, endothelial dysfunction markers deviation and decrease of *NOS3* gene expression (OR = 0.01–0.28; OR 95%CI: 0.002–0.82; $p \leq 0.023$–0.001).

sVCAM-1 as a marker of endothelial dysfunction and inflammation was found increased >1050 ng/ml in EAH patients with *T*-allele of *GNB3* (rs5443) gene by 22.22% ($c^2 = 4.43$; $p = 0.035$) (Table 6). While the rest of the endothelial dysfunction parameters (FMD BA, total NO metabolites), as well as the *NOS3* gene transcriptional activity by the mRNA level, did not demonstrate clear dependence on allele state of *GNB3* (rs5443) gene. It should be noted that structural changes of the CCA with increased IMT and atherosclerotic plaques were more often found in the *T*-allele carriers of *GNB3* gene by 25.0% ($c^2 = 4.68$; $p = 0.03$), 38.89% ($c^2 = 13.57$; $p < 0.001$) and 33.34% ($c^2 = 8.67$; $p = 0.003$), respectively.

Logistic regression analysis indicated that the minor *T*-allele of *GNB3* (825*C*>*T*) gene increases the risk of structural changes of the examined carotid arteries walls in EAH patients: likelihood of CCA IMT >0.9 mm augments almost 3 times (OR 95%CI:1.09–7.74; $p = 0.027$), atherosclerotic plaques on the CCA on one or both sides – practically 10 and 5 times as much (OR 95%CI: 2.55–38.0; $p < 0,001$ and OR 95%CI: 1.61–13.27; $p = 0.003$), respectively (Table 7). Moreover, the chances of high sVCAM-1 (>1050 ng/ml) grew over 3 times (OR 95%CI: 1.06–9.59; $p = 0.032$).

On the contrary, homozygous carriage of *C*-allele of *GNB3* (rs5443) gene is protective and corresponds to the lowest chances in the examined population for the structural elements of the vascular wall changes occurrence (carotid IMT increase), atherosclerotic plaques appearance and sVCAM-1 value elevation (OR = 0.10–0.34; OR 95%CI: 0.03–0.95; $p \leq 0.035$–0.001) (Table 7).

Discussion

Our study was aimed to clarify the risk of endothelial dysfunction and carotid IMT changes depending on guanine nucleotide-binding protein beta-3 (*GNB3*, rs5443) and endothelial nitric oxide synthase (*NOS3*, rs2070744) gene polymorphisms in EAH patients. It is widely accepted that healthy endothelium integrates multi-faceted pathophysiological processes, including endocrine function, metabolism, tissue remodeling, repair, coagulation, inflammation, apoptosis, etc. On the other hand, endothelial dysfunction is the initial step in the pathogenesis of acute and chronic vascular insufficiency, especially atherosclerosis (Incalza et al. 2018; Evans et al. 2021; Hudson and Farkas 2021). However, there is limited evidence related to endothelial dysfunction and genetic predisposition or epigenetic peculiarities in hypertension, coronary artery disease, chronic kidney disease or atherosclerosis as well (Zietzer et al. 2020; Ghafouri-Fard et al. 2021; Hosen et al. 2021; Wu et al. 2021).

The healthy vascular endothelium exerts atheroprotective actions through vasoactive mediators such as NO (Fig. 1). There is evidence that as the endothelium ages, it is exposed to the damaging effects of environmental factors (hypertension, tobacco consumption, dyslipidemia, diabetes mellitus) and diminishing of these protective properties lead to a state

Table 6. Frequency of endothelial dysfunction, carotid "*intima-media*" thickness increase and signs of atherosclerosis depending on the polymorphic variants of *GNB3* (rs5443) gene

		Genotypes of *GNB3* *n*(%)		c^2	*p*
		CC-	*CT*-, *TT*-		
FMD BA	N	3 (8.33)	2 (5.55)	<1.0	>0.05
	↓(<10.0%)	33 (91.67)	34 (94.44)		
IMT CCA	N	19 (52.78)	10 (27.78)	4.68	0.03
	↑ (>0.9 mm)	17 (47.22)	26 (72.22)		
Atherosclerotic plaques on the CCA	Unilateral	19 (52.78)	33 (91.67)	13.57	<0.001
	Bilateral	17 (47.22)	29 (80.56)	8.67	0.003
Total NO metabolites (NO_{2-}/NO_{3-})	N	4 (11.11)	1 (2.78)	<1.0	>0.05
	↓ (<25 μmol/l)	32 (88.89)	35 (97.22)		
sVCAM-1	N	14 (38.89)	6 (16.67)	4.43	0.035
	↑ (>1050 ng/ml)	22 (61.11)	30 (83.33)		
NOS3 gene expression after mRNA	≥0.5 U	9 (25.0)	12 (33.33)	<1.0	>0.05
	<0.5 U	27 (75.0)	24 (66.67)		
n		36	36		

p, significance of differences between groups. For abbreviations, see Table 1.

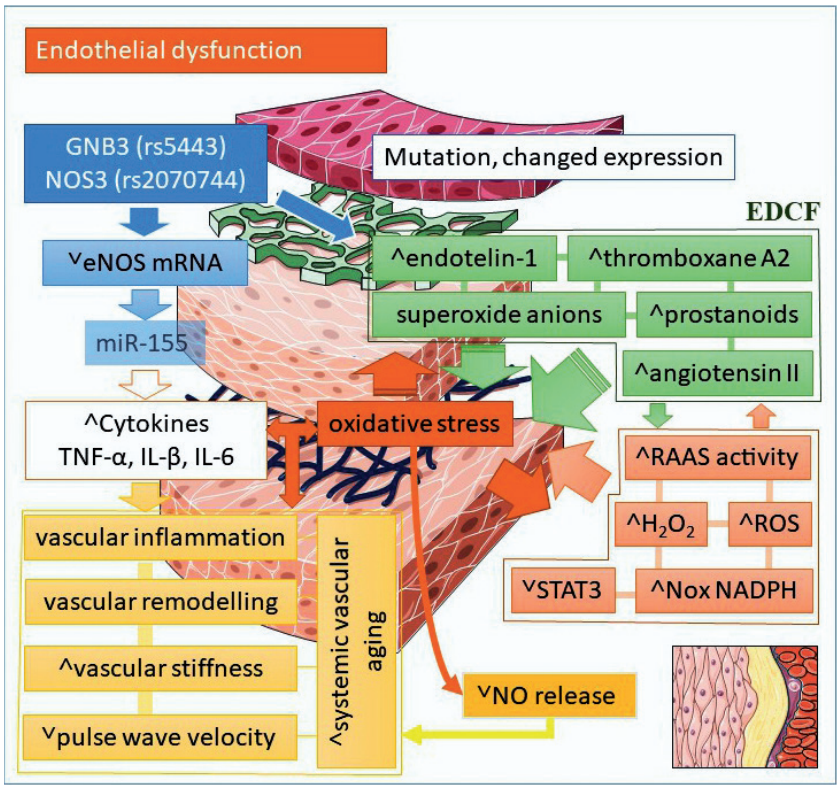

Figure 1. Scheme of interactions and pathways between different molecules, messengers and genes, and their pathogenetic effects in healthy vascular endothelium. EDCF, endothelium-derived contracting factors; ^, growth of; ᵛ, reduction of.

of endothelial dysfunction. Endothelial dysfunction closely correlates with CV risk factors, and may be predictive of incident cardiovascular events. There have been few studies evaluating the heritability of endothelial function, but they have highlighted the genes' role or epigenetic linkage with endothelial dysfunction, pro-inflammatory pathways and the hypertension progression. It was shown that endothelial dysfunction was present in chronic heart failure patients

Table 7. Risk of endothelial dysfunction, carotid "intima–media" thickness increase and atherosclerotic plaques depending on polymorphic variants of *GNB3* (rs5443) gene

		RR	RR 95%CI	OR	OR 95%CI	*p*
T–allele of *GNB3* (825*C*>*T*, rs5443) gene						
↓ FMD BA (<10.0 %)		1.03	0.91–1.17	1.54	0.24–9.85	>0.05
↑ IMT CCA (>0.9 mm)		1.53	1.02–2.28	2.91	1.09–7.74	0.027
Atherosclerotic plaques on the CCA	Unilateral	1.74	1.26–2.40	9.84	2.55–38.0	<0.001
	Bilateral	1.71	1.17–2.50	4.63	1.61–13.27	0.003
↓ Total NO metabolites (<25 μmol/l)		1.09	0.96–1.24	4.37	0.46–41.23	>0.05
↑ sVCAM-1 (>1050 ng/ml)		1.36	1.01–1.84	3.18	1.06–9.59	0.032
↓ *NOS3* gene expression after mRNA (<0.5 U)		0.89	0.66–1.20	0.67	0.24–1.86	>0.05
CC–genotype of *GNB3* (825*C*>*T*, rs5443) gene						
↓ FMD BA (<10.0 %)		0.97	0.86–1.10	0.65	0.10–4.12	>0.05
↑ IMT CCA (>0.9 mm)		0.65	0.44–0.98	0.34	0.13–0.92	0.03
Atherosclerotic plaques on the CCA	Unilateral	0.58	0.42–0.80	0.10	0.03–0.39	<0.001
	Bilateral	0.59	0.40–0.86	0.22	0.08–0.62	0.006
↓ Total NO metabolites (<25 μmol/l)		0.91	0.80–1.04	0.23	0.02–2.15	>0.05
↑ sVCAM- (>1050 ng/ml)		0.73	0.54–0.99	0.31	0.10–0.95	0.035
↓ *NOS3* gene expression after mRNA (<0.5 U)		1.12	0.83–1.52	1.50	0.54–4.18	>0.05

For abbreviations, see Table 1.

and that was partially predicted by ecNOS and AT1R genes' polymorphisms. Moreover, authors urged further investigations to better understand the molecular mechanisms and the clinical implications, particularly to assess whether patients with specific gene polymorphisms would benefit from a more aggressive medical and interventional management (Kose et al. 2014). Individual differences in endothelial function and hence, susceptibility to atherosclerosis, might relate not only to different levels of exposure to risk factors but also to inter-individual differences in the carriage of risk alleles of genes expressed in the vascular endothelium.

Our study revealed that *C*-allele of *NOS3* (rs2070744) gene significantly elevates the risk of atherosclerotic plaques on carotid arteries and the probability of low *NOS3* gene expression, whereas enhancing endothelial dysfunction by both decreasing NO metabolites and growth of sVCAM-1 which promotes inflammatory cells adhesion to the vascular endothelium and facilitates its migration through the endothelial membrane (Tchalla et al. 2018). These findings significantly expand existing understanding of the role of genetic factors in endothelial dysfunction in atherosclerosis development. Presence of *C*-allele may be considered as an independent and reliable risk factor for both atherosclerosis and endothelial dysfunction.

It is interesting to compare obtained results with data of meta-analysis aimed on other genes that may be possibly involved into development of atherosclerosis and endothelial dysfunction, like VEGF (rs699947, rs2010963, and rs3025039) gene polymorphisms and susceptibility to different CV conditions (Ma et al. 2018). Although a subgroup analysis was used by authors to investigate the source of the heterogeneity, the results were interpreted with caution. They found that rs699947, rs2010963, and rs3025020 polymorphisms increased CV events susceptibility, suggesting that these polymorphisms may be risk factors. However, they noticed that rs1570360 polymorphism failed to yield an association with increased CV risk. One possible explanation made by authors for this finding is that the functional polymorphisms of rs699947, rs2010963, and rs3025020 may have more profound effects on angiogenesis than others. Also, authors indicated that their findings were in agreement with some studies but not others, which makes the situation even more confusing. In contrast, our study strongly supports the existence of an association between *NOS3* (rs2070744) gene's polymorphism and occurrence of endothelial dysfunction and atherosclerosis.

Heterotrimeric guanine nucleotide-binding proteins (G-proteins), which integrate signals between receptors and effector proteins, are composed of an alpha, a beta, and a gamma subunit. These subunits are encoded by families of related genes. The *GNB3* gene encodes a beta subunit which belongs to the WD repeat G-protein beta family. Beta subunits are important regulators of alpha subunits, as well as of certain signal transduction receptors and effectors. It is suspected that single-nucleotide polymorphism (C825T) in this gene is associated with EAH and obesity. This polymorphism is also associated with the occurrence of the splice variant *GNB3*-s, which appears to have increased activity. *GNB3*-s is an example of alternative splicing caused by a nucleotide change outside of the splice donor and acceptor sites. Alternative splicing results in multiple transcript variants. Additional alternatively spliced transcript variants of this gene have been described, but their full-length nature is not known. The *GNB3* gene variant could form dimers with the G-protein gamma-3 and gamma-12 subunits, but they were unable to demonstrate functional association between the subunits in an activation assay (Feldman and Hegele 2000; Hengstenberg et al. 2001). Furthermore, declared significance of the *GNB3* genetic variants in EAH remains questioned (Brand et al. 1999). Authors summarize that in their study there was no association of the *GNB3* (825C/T) polymorphism with early onset of EAH, familial history of hypertension, or blood pressure level. They clearly stated that the 825C/T polymorphism of the *GNB3* gene did not contribute in any important way to the risk of EAH or myocardial infarction in two previous studies – Projet d'Etude des Gènes de l'hypertension Artérielle Sévère à modérée Essentielle (PEGASE), a case-control study of moderate to severe hypertension (681 cases and 308 controls), and Etude Cas-Témoins de l'Infarctus du Myocarde (ECTIM), a case-control study of myocardial infarction (564 cases and 633 controls). However, different factors like ethnicity may interfere with these results (Dong et al. 1999) when high frequency of the *T*-allele was observed in population of African origin, and authors provided evidence that the *T*-allele may be a susceptibility factor for the development of hypertension in blacks; given the high frequency of the *T*-allele, even a twofold increased risk of hypertension among the carriers of the mentioned above allele might account for 44% of the cases of hypertension in blacks.

It was shown that the *T*-allele was associated with a splice variant in which nucleotides 498–620 of exon 9 were deleted. This in-frame deletion caused the loss of 41 amino acids and 1 WD repeat domain of the beta subunit. Increased activity of the splice variant was thought to be responsible for the previously observed enhanced signal transduction via pertussis toxin-sensitive G-proteins in lymphoblasts and fibroblasts from selected patients with essential hypertension. The *T*-allele in homozygous or heterozygous state was found in 44% of normotensive subjects and in 53.1% of hypertensive subjects (Siffert et al. 1998).

Our findings suggest that *T*-allele of *GNB3* (rs5443) gene significantly increases the risk of the structural changes of the carotid arteries walls including development of atherosclerotic plaques and growth of carotid IMT. While this fact doesn't directly indicate the growing risk of hypertension

chronic heart failure or myocardial infarction it explains possible mechanisms of *GNB3* (rs5443) polymorphism association with CV pathology. In addition, our study provides evidence that the *T*-allele of *GNB3* (rs5443) gene enhances the triple probability of high sVCAM-1 levels (>1050 ng/ml) compared to control suggesting its significance in EAH.

Conclusions

The risk of severe EAH course (SBP≥160/DBP≥100 mmHg) is associated with the structural changes of the carotid arteries (carotid IMT (>0.9 mm) increase the likelihood more than 3.5 times (OR 95%CI: 1.28–10.23; $p = 0.012$), atherosclerotic plaques on the CCA – 4 and 3.5 times as much (OR 95%CI: 1.18–13.59; $p = 0.018$ and OR 95%CI: 1.23–10.71; $p = 0.014$) and decreased *NOS3* gene transcriptional activity by the mRNA level (<0.5 U) – 3 times as much (OR 95%CI: 1.0–9.66; $p = 0.042$), respectively), but not depend on separate endothelial dysfunction markers (FMD BA, total NO metabolites or sVCAM-1). Moderate and severe endothelial dysfunction grades (2^{nd}, 3^{rd} degrees) enhance the risk of severe EAH course over 3 and 5.5 times (OR 95%CI: 1.13–9.34; $p = 0.025$ and OR 95%CI: 1.96–14.45; $p < 0.001$).

C-allele of *NOS3* (rs2070744) gene elevates the risk of atherosclerotic unilateral and bilateral plaques on the CCA over 3.5 times (OR 95%CI: 1.24–11.20; $p = 0.019$ and OR 95%CI: 1.22–10.18; $p = 0.018$), endothelial dysfunction – by decrease of total NO metabolites (<25 μmol/l) and sVCAM-1 value raise (>1050 ng/ml) almost 12 and 4 times (OR 95%CI: 1.23–112.7; $p = 0.023$ and OR 95%CI: 1.24–11.20; $p = 0.019$), respectively. Moreover, *C*-allele of *NOS3* gene heighten the probability of low *NOS3* gene expression by the mRNA level (<0.5 U) 69 times as much (OR95%CI: 17.72–520.0; $p < 0.001$).

The minor *T*-allele of *GNB3* (rs5443) gene increases the risk of the structural changes of the carotid arteries walls: by carotid IMT (>0.9 mm) almost 3 times (OR 95%CI: 1.09–7.74; $p = 0.027$), atherosclerotic plaques on the CCA – almost 10 and 5 times as well (OR 95%CI: 2.55–38.0; $p < 0.001$ and OR 95%CI: 1.61–13.27; $p = 0.003$). Besides, the *T*-allele of *GNB3* (rs5443) gene enhances the probability of high sVCAM-1 levels (>1050 ng/ml) over 3 times (OR 95%CI: 1.06–9.59; $p = 0.032$).

Conflict of interest. The authors declare that they have no competing interests.

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