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Assessment of the Level of Matrix Metalloproteinases, VEGF and MicroRNA-34a in Patients With Non-obstructive and Obstructive Lesions of the Coronary Arteries

Aim	To assess the levels of matrix metalloproteinases (MMP), vascular endothelial growth factor (VEGF), and miRNA-34a expression in patients with ischemic heart disease (IHD) and obstructive and nonobstructive coronary artery (CA) disease.
Material and methods	This cross-sectional observational study included 64 patients with IHD (diagnosis verified by coronary angiography or multislice computed tomography coronary angiography), of which 33 (51.6%) were men aged 64.9±8.1 years. 20 patients had nonobstructive CA disease (stenosis <50%), and 44 had hemodynamically significant stenoses. The control group consisted of 30 healthy volunteers. MMP- 1, -9, -13, and -14, miRNA-34a, and VEGF were measured in all patients.
Results	The concentration of MMP-1 was significantly higher in patients with ischemia and nonobstructive CA disease (INOCAD) (p=0.016), and the concentration of MMP-9 was the highest in the group with obstructive CA disease (p<0.001). The concentrations of MMP-13 and MMP-14 did not differ significantly between the groups. The highest VEGF concentrations were observed in the INOCAD group (p<0.001). The expression of miRNA-34a significantly differed between the IHD groups with different types of CA disease and controls (p<0.001). Patients with hemodynamically significant stenosis showed moderate relationships between the concentrations of MMP-14 and VEGF (ρ=0.418; p=0.024), as well as between VEGF and miRNA-34a (ρ=0.425; p=0.022). Patients with INOCAD had a significant negative correlation between the concentrations of MMP-13 and VEGF (ρ= –0.659; p=0.003). Correlation analysis showed in all IHD patients a moderate relationship of the concentrations of MMP-1 and MMP-14 with VEGF (p=0.449; p=0.002 and p=0.341; p=0.019, respectively). According to ROC analysis, a MMP-9 concentration above 4.83 ng/ml can be a predictor for the presence of hemodynamically significant CA obstruction in IHD patients; a VEGF concentration higher than 27.23 pg/ml suggests the absence of hemodynamically significant CA stenosis.
Conclusion	IHD patients with INOCAD had the greatest increase in MMP-1, whereas patients with obstructive CA disease had the highest level of MMP-9. According to our data, concentrations of MMP-9 and VEGF can be used to predict the degree of CA obstruction. The expression of miRNA-34a was significantly higher in IHD patients with INOCAD and CA obstruction than in the control group, which suggested a miRNA-34a contribution to the development and progression of coronary atherosclerosis. In the future, it may be possible to use this miRNA as a diagnostic marker for IHD.

Keywords	Ischemic heart disease; matrix metalloproteinase-1, -9, -13, -14; miRNA-34a; vascular endothelial growth factor (VEGF); nonobstructive coronary artery disease
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Introduction

According to statistics, it can be estimated reduction in annual mortality from coronary artery disease (CAD) in the global population since 2010, with a decrease of 19.2% by 2020, while the actual number of deaths increased by 0.9%.

According to the Russian Federal State Statistics Service (Rosstat), the mortality rate due to cardiovascular diseases will be 11.1% lower by the end of 2022. However, the incidence of CAD remains extremely high. In 2020, approximately 244.1 million people worldwide had CAD and 8.95 million people died [1]. The high mortality rate is caused by a number of reasons, among which we can highlight the difficulties in diagnosing CAD (the presence of silent ischemia and other features of the disease course),

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which is associated with low patient awareness of their diagnosis.

The search for new biomarkers will make it possible in the future to use them in real clinical practice for the diagnosis of various forms of CAD and possibly as therapeutic vectors. Matrix metalloproteinases (MMPs), which are involved in the pathogenesis of atherosclerotic plaque formation and growth, may play a role as potential biomarkers for predicting future atherothrombotic cardiovascular events [2]. MMPs play a key role in all stages of atherosclerosis development and are involved in the regulation of inflammation, endothelial dysfunction, smooth muscle cell migration, vascular calcification, extracellular matrix degradation, plaque destabilization [3]. The available evidence supports a key role for MMPs in the pathogenesis of atherosclerosis and the development of plaques in advanced stages of the disease, with increased MMP activity increasing the risk of plaque rupture. Degradation of extracellular matrix protein is catalyzed by MMPs and is involved in vascular smooth muscle cell (VSMC) migration, leading to plaque instability. Elevated levels of MMP-2 and MMP-9 in peripheral blood in acute coronary syndrome (ACS) may have potential as non-invasive tests to determine plaque destabilization and identify patients at high risk of cardiovascular complications.

MMP-1 expression is increased in inflammatory and autoimmune diseases by pro-inflammatory cytokines tumor necrosis factor-α and interleukin-1 (IL-1) [4]. MMPs are involved in neoangiogenesis by penetrating into the extracellular matrix, leading to tissue remodeling. Cross-interaction between VSMCs and macrophages increases the synthesis of angiogenic factors (vascular endothelial growth factor (VEGF), IL-1 (a cytokine that stimulates VEGF secretion) and MMPs [5]. Furthermore, MMP expression was found to be controlled by several miRNAs.

The regulation of extracellular matrix components by miRNAs is critical in the process of plaque destabilization [6]. Some miRNAs were shown to be of diagnostic value in CAD patients, but there are still many potential biomarker candidates. According to few studies conducted in the Russian Federation, the level of miRNA expression in CAD patients may become a new marker for predicting the degree of coronary artery stenosis. The study of expression levels and the search for specific miRNAs will make it possible to use them in clinical practice as components of the diagnosis of various forms of CAD and possibly as the basis for targeted therapy.

Objective

Evaluate the levels of MMP, VEGF and miRNA-34a expression in CAD patients with obstructive and nonobstructive coronary artery lesions.

Material and Methods

Cross-sectional observational study included 64 patients aged 45–75 years with documented stable CAD diagnosed according to clinical guidelines (2020) who signed informed consent [7]. The study was conducted in accordance with the principles of the Declaration of Helsinki (extract from the protocol of the Ethics Committee #01–21 dated 22.01.2021). The presence of myocardial ischemia in hospitalized patients with the clinical picture of stable angina pectoris or its equivalent was demonstrated by clinical tests: stress echocardiography or single-photon emission computed tomography during exercise testing.

Patients were divided into 2 groups according to the results of coronary angiography or multislice computed tomography of the coronary arteries: 20 patients with nonobstructive coronary artery lesions (stenosis <50% or normal coronary arteries); 44 patients with obstructive CAD (presence of hemodynamically significant coronary artery stenosis). The control group (n = 30) consisted of healthy volunteers without cardiovascular diseases (CVD) or cardiovascular risk factors.

Patients with diabetes mellitus, ACS, acute myocardial infarction within the previous 3 months and stroke, chronic heart failure NYHA class III–IV, autoimmune diseases and cancer, severe hepatic and renal failure were excluded.

All patients underwent routine clinical blood and urine tests, biochemical tests including lipid profile, glucose and uric acid levels.

Blood plasma samples were frozen in cryotubes at –80°C after centrifugation. The samples were centrifuged for 20 minutes with EDTA K3 as anticoagulant. Enzyme-linked immunosorbent assay (ELISA) was performed on an Adaltis Personal Lab analyzer using Cloud-Clone Corp. kits to estimate MMP and VEGF levels. Coefficient of variation (CV) of the sets was 10% and 12%, respectively.

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA, including ncRNA, was recovered from samples using Qiazol according to the manufacturer's protocol. A NanoDrop 2000 microvolume spectrophotometer was used to assess the concentration and purity of the recovered RNA. The recovery process was repeated for each sample until a sufficient amount of RNA was obtained to perform the following steps.

The cDNA was obtained by RT-PCR using the MiScript II RT Kit according to the manufacturer's protocol. 300 ng of total RNA recovered from each sample was used to obtain cDNA.

Expression in tissue and plasma was quantified by real-time PCR (RT-PCR) using the CFX96 Real-Time PCR Detection System. Expression levels were determined in

triplicate for miRNA analyzed as well as for the exogenous control cel-miR-39-3p using the MiScript SYBR Green PCR Kit, the pre-synthesized miScript Primer Assay for the control and primers selected in the laboratory for miRNA (miR-34a – 5' – TGGCAGTGTCTTAGCTGGTTGT-3'). The resulting Ct values were normalized relative to the exogenous control cel-miR-39-3p and analyzed using the 2-ΔCt method. Results are expressed as relative expression units (REU) [8].

Statistical analysis

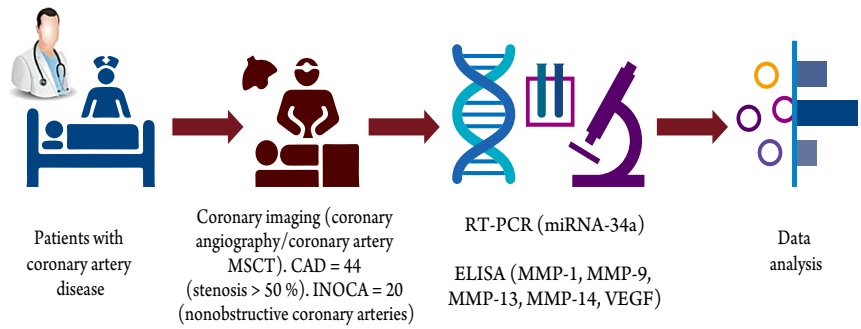
Statistical analysis of the data obtained was carried out in StatTech v. 3.1.10 (StatTech, Russia) and using the free Python computing environment (v. 3.11). The distribution of the parameters was evaluated using the Shapiro-Wilk test (n < 50) or the Kolmogorov-Smirnov test (n > 50). Arithmetic mean (M) and standard deviation (SD), 95% confidence interval (CI) were used to describe normally distributed quantitative variables. Non-normally distributed quantitative data were described using the median (Me) and the lower and upper quartiles [Q1 – Q3]. Two groups with non-normal distribution of quantitative indices were compared using the Mann-Whitney U test, three or more groups were compared using the Kruskal-Wallis test, and post hoc comparisons were made using the Bonferroni test. The direction and strength of a correlation between two quantitative indices were estimated using Spearman's rank correlation coefficient (for non-normal distribution). ROC curve analysis was performed to estimate the diagnostic power of quantitative indicators in predicting a specific outcome. The quantitative sign cut-off was determined by the highest value of the Youden index. The differences were considered statistically significant at $p < 0.05$.

Results

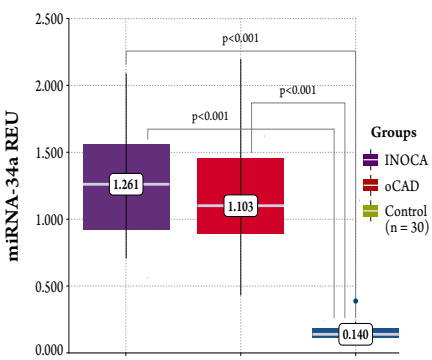
The general clinical and demographic characteristics of the subjects are summarized in Table 1. The study groups were comparable in terms of basic clinical and demographic parameters (age, body mass index). Female patients predominated in a ratio of 3:1 in the INOCA (ischemia with nonobstructive coronary arteries) group. All patients received recommended therapy according to national and international clinical guidelines.

The differences in total cholesterol (TC) and low-density lipoprotein cholesterol (LDL–C) levels between the groups are most likely due to the fact that obstructive CAD patients treated with higher doses of statins had better results in reducing TC and LDL–C levels. Statistically significant differences ($p = 0.02$) were found between the groups according to the type of coronary lesion in

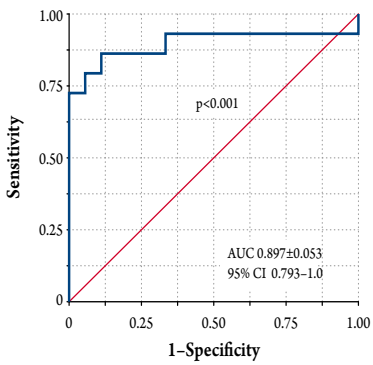
Central illustration. Assessment of the Level of Matrix Metalloproteinases, VEGF and MicroRNA-34a in Patients With Non-obstructive and Obstructive Lesions of the Coronary Arteries



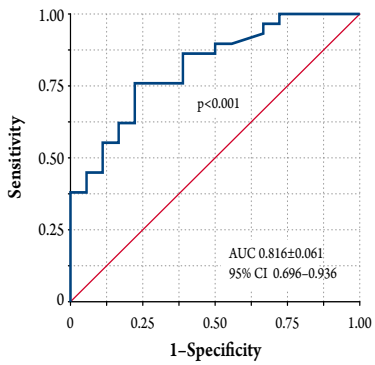
Parameter	Group	Me	[Q1; Q3]	p
MMP-1, ng/mL	INOCA	101,550	[67,950; 254,400]	0.021 $p_{oCAD-INOCA}$ = 0.016
	oCAD	66,910	[36,410; 171,740]	
	Control	67,500	47,790; 111,300	
MMP-9, ng/mL	INOCA	0,960	0,782; 1,163	<0.001 $p_{oCAD-INOCA}$ < 0.001 $p_{Control-INOCA}$ = 0.007
	oCAD	0,770	0,710; 1,270	
	Control	1,001	0,750; 1,310	
MMP-13, ng/mL	INOCA	37,17	30,55; 41,83	0.088
	oCAD	10,75	7,43; 22,53	
	Control	35,03	10,50; 41,62	
MMP-14, ng/mL	ИНОКА	0,960	0,782; 1,163	0.667
	оИБС	0,770	0,710; 1,270	
	Контроль	1,001	0,750; 1,310	
VEGF, pg/mL	ИНОКА	37,17	30,55; 41,83	<0.001 $p_{oCAD-INOCA}$ < 0.001 $p_{Control-oCAD}$ = 0.003
	оИБС	10,75	7,43; 22,53	
		35,03	10,50; 41,62	



Levels of miRNA-34a expression in the study groups



ROC curve characterizing the dependence of the probability of coronary lesion type on VEGF levels



ROC curve characterizing the dependence of the probability of coronary lesion type on MMP-9 levels

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Table 1. Key clinical and demographic characteristics of the examined patients

Parameter	CAD (all, n = 64)	INOCA (n = 20)	oCAD (n = 44)	Control (n = 30)	p
Male patients, n (%)	33 (51.6)	5 (25)	28 (63.6)	10 (33.3)	0.004 $p_{INOCA-oCAD}$ = 0.012 $p_{INOCA-Control}$ = 0.021
Female patients, n (%)	31 (48.4)	15 (75)	16 (36.4)	20 (66.7)	
Age, years	64.9 ± 8.1	66.5 [62.8; 71.2]	64.0 [56.5; 71.0]	28.5 [26.0; 39.2]	<0.001 $p_{Control-INOCA}$ <0.001 $p_{Control-oCAD}$ <0.001
BMI, kg/m2	26.92 ± 3.69	26.20 [25.67; 30.40]	26.23 [24.68; 28.68]	21.95 [20.75; 25.23]	< 0.001 $p_{Control-INOCA}$ = 0.003 $p_{Control-oCAD}$ = 0.003
Smoking, %	9 (14)	3 (15.0)	6 (13.6)	—	0.879
Hemoglobin, g/L	142 [133; 152]	142 [134; 151]	144 [133; 152]	136 [129; 152]	0.459
Glucose, mmol/L	5.5 [5.17; 5.8]	5.60 [5.2; 6.21]	5.40 [5.1; 5.63]	4.9 [4.59; 5.35]	<0.001
Creatinine, μmol/L	89 [78.2; 99.2]	83.4 [74.8; 96.3]	89.80 [81; 101.8]	82 [77.7; 87]	0.106
Total cholesterol, mmol/L	3.96 [3.47; 4.97]	5.11 ± 1.51	3.85 ± 0.95	4.87 ± 0.77	<0.001 $p_{INOCA-oCAD}$ = 0.005 $p_{oCAD-Control}$ <0.001
LDL-C, mmol/L	2.36 [1.85; 2.97]	2.89 [4.34; 3.62]	2.12 [1.79; 2.48]	2.54 [2.28; 3.21]	<0.001 $p_{oCAD-INOCA}$ <0.001 $p_{Control-oCAD}$ =0.049
HDL-C, mmol/L	1.15 [1.02; 1.36]	1.27 [1.06; 1.37]	1.11 [1.02; 1.33]	1.62 [1.35; 1.9]	<0.001 $p_{Control-INOCA}$ = 0.015 $p_{Control-oCAD}$ <0.001

CAD, coronary artery disease; INOCA, ischemia with nonobstructive coronary arteries; oCAD, coronary artery disease with coronary artery obstruction; BMI, body mass index; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol.

Table 2. Biomarker concentrations by groups

Parameter	Group	Me	[Q1; Q3]	p
MMP-1, ng/mL	INOCA	101.550	[67.950; 254.400]	0.021 $p_{oCAD-INOCA}$ = 0.016
	oCAD	66.910	[36.410; 171.740]	
	Control	67.500	47.790; 111.300	
MMP-9, ng/mL	INOCA	0.960	0.782; 1.163	<0.001 $p_{oCAD-INOCA}$ < 0.001 $p_{Control-INOCA}$ = 0.007
	oCAD	0.770	0.710; 1.270	
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MMP-13, ng/mL	INOCA	37.17	30.55; 41.83	0.088
	oCAD	10.75	7.43; 22.53	
	Control	35.03	10.50; 41.62	
MMP-14, ng/mL	ИНОКА	0,960	0,782; 1,163	0.667
	оИБС	0,770	0,710; 1,270	
	Контроль	1,001	0,750; 1,310	
VEGF, pg/mL	ИНОКА	37,17	30,55; 41,83	<0.001 $p_{oCAD-INOCA}$ < 0.001 $p_{Control-oCAD}$ = 0.003
	оИБС	10,75	7,43; 22,53	
		35,03	10,50; 41,62	

MMP, matrix metalloproteinase; INOCA, ischemia with nonobstructive coronary arteries; oCAD, coronary artery disease with coronary artery obstruction; VEGF, vascular endothelial growth factor.

the analysis of the daily doses of statins. There were no differences between the groups in the administered drug therapy.

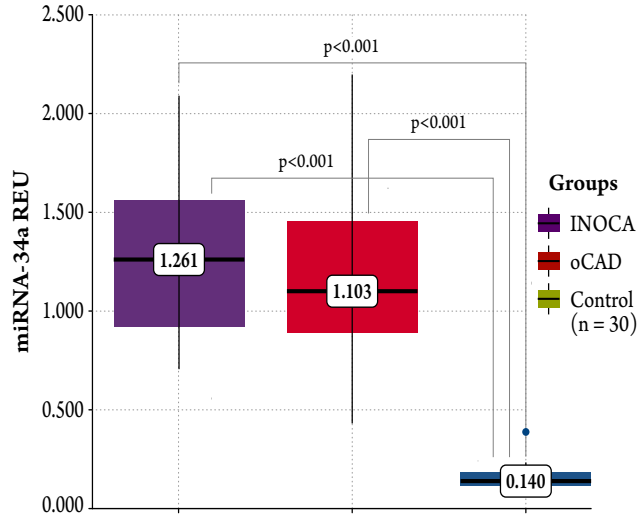
According to the examination results, MMP-1 levels were significantly higher in patients with INOCA ($p = 0.016$), while MMP-9 levels were maximal in the group with obstructive coronary artery lesions ($p < 0.001$). The levels of MMP-13 and MMP-14 did not differ significantly between the groups (Table 2). The highest VEGF levels were observed in the INOCA group ($p < 0.001$).

When comparing miRNA-34a expression, a significant difference was observed between groups of patients with different forms of CAD and the control group (Figure 1).

According to the correlation analysis, moderate correlations between MMP-14 and VEGF levels ($\rho = 0.418$; $p = 0.024$) and between VEGF and miRNA-34a ($\rho = 0.425$; $p = 0.022$) were observed in patients with hemodynamically significant stenosis. Patients with INOCA showed a significant negative correlation between MMP-13 and VEGF ($\rho = –0.659$; $p = 0.003$). Correlation analysis of the parameters in all CAD patients showed a moderate relationship between MMP-1 and MMP-14 levels and VEGF ($\rho = 0.449$; $p = 0.002$ and $\rho = 0.341$; $p = 0.019$, respectively).

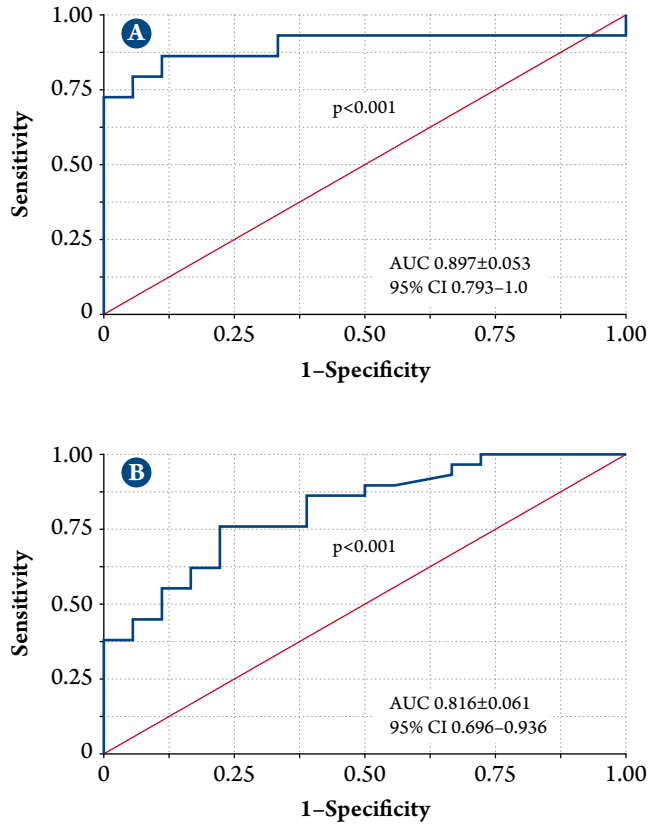
ROC curves were constructed to assess the diagnostic significance of VEGF and MMP-9 levels (Figure 2).

Figure 1. Levels of miRNA-34a REU expression in the study groups



INOCA, ischemia with nonobstructive coronary arteries; oCAD, coronary artery disease with coronary artery obstruction.

Figure 2. ROC curve characterizing the dependence of the probability of coronary lesion type on VEGF (A) and MMP-9 (B) levels



A – the cut-off VEGF level corresponding to the highest Youden index value was 27.23 pg/mL. CAD with hemodynamically significant stenosis was predicted when VEGF was below this level; the sensitivity and specificity of the model were 86.2 % and 88.9 %, respectively;

B – the cut-off MMP-9 level corresponding to the highest Youden index was 4.83 ng/ml, obstructive CAD was predicted when the MMP-9 level was higher than or equal to this value, the sensitivity and specificity of the model were 75.9 % and 77.8 %, respectively. MMP, matrix metalloproteinases; VEGF, vascular endothelial growth factor.

Discussion

According to our findings, there was a significant difference in the levels of MMPs in the study groups, with significantly higher levels of MMP-1 in patients with INOCA and MMP-9 in patients with coronary artery obstruction.

Elevated MMP-1 levels were found in CAD patients in a series of studies. However, some studies did not report such results [9–11]. It should be emphasized that the highest levels of MMP-1 were found in patients with unstable angina pectoris [8]. Lehrke et al [12] found that MMP-1 levels correlate with the presence of unstable plaques, which are mainly found in patients with ACS and unstable angina [13–15]. Patients with calcified and, to a lesser extent, mixed coronary plaques also showed similar associations. The absence of patients with ACS and unstable angina in our study may explain the obtained results. Notably, statins exhibit pleiotropic effects in vivo, including effects on signaling mechanisms leading to inhibition of MMP activity [16].

The level of MMP-9 is a strong independent predictor of plaque damage in patients with stable CAD. MMP-9 levels positively correlated with the size of the necrotic core of a coronary plaque and being associated with adverse cardiovascular outcomes, including future cardiovascular mortality [5, 17–19].

In our study, the lowest VEGF levels were found in patients with obstructive CAD. The role of VEGF in the pathogenesis of CAD is known to be controversial. It induces angiogenesis and is also involved in the process of atherogenesis [20]. According to Eaton et al. [21], there was a correlation between higher VEGF levels and mortality in CAD (p = 0.0004). In the study by Bonanni et al. [22], which included a small number of patients, lower VEGF expression was observed in the INOCA group in contrast to the group with hemodynamically significant stenosis. Opposite results were obtained in a study by Mirhafez et al. [23], which included 426 patients (93 patients with INOCA, 196 patients with obstructive CAD, 48 patients with a history of coronary artery bypass grafting, and 89 patients in the control group). It turned out that as the severity of atherosclerosis increased, the levels of VEGF decreased, which is consistent with our findings.

In our study, miRNA-34a expression was significantly higher in patients with various forms of CAD compared

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to the control group ($p < 0.001$). Tabuchi et al. and Han et al. reported similar results [24, 25]. Levels of miRNA-34a increase with age, inducing VSMC senescence through modulation of its target Sirtuin-1 (SIRT1) and contributing to the progression of vascular calcinosis [26, 27]. According to a meta-analysis performed by Kim et al [28], high levels of miRNA-34a were associated with the occurrence of adverse cardiovascular outcomes. This suggests an important role of the expression of this miRNA in the process of atherogenesis.

INOCA patients showed significant negative correlations between MMP-13 and VEGF. Most authors suggest that VEGF induces the expression of MMP-1, MMP-3, and MMP-13 [29]. In the animal model experiments, the expression level of MMP-13 was found to differ between arteries (aorta, carotid and femoral arteries) and veins; therefore, it is difficult to establish the potential prognostic significance of its blood levels [30, 31]. Because the relationship we found is not straightforward, further study of this question is required.

According to the correlation analysis, a moderate correlation between MMP-14 and VEGF levels was observed in patients with hemodynamically significant stenosis. A similar correlation was found in the general group of CAD patients (INOCA and coronary artery obstruction). The proatherogenic role of MMP-14 has been demonstrated in many studies. VEGF, which induces angiogenesis in plaques, is known to play a critical role in the development of plaque instability [32]. In the group of patients with coronary artery obstruction, a correlation was found between VEGF levels and miRNA-34a expression. This miRNA plays an important role in the pathogenesis of CVDs by affecting many important biological and cellular functions such as differentiation, proliferation, and apoptosis of cardiovascular cells [33]. However, the main limitation of the use of miRNAs as diagnostic or prognostic markers in CAD is the influence of age-related factors on their expression, which requires further research [34].

Correlation analysis of the parameters in all patients with CAD (INOCA and obstructive coronary artery lesion) showed a moderate relationship between MMP-1 and VEGF levels. A similar correlation was shown in an animal model of aortic plaque [35]. MMP-1 expression is increased by proinflammatory cytokines in inflammatory and autoimmune diseases. The expression of MMP-1 and MMP-9 correlated with severe anterior descending artery stenosis in patients with CAD [36].

Based on the results of the ROC analysis, we concluded that MMP-9 and VEGF serve as predictors of coronary artery obstruction. Similar results were obtained by Caselli et al. [37] in a prospective observation of patients with obstructive CAD: MMP-9 was a predictor of progression of atherosclerotic stenosis. Thus, MMPs not only contribute to the progression of coronary atherosclerosis, but also lead to plaque destabilization, rupture, and the development of adverse cardiovascular outcomes.

Mirhafez et al [23] found that low serum VEGF levels (cutoff 37.18 pg/mL) with a sensitivity of 92.1%, specificity of 99.2% and prognostic value of a positive test result of 100% were associated with the severity of CAD in patients with hemodynamically significant stenosis. Therefore, this indicator can be recommended as a diagnostic marker for the degree of coronary artery stenosis [23].

Conclusion

Matrix metalloproteinase-9 activation predominated in patients with obstructive coronary artery disease, whereas matrix metalloproteinase-1 activity was higher in the group with nonobstructive coronary artery disease. It should be noted that the level of matrix metalloproteinase-9 greater than 4.83 ng/mL may be a predictor of hemodynamically significant coronary artery obstruction in patients with coronary artery disease (sensitivity 75.9%, specificity 77.8%).

The maximum level of vascular endothelial growth factor was observed in patients with nonobstructive coronary artery disease, and according to ROC analysis, the absence of hemodynamically significant coronary artery stenosis can be assumed (sensitivity 86.2%, specificity 88.9%) if the value is higher than 27.23 pg/mL.

The expression of miRNA-34a was statistically significantly higher in patients with nonobstructive coronary artery disease and patients with coronary artery obstruction compared to the control group, indicating its contribution to the development and progression of coronary atherosclerosis. The potential use of this miRNA as a diagnostic marker for coronary artery disease may be possible in the future.

Further investigation of the role of matrix metalloproteinases, vascular endothelial growth factor and their epigenetic regulation in patients with different variants of coronary artery disease to screen for high-risk groups and to clarify their significance as prognostic markers seems reasonable.

Funding

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No conflict of interest is reported.

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