ГЕНОТИПЫ И СЫВОРОТОЧНЫЕ УРОВНИ АПОЛИПОПРОТЕИНА Е И ПАРАОКСОНАЗЫ 1 ПРИ КАЛЬЦИНИРОВАННОМ СТЕНОЗЕ КЛАПАНА АОРТЫ

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Цель. Аполипопротеин Е (АпоЕ) и параоксоназа 1 (ПОН1) участвуют в регуляции уровня липопротеинов крови, как и в их гидролизе и окислении. Мы предположили, что индивидуальные аллели генов *apoE* и *PON1*, вместе с изменениями концентрации данных молекул, вносят вклад в развитие кальцинированного стеноза клапана аорты (КСКА).

Материал и методы. Группа исследования включила 108 участников с КСКА и 46 пациентов без признаков стеноза клапана аорты. Сывороточные уровни АпоЕ и ПОН1 измеряли методом ELISA, мутацию Leu28Pro в гене *apoE* (rs429358) и мутацию Gln192Arg в гене *PON1* (rs662) выявляли методом ПЦР SNP-EXPRESS электрофореза.

Результаты. Повышенные уровни АпоЕ (0,05 vs 0,03 мкг/л, p<0,001) и ПОН1 (4,8 vs 3,4 мкг/мл, p<0,05) были выявлены у пациентов с КСКА. Частоты полиморфизмов аллелей генов АпоЕ и ПОН1 были одинаковы в обеих группах. Аллель 28Pro гена АпоЕ был ассоциирован с повышенным уровнем липопротеинов низкой плотности у группы с КСКА (3,9±1,05 vs 3,13±1,08 ммоль/л, p<0,02) и общего холестерина — у контроля (6,2; 6,5 vs 5,11±0,89 ммоль/л, p<0,05). Генотип *PON1* не оказывал влияния на обмен липидов при КСКА. Контрольная группа с аллелем 192Gln показала сниженный уровень АпоЕ (0,02 vs 0,05 мкг/л, p<0,01) и повышенную концентрацию в сыворотке ПОН1 (4,1 vs 3,3 мкг/мл, p<0,01).

Заключение. У пациентов с КСКА повышен сывороточный уровень АпоЕ и ПОН1; уровень АпоЕ — независимый предиктор кальциноза аорты. Маркеры полиморфизмов Gln192Arg гена *PON1* и Leu28Pro гена *apoE* не ассоциированы с КСКА.

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CAVS — calcific aortic valve stenosis, IHD — ischemic heart disease, apoE — apolipoprotein E, PON1 — paraoxonase 1, LDL — low-density lipoproteins, VLDL — very low-density lipoproteins, HDL — high-density lipoproteins.

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GENOTYPES AND SERUM LEVELS OF APOLIPOPROTEIN E AND PARAOXONASE 1 IN CALCIFIC AORTIC VALVE STENOSIS

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Aim. Apolipoprotein E (apoE) and paraoxonase 1 (PON1) participate in regulation of blood lipoprotein levels, as well as in their hydrolysis and oxidation. We assumed that individual alleles of the *apoE* and *PON1* genes, along with the changes in concentrations of these substances, contribute to the development of calcific aortic valve stenosis (CAVS).

Material and methods. The study group included 108 patients with CAVS and 46 patients without any signs of the aortic valve lesions were determined. The serum apoE and PON1 levels were measured by ELISA, the Leu28Pro mutation in the *apoE* (rs429358) gene and the Gln192Arg mutation in the *PON1* (rs662) gene were detected using PCR SNP-EXPRESS electrophoresis detection scheme.

Results. Increased serum levels of apoE (0,05 vs 0,03 μg/l, p<0,001) and PON1 (4,8 vs 3,4 μg/ml, p<0,05) were detected in CAVS patients. The frequencies of allelic polymorphisms of the apoE and PON1 genes were similar in the two groups. 28Pro allele of the apoE gene was associated with increased level of low density lipoproteins in CAVS (3,9±1,05 vs 3,13±1,08 mmol/l, p<0,02) and total cholesterol in the controls (6,2; 6,5 vs 5,11±0,89 mmol/l, p<0,05). The *PON1* genotype had no effect on lipid metabolism in CAVS patients. Controls with 192Gln allele demonstrated decreased blood levels of apoE (0,02 vs 0,05 μg/l, p<0,01) and increased PON1 serum concentration (4,1 vs 3,3 μg/ml, p<0,01).

Conclusions. CAVS patients have increased serum levels of apoE and PON1; the apoE level is an independent predictor of aortic calcification. Polymorphic markers Gln192Arg of the *PON1* gene and Leu28Pro of the *apoE* gene are not associated with CAVS.

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Key words: apoliporotein E, paraoxonase 1, gene polymorphism, calcific aortic stenosis.

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Introduction

Calcific aortic valve stenosis (CAVS) is the progressive non-rheumatic thickening and calcification of aortic semilunar cusps. This pathology is known to be triggered by hyperlipidemia and local inflammatory process.

A thorough analysis of the lipid profile in the CAVS patient population revealed increased levels of total cholesterol and atherogenic lipid fractions [1]. The total cholesterol level in these patients is higher even than that in patients with ischemic heart disease (IHD) after

coronary bypass surgery. However, only 40% of CAVS patients have hemodynamically significant coronary artery stenosis, while aortic stenosis is revealed in only 2% of patients with severe IHD [2].

Oxidized lipoproteins are deposited on the valve cusps, where they interact with T-cells and macrophages. This interaction initiates expression of inflammatory cytokines followed by valve matrix remodeling and formation of fibrotic and calcification areas [3].

Apolipoprotein E (apoE) regulates the plasma level of lipoproteins and triglycerides. The key role of apoE is transferring circulating lipoproteins from plasma into the cells. Furthermore, apoE participates in vascular wall inflammation by presenting lipid antigens in the immune [4]. Oxidation of low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL) in plasma is inhibited by substances with antioxidant activity of high-density lipoproteins (HDL). Antioxidant and anti-atherogenic activity of HDL is attributed to paraoxonase 1 (PON1). PON1 inhibits lipid hydrolysis in LDL, differentiation of monocytes to macrophages, entrapment of oxidized LDL by macrophages, and conversion of macrophages to foam cells [5]. The role of apoE and PON1 in the development of aortic calcification is controversial; however, association of their biological functions with lipid metabolism and vascular endothelial injury may indicate their involvement in the disease mechanisms.

Polymorphisms of the *apoE* and *PON1* genes causing development of cardiovascular pathology have been reported. *PON1* 192Arg (rs662, Gln192Arg) carriers have an increased risk of coronary atherosclerosis and acute coronary events [6], while *apoE* Leu28Pro polymorphism (rs429358) is associated with lipid metabolism abnormalities [7].

There is a concept that CAVS is a genetically determined disease; gene candidates of this pathology have been reported sporadically [8]. Our hypothesis is that some alleles of the *apoE* and *PON1* genes, along with the changes in concentrations of these substances, may cause development of CAVS. We studied frequency of allelic polymorphisms of the *PON1* (rs662) and *apoE* (rs429358) genes and measured PON1 and apoE levels in blood serum of CAVS patients and in patients without any signs of aortic valve lesions.

Table 1

Demographic and clinical profile of the study participants

Indicator	CAVS patients (n=108)	Control group (n=46)	p
Age, years	72.5±7.5	70.1±5.9	0.09
Males	49 (45%)	24 (49%)	0.55
BMI (kg/m^2)	29.6±4.5	29.1±3.7	0.11
Concomitant pathology			
Hypertension	105 (95%)	42 (87.5)	0.23
Angina	90 (82%)	37 (80%)	0.84
Diabetes mellitus	14 (13%)	6 (13%)	0.80
Drug therapy:			
acetylsalicylic acid	69 (63%)	36 (78%)	0.11
warfarin	30 (27%)	8 (17%)	0.24
statins	61 (56%)	32 (67%)	0.18
β-adrenoblockers	66 (60%)	34 (74%)	0.18
calcium channel blockers	38 (35%)	14 (30%)	0.70
ACE inhibitors	46 (42%)	26 (54%)	0.16
angiotensin II receptor blockers	35 (32%)	14 (30%)	0.95
Echocardiographic data:			
LVEDD, cm	5.62±0.62	5.36±0.44	0.054
LVESD, cm	3.96±0.68	3.71±0.39	0.010
LVPW, cm	1.05±0.1	1.0±0.06	0.001
IVSd, cm	1.25±0.18	1.1±0.17	0.062
EDV, cm^3	158.44±46.9	137.2±27.61	0.005
ESV, cm^3	73.35±32	58.8±17.88	0.057
SV, cm^3	86.77±17.4	78.2±1.32	0.042
LA, cm	4.73±0.82	4.21±0.56	0.002
EF, %	55.1±6.41	57.67±4.27	0.049
Separation of AV, mm	13.53±5.58	19.53±0.97	0.0001
Transaortic flow velocity, m/s	219.16±93.01	119.73±19.83	0.0001

Abbreviations: BMI — body mass index; ACE — angiotensin converting enzyme, LVEDD — left ventricular end-diastolic dimension, LVESD — left ventricular end-systolic dimension, LVPW — left ventricular posterior wall, IVSd — interventricular septal thickness at diastole, EDV — end-diastolic volume, ESV — end-systolic volume, SV — stroke volume, LA — left atrium, EF — ejection fraction, AV — aortic valve.

Table 2

Genotypes and alleles of polymorphic markers of the *PON1* and *apoE* genes in patients with calcific aortic valve stenosis and in the control group

Polymorphism	Genotype	CAVS patients (n=108)	Control group (n=46)	p
apoE gene (rs429358)				
	Leu28Leu	100 (93%)	44 (96%)	0.8*
	Leu28Pro	7 (6%)	2 (4%)	
	Pro28Pro	1 (1%)	0	
	Allele frequencies			
	28Leu	207 (96%)	90 (98%)	0.59
	28Pro	9 (4%)	2 (2%)	
PON1 gene (rs662)				
	Arg192Arg	65 (60%)	24 (50%)	0.364
	Gln192Arg	34 (31%)	20 (43%)	
	Gln192Gln	9 (9%)	2 (7%)	
	Allele frequencies			
	192Arg	164 (76%)	68 (71%)	0.81
	192Gln	52 (24%)	24 (29%)	

Note: * — Since the control group contained no carriers of the Pro28Pro genotype, the Pro28Pro and Leu28Pro genotypes were combined during statistical processing.

Material and methods

Study population. This was an open, non-randomized, comparative case-control study. Study group included 108 patients with calcific aortic valve stenosis (≥65 years) who received hospital care at the Regional Clinical Center of Cardiology in 2010-2012. Aortic valve calcification was confirmed by transthoracic echocardiography. Aortic stenosis was diagnosed in accordance with the recent international guidelines [9]. The exclusion criteria were congenital anomalies of aortic valve and/or past history of surgical correction of malformations, chronic rheumatic heart disease, infra- or supravalvular aortic stenosis, chronic kidney disease, parathyroid gland disorders, and neoplasms. The control group included 46 patients without any signs of aortic valve lesions matched for sex, age, concomitant cardiovascular pathology, and received therapy. The study followed the principles of the Declaration of Helsinki and Good clinical practice and complied with all local regulations. The study was approved by the Ethics Committee of the Stavropol State Medical University. All the patients provided written informed consent.

Demographic and clinical profile of the enlisted patients is shown in Table 1. Mean age of CAVS patients was 72.5±7.5 years. CAVS patients were generally characterized by a more pronounced heart chamber remodeling: left ventricular hypertrophy and dilatation with moderately reduced ejection fraction and increased left atrial and left ventricular size (Table 1).

Laboratory testing. The serum apoE and PON1 levels were measured by ELISA using AssayMax Human Apolipoprotein E ELISA Kit (Assaypro, USA) and Human serum paraoxonase 1 (PON1) ELISA Kit (Aviscera Bioscience Inc., USA) in accordance with the manufacturer's protocols.

DNA was isolated from whole blood leukocytes using a DNA-sorb-C kit (Central Research Institute of Epidemiology, Federal Service on Customers' Rights Protection and Human Wellbeing Surveillance, Russia). The Leu28Pro mutation in the *apoE* (rs429358) gene and the Gln192Arg mutation in the *PON1* (rs662) gene were detected with commercially available kits using PCR SNP-EXPRESS electrophoresis detection scheme (Research and Production Company Litekh, Russia).

Statistical analysis. Statistical analysis was performed using IBM SPSS Statistics 21 for Windows (IBM SPSS Inc., USA). For the normal distribution, indicators were shown as the mean values and standard deviation (M±σ); the differences between groups were assessed using single-factor dispersion analysis and Fischer's test. The non-normally distributed data were represented as a median and interquartile range (Me (Q1-Q3)); the differences between groups were analyzed using the Mann–Whitney U-test. The Spearman' rank correlation coefficient *r* was calculated. The qualitative indicators were represented as absolute values (%). The chi-squared test and Fischer's exact test were used to compare fractions. Stepwise binary logistic regression with calculation of the Wald statistics was used to reveal independent predictors of CAVS. Differences at $p<0.05$ were considered to be significant.

Results

No significant differences in the mean levels of total cholesterol, cholesterol fractions, and triglycerides were found in both groups. Study group patients were characterized by lower serum Ca^{2+} levels (1.17±0.06 vs 1.25±0.14 mmol/l, p=0.0005) and elevated apoE levels (0.05 (0.03-0.09) vs 0.03 (0.02-0.04) μg/l, p=0.0001) and

Table 3

Concentrations of lipid fractions, PON1, and apoE in blood serum of patients with different *apoE* genotypes

Indicator	*ApoE* genotype					
	CAVS patients			Control group		
	Leu28Leu (n=100)	Leu28Pro Pro28Pro (n=8)	p	Leu28Leu (n=44)	Leu28Pro Pro28Pro (n=2)*	p
Total cholesterol, mmol/l	4.91±1.28	5.68±1.3	0.13	5.11±0.89	6.2; 6.5	0.04
Triglycerides, mmol/l	1.52±0.82	1.25±0.29	0.62	1.59±0.67	2.0; 2.6	0.12
LDL, mmol/l	3.13±1.08	3.9±1.05	0.02	3.28±0.91	3.3; 3.6	0.64
HDL, mmol/l	1.09±0.25	1.07±0.15	0.91	1.13±0.24	1.04; 1.1	0.74
VLDL, mmol/l	0.71±0.51	0.57±0.13	0.78	0.72±0.3	1.02; 1.15	0.13
ApoE, μg/l	0.05 (0.04-0.09)	0.03 (0.02-0.04)	0.16	0.03 (0.02-0.04)	0.028; 0.032	0.27
PON1, μg/ml	4.5 (3.4-6.4)	5.1 (3.1-6.8)	0.31	3.5 (3.2-4.2)	3.9; 4.1	0.77

Note: * — Since the number of patients per group is small, the exact values for each patient are given.

Table 4

Concentration of lipid fractions, PON1 and apoE in blood serum of patients with different *PON1* genotypes

Indicator	PON1 genotype					
	CAVS patients			Control group		
	Arg192Arg (n=65)	Gln192Arg Gln192Gln (n=43)	p	Arg192Arg (n=24)	Gln192Arg Gln192Gln (n=22)	p
Total cholesterol, mmol/l	4.96±1.33	4.96±1.24	0.95	5.5±0.87	4.86±0.86	0.02
Triglycerides, mmol/l	1.48±0.84	1.54±0.75	0.52	1.86±0.6	1.41±0.69	0.01
LDL, mmol/l	3.17±1.02	3.19±1.19	0.99	2.98±0.9	2.59±0.8	0.03
HDL, mmol/l	1.1±0.26	1.08±0.2	0.69	1.09±0.18	1.16±0.27	0.68
VLDL, mmol/l	0.7±0.58	0.69±0.36	0.63	0.85±0.27	0.64±0.31	0.01
ApoE, μg/l	0.05 (0.03-0.09)	0.05 (0.03-0.1)	0.85	0.05 (0.02-0.06)	0.02 (0.02-0.03)	0.01
PON1, μg/ml	4.5 (3.3-6.6)	4.5 (3.5-6.3)	0.94	3.3 (2.7-3.9)	4.1 (3.2-7.4)	0.01

PON1 (4.8 (3.57-6.8) vs 3.4 (3.1-4.1) μg/ml, p=0.018) as compared to patients without CAVS.

In the study group a weak negative correlation between PON1 concentration with the levels of LDL (r=–0.24, p=0.04) and VLDL (r=–0.27, p=0.02) and a weak positive correlation with the apoE level (r=0.24, p=0.04) were observed; apoE showed a weak negative correlation with the level of calcium ions (r=–0.28, p=0.04). Control group showed a moderate negative correlation between the serum level of apoE and the levels of total cholesterol (r=–0.42, p=0.02) and LDL (r=–0.43, p=0.01).

Table 2 shows frequencies of genotypes and alleles of the *apoE* and *PON1* genes in the study population. Allele frequencies of the genes under study did not differ between the groups and corresponded to the data of global populations according to the dbSNP database.

An analysis of the lipid profile of blood, serum concentrations of PON1 and apoE depending on *apoE* genotype showed a statistically significant increase in LDL in CAVS patients with 28Pro allele (3.9±1.05 vs 3.13±1.08 mmol/l, p=0.02); in the control group the presence of this allele was accompanied by an increase in the total cholesterol level (6.2; 6.5 vs 5.11±0.89 mmol/l, p=0.04) (Table 3).

PON1 genotype in CAVS patients had no significant effect on blood lipid levels. Significantly lower levels of total cholesterol (4.86±0.86 vs 5.5±0.87 mmol/l, p=0.02), triglycerides (1.41±0.69 vs 1.86±0.6 mmol/l, p=0.01), and VLDL (0.64±0.31 vs 0.85±0.27 mmol/l, p=0.01) were observed in the control group when the 192Gln allele was present in the genotype. Furthermore, carriers of this allele were characterized by a relatively higher PON1 concentration (4.1 (3.2-7.4) vs 3.3 (2.7-3.9) μg/ml, p=0.01) and reduced apoE level (0.02 (0.02-0.03) vs 0.05 (0.02-0.06) μg/l, p=0.01) (Table 4).

Regression analysis with serum concentrations of blood lipids, apoE, PON1, and Ca^{2+} used as covariates enabled us to ascertain that apoE concentration is an independent predictor of CAVS (Odds ratio 7.04 (95% CI 2.54; 19.47), p=0.004).

Discussion

ApoE is often considered to be a lipoprotein inhibiting the development of both atherosclerosis and aortic valve calcification. The apoE knockout animals have severe hyperlipidemia resulting in the formation of atherosclerotic plaques and sclerotic aortic valve changes that are morphologically similar to the changes in the calcified

aortic valve. However, results of experiments on mice cannot be reliably extrapolated to humans. Aortic valves in mice and humans have different morphological structure and laboratory induced sclerosis of the aortic valve in transgenic animals can be reproduced under natural conditions in extremely rare cases. The high plasma level of apoE in a human population is associated with the risk of such conditions as Alzheimer disease and cardiovascular death [10]. The results of our study show that development of CAVS can be associated with an increased apoE level. The mechanisms of this relationship remain unclear. It has been shown that apoE level in young and middle-aged people correlates with the levels of total cholesterol, HDL, and LDL. However, CAVS most often develops in older people, while the correlation between the levels of apoE and plasma lipids in this population becomes not so clearly pronounced. The *apoE* genotype (rs429358) also affects blood lipid concentration. Since *apoE* and its allele polymorphism participate in lipid metabolism, it would be reasonable to assume that they contribute to progression of CAVS by inducing hyperlipidemia. In the population studied, lipid profile parameters were not significantly higher than the normal values and did not differ between the groups. The lack of expected differences might be due to the fact that most of our patients received hypolipidemic therapy. Another mechanism through which apoE may induce CAVS is related to the proinflammatory activity of this lipoprotein. Plasma apoE tightly binds to lipid antigens and promotes their presentation on LDL receptors of antigen-presenting cells of the vascular wall (macrophages, T cells), thus inducing endocytosis of lipid particles [11]. The subsequent inflammatory process results in elimination of lipid antigens from systemic circulation. Close correlation between the levels of this lipoprotein and the high-sensitivity C-reactive protein may be indicative of proinflammatory activity of apoE. It is likely that apoE interacts with macrophages and T cells that infiltrate aortic valve cusps to initiate and promote persistence of the local inflammatory process, which is accompanied by secretion of proinflammatory cytokines and matrix metalloproteinases, destruction of intercellular matrix, and eventually aortic valve sclerosis.

It has been reported that aortic valve calcification in patients without chronic kidney disease is accompanied by reduced serum calcium level [12]. In our study, the level of calcium ions in CAVS patients was reduced and showed a negative correlation with apoE. Expression of apoE by macrophages is known to be inhibited by chelated intracellular calcium, while high extracellular apoE concentrations promote increased calcium level inside the cell through the feedback mechanism. It can be assumed that a decrease in plasma calcium level results from its macrophage clearance; however, this hypothesis needs further experimental data.

The study group was also characterized by an increased paraoxonase 1 level compared to the control group. PON1 exhibits anti-atherogenic properties. Reduced serum level and activity of this enzyme is observed in patients with ischemic heart disease and atherosclerotic peripheral vascular disease. No relationship was found between the PON1 level and aortic calcification or arterial calcification in patients with diabetes mellitus [13]. Research is currently focused on the effect of PON1 on the emergence and progression of CAVS. Moura et al. have reported increased PON1 activity in CAVS patients, which was associated with the progression of aortic stenosis. Meanwhile, Cagirci et al. have reported quite an opposite results [14]. The level and activity of PON1 are known to largely depend on Gln192Arg polymorphism of the *PON1* gene. This locus ensures production of two alloenzymes: with either high or low activity depending on arginine or glutamine at position 192, respectively. The Gln192Gln genotype is also associated with the increased levels of total cholesterol, low-density lipoproteins, and triglycerides. In our study, similar trends were revealed in the control group of patients. However, the *PON1* genotype in CAVS patients had no effect on blood lipids and PON1 concentration. Apolipoprotein A1 on the surface of low-density lipoproteins is known to participate in PON1 activation. An apoE molecule also exhibits nanomolar binding affinity for a PON1 molecule; identically to apoA1, it can enhance stability and activity of this enzyme [15]. Increased PON1 concentration in CAVS patients may result from the high apoE level, which is confirmed by the direct correlation between plasma levels of these molecules in the examined population.

The main limitation of this study is the small size of study population. Further studies with larger groups are needed.

Since the patients were selected for the study at the Center of Cardiology, assessed patients frequently had concomitant cardiovascular diseases (IHD, peripheral atherosclerosis, and arterial hypertension), which could have affected the measured parameters. However, taking into account epidemiology of cardiovascular diseases in Russia, such problems as the lack of complaints and laboratory abnormalities in patients with hemodynamically insignificant atherosclerosis during the routine clinical examination, unavailability of routine invasive procedures for screening purposes, make it difficult to select almost healthy elderly individuals to comprise the control group, as well as to find otherwise healthy patients with aortic calcification.

No final conclusions regarding possible contribution of candidate genes to CAVS pathogenesis and progression can be drawn from analyzing single polymorphism of each gene. The studied mutations may be located in several loci; both point mutations and their combinations can change the structure and activity of protein synthesis and biological properties of proteins of interest. Further fundamental studies of the combination of point

mutations of *apoE* and *PON1* and their products and a prospective study of the natural history of CAVS are needed.

The results demonstrated that CAVS patients are characterized by the increased apoE and PON1 levels in blood serum; the apoE level turned out to be an independent predictor of the development of aortic calcification. Polymorphic markers Gln192Arg of the *PON1* gene (rs662) and Leu28Pro of the *apoE* gene (rs429358) are not associated with CAVS.

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