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MicroRNAs and Oxidative Stress Markers as Additional Diagnostic Criteria for Coronary Heart Disease

Abstract

The aim. To examine the significance of microribonucleic acids (miRNAs) and oxidative stress markers in predicting the onset of atherosclerosis and the connection between oxidative stress levels and miRNAs in individuals with coronary heart disease.

Materials and methods. Initially, 40 patients were divided as follows: 10 subjects without any lesions in coronary arteries (group 0), 4 patients with non-stenotic atherosclerosis (group 1), and 26 patients with significant multivessel atherosclerotic lesions (group 2). Various biochemical parameters were analyzed, including miRNA expression levels and common oxidative stress markers.

Results. The groups were comparable in terms of the patients' age, but there was unequal distribution of males and females in the angio-groups as per Fisher's exact test. We also analyzed the data separately for females, but no significant difference was found. There were significant differences in miRNA-122 levels, N-terminal prohormone of brain natriuretic peptide levels, lipid profiles, and oxidative stress markers between group 0 and groups with atherosclerotic lesions. Specifically, miRNA-122 levels were elevated in group 0, along with N-terminal prohormone of brain natriuretic peptide, triglycerides, ratio of triglycerides to high-density lipoprotein cholesterol, and oxidative stress markers. Conversely, compared to group 0, total cholesterol, high-density lipoprotein cholesterol, bilirubin, and specific glutathione levels decreased in patients with coronary lesions.

Conclusions. The study demonstrated the potential of miRNAs, particularly miRNA-122, as predictive biomarkers for atherosclerosis. Further research with larger cohorts is warranted to validate these findings and explore additional miRNA candidates and therapeutic interventions for cardiovascular diseases.

Keywords: *miRNA-122, glutathione, lipoprotein, biomarker, atherosclerosis, ischemic heart disease.*

Introduction. Atherosclerosis is a chronic inflammatory vascular disease characterized by disrupted cholesterol metabolism, atypical inflammatory response, endothelial dysfunction, apoptosis, vascular smooth muscle cell proliferation and lipid deposition in arterial walls [1]. An atherosclerotic lesion can provoke coronary artery disease, stroke, peripheral artery disease, or renal pathology, depending on the arteries affected. Therefore, there is a need to identify a reliable biomarker/set of biomarkers for early detection of the possible presence of atherosclerosis and progression of vascular lesions for the prevention of cardiovascular disease and successful treatment.

The occurrence of atherosclerotic plaques is intrinsically linked with an imbalance of different fractions of lipoproteins. The reduction of high-density lipoproteins (HDL), elevated triglycerides (TG) or increased low-density lipoproteins (LDL) shows negative effect on the progression of atherosclerotic lesions. At the same time, previous studies showed the importance of not only definite fractions of lipids but also ratios thereof. For example, a TG/HDL ratio is associated and has been positively correlated with revealed coronary atherosclerosis [2].

On the other hand, one of the well-established biochemical markers which have an antioxidative capacity is

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bilirubin. Earlier studies have proved that elevated serum bilirubin may be one of protecting substances for coronary arteries and peripheral arteries against atherosclerosis [3].

Microribonucleic acids (miRNAs) are small non-coding ribonucleic acids (RNAs) that regulate the expression of different genes and, thus, take part in multiple signaling pathways and cell-to-cell communication [4]. Since miRNAs control the functioning of cardiovascular system cells, they can provide additional information for the understanding of pathogenic processes such as inflammation, atherosclerotic lesions, tissue fibrosis, myocardial hypertrophy and fundamental causes of diseases such as myocardial infarction, heart failure, and multiple varieties of arrhythmias. Furthermore, the target genes of these miRNAs participate in several pathways associated with metabolic diseases. In addition, interactions between miRNA and long non-coding RNAs, as well as miRNA and small molecules were found, suggesting that some molecules can modulate gene expression indirectly [5]. Moreover, the pathogenesis of numerous diseases can usually be traced by common oxidative stress markers such as superoxide dismutase, malondialdehyde, advanced glycation end product (AGE) levels, peroxidase and catalase activity, as well as by reduced glutathione (GSH) to oxidized glutathione (GSSG) ratio, and quantities of different types of miRNAs in blood [6]. These parameters may be predictors of endothelial dysfunction, atherosclerosis, organ failure, and cardiovascular complications [7]. Thus, it is essential to study the interconnection between early indicators of oxidative stress and miRNAs for a deeper understanding of pathological processes that could be used to prevent atherosclerosis and improve treatment.

In this study, we investigated whether miRNA-122, miRNA-27a, and miRNA-29a could be relevant predictors for atherosclerosis. The second research question was whether there is a relationship between the level of oxidative stress and miRNA quantities in patients with coronary heart disease. The unveiling of such dependencies between the biomarkers mentioned above and atherosclerosis can be applied to the assessment of the cardiovascular system condition.

The aim. To examine the significance of miRNAs and oxidative stress markers in predicting the onset of atherosclerosis and the connection between oxidative stress levels and miRNAs in individuals with coronary heart disease.

Materials and methods. *Ethics aspects.* The ethics committees of the Center Review Board approved the study protocol. All the participants gave their written informed consent. The Declaration of Helsinki (2000) and applicable national standards regarding their participation in research have been taken into account.

Patient selection. Initially, 40 patients were recruited from the Cardiological Department of the Ukrainian Children's Cardiac Center, Clinic for Adults (Kyiv, Ukraine) in 2019. Based on the results of coronary angiography, the patients were divided as follows: 10 subjects without lesions in coronary vessels (group 0), 4 patients with non-stenotic atherosclerosis (group 1), and 26 patients with significant multivessel atherosclerotic lesions (group 2).

All the patients underwent the same diagnostic procedure which included both clinical examination and laboratory testing. Venous blood samples were taken from each subject and centrifuged before testing. Biochemical analyses, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, TG, total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C), were conducted using an AU 480 chemistry analyzer (Beckman Coulter, Brea, CA, United States). All the tests were performed according to the manufacturers' instructions. The N-terminal prohormone of brain natriuretic peptide (NT-proBNP) level was analyzed using an enzyme-linked fluorescence assay, automatic miniVIDAS® (bioMerieux, Craponne, France).

Collection and storage of samples for miRNA measurement. Blood samples were collected in 10 mL vacutainers during clinical assessments. The blood was left at room temperature to clot for 15-30 minutes. Afterwards, the samples were centrifuged at 1500 x g for 10 minutes at +4 °C to remove the clot. The supernatant was transferred into clean tubes and stored initially at -76 °C, then at -20 °C until use [8].

Measurement of miRNA expression. The levels of three circulating miRNAs in serum were identified by quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR). Briefly, before extracting the RNA, the serum was gradually thawed, centrifuged at 10000 x g for 10 min at 4 °C. Isolation and purification of RNA were performed according to the manufacturer's manual for the RIBO-prep commercial kit (AmpliSens). To each sample, the spike-in control (Caenorhabditis elegans) was added. The samples of purified RNA were stored at -20 °C.

Subsequent reactions of polyadenylation and first strand complementary DNA (cDNA) synthesis were performed using the 1st-Strand cDNA Synthesis Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's manual. The qRT-PCR reaction mixture was prepared using a commercial PCR reagent kit (Syntol) with the addition of the SybrGREEN 1 intercalating agent. The expression of three human-specific miRNAs was measured using particular primers: miRNA-27a-3p (5'-UUCACAGUGGCUAAGUUCCGC-3'), miRNA-29a-3p (5'-AGCACCAUCUGAAAUCGGUUA-3'), miRNA-122 (5'-UGGAGUGUGACAAUGGUGUUUG-3'). Regardless of the type of miRNA, the Universal Reverse Primer was added to each reaction. Caenorhabditis elegans miRNA-39p (cel-miRNA-39) was used as an external control (spike-in) for each sample to normalize the efficiency of miRNA extraction and cDNA synthesis. This is a widely used practical method in the

measurement of circulating miRNAs. The reverse transcription reaction was performed using the Bio-Rad iCycler MyIQ thermal cycler (Bio-Rad Laboratories, Foster City, CA, USA). The thermal cycling profile was as follows: 95 °C for 5 minutes; 59 cycles: 95 °C for 10 s, annealing temperature 60-69 °C for 15 s, 72 °C for 30 s, signal acquisition. For each of these miRNAs, the appropriate annealing temperature was determined for amplification (60 °C for miRNA-122, 62 °C for miRNA-29a and miRNA-27a, and 69 °C for miRNA-39p). qRT-PCR reactions were performed in duplicates for all the samples. Relative expression levels of miRNAs were calculated using the Cy0 normalization method, which was shown to be more accurate for quantifications [9].

Serum protein concentration measurement. The Bradford protein assay with minor modifications was used to quantify the protein concentration in the samples. The Bradford working solution was prepared by mixing Coomassie Brilliant Blue G-250 with 50 mL 95% ethanol and 100 mL 85% (w/v) of phosphoric acid and adjusting the volume to 1 L with distilled water [10].

The standard solutions of different concentrations (in mg/mL) of bovine serum albumin mixed with 0.9% NaCl buffer were prepared in order to build a calibration curve. To perform the Bradford protein assay, the 3 μL of the sample were pipetted into a 96-well plate and 172 μL of the Bradford working solution were subsequently added. The plate was incubated for 5 minutes, then the color intensity was measured spectrophotometrically (Thermo Scientific Varioskan Flash) at a wavelength of 595 nm against the blank control which contained 3 μL of 0.9% NaCl buffer. The exact protein concentration was determined according to the constructed calibration curve of absorbance versus micrograms of protein.

Oxidative stress evaluation. The concentration of serum proteins, modified by nonspecific glycation – AGEs, was assessed by spectro-photofluorimetry according to the protocol described to measure the fluorescence of AGEs. The serum samples were centrifuged at 20000 x g for 10 min at 4 °C to precipitate debris. The trichloroacetic acid (50 μL) was added to deproteinize the aliquot of serum (200 μL). Then 150 μL of Milli Q H_2O and 350 μL of chloroform were added, vortexed for 60 seconds and centrifuged at 14000 rpm. 200 μL of the supernatant was pipetted into each well in triplicate. After excitation at 355 nm, the fluorescence intensity was measured at 440 nm. The results were expressed as arbitrary units and corrected for serum protein levels (measured by absorptiometry at 280 nm) in serum samples [11].

The standard procedure was used to assess GSH/GSSG content in serum in the presence of imidazole compounds [12]. Briefly, 10 μL of the sample were mixed with 100 μL of buffered formaldehyde (1:4 [v/v] 37% formalin: 0.1 M Na_2HPO_4). 3-5 minutes later 1 mL of phosphate-EDTA buffer (0.1 M Na_2HPO_4 with 5 mM EDTA) was added followed by 100 μL of o-phthalaldehyde. Eppendorf tubes with mixtures were kept in the dark place for 45 minutes at room temperature. To conduct the same reaction at pH 13, which allows GSSG to be measured, 0.1 M NaOH was used instead of phosphate-EDTA buffer. After 45 minutes at room temperature, the fluorescence was measured in Thermo Scientific Varioskan Flash. The excitation wavelength was 345 nm, and the fluorescence wavelength was 425 nm. The calibration curve was built using the measurements taken from the following GSH and GSSG concentrations: 1000, 500, 200, 100, 50 μM and 100, 50, 10, 5, and 1 μM, respectively.

Statistical analysis. Statistical analysis was performed using Statistica 8.0 (Stat Soft Inc.) and SPSS Statistics (v. 26, IBM Corporation) software. The Shapiro-Wilk test was used to check the normal distribution of analyzed parameters. Further statistical analysis was performed with non-parametric tests (median with I and III quartiles). The equality of male and female proportions in groups was assessed with Fisher's exact test. Spearman's test was used for correlations assessment. The significance of miRNA associations as other biochemical parameters across angio-groups was tested by the Jonckheere-Terpstra (JT) trend test and Kruskal-Wallis ANOVA test. The comparison of control individuals with patients of angio-groups 1 and 2 was performed using Mann-Whitney U Test.

Results. There was no statistical difference in age between the study groups. According to Fisher's exact test, proportions of males and females were not equal in the angio-groups. To analyze the characteristics of the participants, non-parametric tests were used. The measured parameters of all the participants are represented in Table 1. The significance is shown for the difference between the group 0 and all the patients with atherosclerotic coronary vessel lesions (groups 1 and 2 combined).

Statistically significant variance between two groups is observed for the values of miRNA-122, NT-proBNP, TC, TG, HDL-C, non-HDL-C, TG/HDL-C, bilirubin, GSH/GSSG, GSH/total glutathione and GSSG/total glutathione (Table 1). Specifically, the miRNA-122, NT-proBNP, TG, TG/HDL-C, GSH/GSSG, GSH/total glutathione values are higher in patients with atherosclerosis than in those without it. At the same time, the levels of TC, HDL-C, bilirubin, and GSSG/total glutathione are decreased in the groups 1 and 2 compared to group 0.

Where the number of women in the study was much higher than men, we have checked the obtained results for significance for females separately (Kruskal-Wallis test), but no significant difference was detected.

In order to check the obtained results for possible higher significance between parameters among all angio-groups, we compared all of them separately with the JT trend test (Table 2).

Our research has found notable variations among parameters such as miRNA-122, TC, HDL-C, LDL-C,

Table 1

Comparison of patients of group 0 with those of groups 1 and 2 using Mann-Whitney U test

Parameter	Group 0 (n=10)	Groups 1&2 (n=30)	Mann-Whitney U test*
M/F	6/4	4/26	
Age	66 (77-63)	65 (59-70)	0.569
miRNA-27a	0.106 (0.079-0.149)	0.121 (0.083-0.217)	0.475
miRNA-29a	0.053 (0.035-0.151)	0.057 (0.040-0.113)	0.939
miRNA-122	0.029 (0.010-0.047)	0.090 (0.023-0.236)	**0.012**
NT-proBNP, pg/mL	118.2 (40.0-206.0)	193.8 (121.6-429.5)	**0.033**
TC, mmol/L	196.9 (173.7-212.3)	154.4 (119.7-181.4)	**0.012**
TG, mmol/L	92.9 (60.2-106.2)	121.2 (92.9-172.6)	**0.014**
HDL-C, mmol/L	44.8 (44.1-48.7)	37.1 (33.6-42.5)	**0.001**
LDL-C, mmol/L	123.7 (105.9-147.3)	88.7 (68.1-118.7)	0.10
non-HDL-C, mmol/L	143.3 (128.8-168.2)	116.3 (79.1-147.4)	**0.036**
TC/HDL-C	4.07 (3.56-4.56)	4.14 (3.27-4.81)	0.939
TG/HDL-C	1.93 (1.34-2.09)	3.45 (2.29-4.42)	**0.001**
LDL-C/HDL-C	2.57 (2.11-3.14)	2.43 (1.80-2.85)	0.450
non-HDL-C/HDL-C	3.07 (2.56-3.56)	3.14 (2.27-3.81)	0.914
ALT, U/L	20.9 (16.4-34.0)	29.6 (18.4-42.1)	0.331
AST, U/L	19.9 (16.7-23.9)	23.1 (17.9-32.9)	0.488
AST/ALT	0.80 (0.66-1.19)	0.84 (0.67-1.18)	0.939
Bilirubin, μmol/L	15.7 (12.8-24.1)	12.3 (11.5-16.0)	**0.039**
GSH, μmol/L	503.2 (471.8-510.8)	549.6 (484.4-616.9)	0.116
GSSG, μmol/L	499.1 (450.0-540.9)	497.3 (467.8-514.9)	0.890
GSH/GSSG	0.985 (0.932-1.132)	1.154 (1.043-1.242)	0.050
Total glutathione, μmol/L	1479.6(1388.7-1586.3)	1549.0(1409.3-1630.9)	0.569
GSH/total glutathione	0.330 (0.318-0.361)	0.366 (0.343-0.383)	0.050
GSSG/total glutathione	0.335 (0.319-0.341)	0.317 (0.308-0.329)	0.050
AGEs, mg/mL	0.008 (0.004-0.013)	0.005 (0.004-0.007)	0.127

Note. All the characteristics (except "M/F") are represented as medians (Q1–Q3). The values marked in bold indicate significance (*p*-value 0.05). *asymptotic significance (2-sided test).

TG/HDL-C, and bilirubin. Additionally, we used the JT test to compare these parameters in pairs among different angio-groups. The most significant differences were observed between the control group and patients with multivessel atherosclerosis (Table 3).

The intercorrelation of the studied parameters was analyzed with a Spearman correlation test and visualized with a heat map (Fig. 1).

For example, significant negative correlations have been found between angio-groups in TC, HDL, LDL, and bilirubin, while positively correlating parameters were sex, miRNA-122, and TG/HDL ratio. When investigating the control and the comparison group (1&2 angio-groups together) even more correlations have been revealed, specifically, positive correlations for NT-proBNP (r=0.34, p=0.03), TG (r=0.39, p=0.01), GSH/GSSG and GSH/total glutathione (r=0.32, p=0.05). Negatively correlated were non-HDL (r=-0.34, p=0.03) and GSSG/total glutathione (r=-0.32, p=0.05). We observed that sex had negative correlation with TC (r=-0.41, p=0.01), HDL (r=-0.48, p=0.002), LDL (r=-0.37, p=0.02), and positively correlated with non-HDL (r=0.36, p=0.02). The age of the participants negatively correlated with ALT and AGEs levels, while positively correlated with HDL. The expression of miRNA-27a strongly correlated with miRNA-29a (r=0.79, p<0.001), and these two miRNAs moderately correlated with GSH (r=0.38, p=0.02, and r=0.31, p=0.05 respectively); miRNA-29a also negatively correlated with AGEs level (r=-0.38, p=0.02). At the same time, a positive correlation between angio-groups and miRNA-122, which is statistically significant (*r*=0.38, *p*=0.02), and negative correlations between this type of miRNA, GSSG, and AGEs were found. Additionally, TC levels negatively correlated with angio-groups and male gender, and positively correlated with lipidogram parameters: HDL (r=0.61, p<0.001), LDL/HDL (r=0.76, p<0.001), TC/HDL (r=0.68, p<0.01). Moreover, this was most noted with LDL (r=0.96, p<0.001) and non-HDL (r=0.99, p<0.001).

On the other hand, TG negatively correlated with HDL (r=-0.37, p=0.02), and positively correlated with TG/HDL

Table 2

Comparison of characteristics of participants across angio-groups, Jonckheere-Terpstra trend test

Parameter	Group 0 (n=10)	Group 1 (n=4)	Group 2 (n=26)	JT Test*
M/F	6/4	1/3	3/23	
Age	66 (77-63)	64 (57-72.5)	65 (59-70)	0.625
miRNA-27a	0.106 (0.079-0.149)	0.159 (0.100-0.232)	0.118 (0.083-0.217)	0.667
miRNA-29a	0.053 (0.035-0.151)	0.091 (0.051-0.127)	0.054 (0.004-0.112)	0.889
miRNA-122	0.029 (0.010-0.047)	0.078 (0.027-0.163)	0.197 (0.225-0.239)	**0.017**
NT-proBNP, pg/mL	118.2 (40.0-206.0)	160.9 (123.2-444.3)	240.3 (96.0-429.5)	0.059
TC, mmol/L	196.9 (173.7-212.3)	158.3 (123.5-173.7)	152.5 (119.6-181.4)	**0.037**
TG, mmol/L	92.9 (60.2-106.2)	146.0 (139.4-150.9)	109.7 (90.3-182.3)	0.085
HDL-C, mmol/L	44.8 (44.1-48.7)	39.2 (33.6-41.6)	36.7 (33.6-44.1)	**0.005**
LDL-C, mmol/L	123.7 (105.9-147.3)	88.6 (58.0-103.4)	88.7 (68.1-120.6)	**0.044**
non-HDL-C, mmol/L	143.3 (128.8-168.2)	119.0 (89.8-132.1)	115.7 (79.1-150.5)	0.090
TC/HDL-C	4.07 (3.56-4.56)	4.03 (3.60-4.20)	4.22 (3.27-4.99)	0.627
TG/HDL-C	1.93 (1.34-2.09)	3.85 (3.54-4.27)	3.19 (2.13-4.42)	**0.017**
LDL/HDL	2.57 (2.11-3.14)	2.25 (1.65-2.50)	2.49 (1.80-2.95)	0.770
non-HDL-C/HDL-C	3.07 (2.56-3.56)	3.03 (2.60-3.20)	3.22 (2.27-3.99)	0.607
ALT, U/L	20.9 (16.4-34.0)	26.8 (16.1-37.5)	30.5 (18.4-42.1)	0.232
AST, U/L	19.9 (16.7-23.9)	24.4 (14.7-34.9)	23.1 (18.1-32.1)	0.470
AST/ALT	0.80 (0.66-1.19)	0.96 (0.69-1.36)	0.83 (0.67-1.18)	0.889
Bilirubin, μmol/L	15.7 (12.8-24.1)	13.6 (12.7-31.1)	12.1 (11.4-16.0)	**0.011**
GSH, μmol/L	503.2 (471.8-510.8)	640.4 (550.7-666.6)	544.2 (484.2-600.8)	0.374
GSSG, μmol/L	499.1 (450.0-540.9)	511.8 (496.4-536.8)	494.4 (454.9-514.3)	0.487
GSH/GSSG	0.985 (0.932-1.132)	1.221 (1.100-1.251)	1.123 (1.043-1.242)	0.133
Total glutathione, μmol/L	1479.6 (1388.7-1586.3)	1664.4 (1543.5-1740.3)	1539.8 (1399.8-1618.2)	0.934
GSH/total glutathione	0.330 (0.318-0.361)	0.379 (0.354-0.385)	0.360 (0.343-0.383)	0.133
GSSG/total glutathione	0.335 (0.319-0.341)	0.310 (0.308-0.323)	0.320 (0.308-0.329)	0.133
AGEs, mg/mL	0.008 (0.004-0.013)	0.004 (0.003-0.006)	0.005 (0.004-0.007)	0.459

Note. All characteristics (except "M/F") are represented as medians (Q1–Q3). The values marked in bold indicate significance (p-value 0.05). *asymptotic significance (2-sided test).

Table 3

Pairwise comparisons (JT test) of measured parameters across angio-groups

Angio-groups compared	miRNA-122	TC	HDL-C	LDL-C	TG/HDL-C	Bilirubin
0 and 1	0.304	0.058	**0.016**	0.051	0.016	1.000
0 and 2	**0.020**	**0.035**	**0.006**	**0.030**	**0.005**	**0.036**
1 and 2	1.000	1.000	1.000	0.906	0.493	0.191

Note. The values marked in bold indicate significance (*p*-value 0.05). Significant values are adjusted by Bonferroni correction for multiple tests.

(r=0.92, p<0.001), non-HDL/HDL (r=0.42, p=0.01), and ALT (r=0.32, p=0.04); positive correlation was also observed between ALT and AST levels (r=0.69, p<0.001).

The Spearman correlation test was also done for all parameters for the female group only to evaluate the sex-specific difference. Interestingly, no notable differences were seen, except for the newly occurred significant positive correlation between HDL and miRNA-29a levels (r=0.39, p=0.03).

Binary logistic regressions have been built for all parameters that correlated with the variable control/experiment (Table 4). Statistically significant values have been obtained for NT-proBNP odds ratio [OR] 1.01, confidence interval [CI]: 1-1.02), TC (OR 0.98, CI: 0.96-1), TG (OR 1.03, CI: 1-1.05), HDL-C (OR 0.83, CI: 0.72-0.96), LDL-C (OR 0.98, CI: 0.96-1), TG/HDL-C (OR 4.48, CI: 1.37-14.62). It should be noted that other parameters, namely miRNA-122, non-HDL-C, GSH/GSSG, GSH/total glutathione and GSSG/total

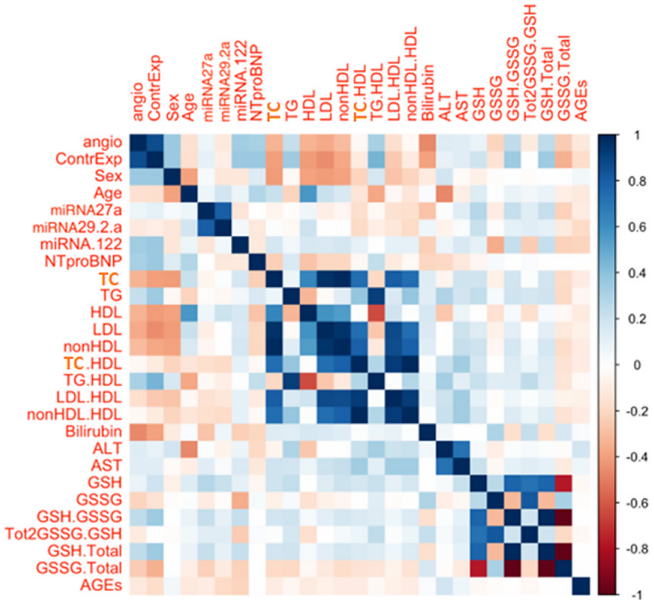

Fig. 1. *Correlation heat map of biochemical parameters, miRNAs, and group characteristics: darker cells indicate significant positive correlations. Pale colors are non-significant correlations; p-value 0.05*

glutathione but supposedly may change in the case of the greater study cohort. Also, an unexpected observation has been detected, namely, AGEs negatively correlated with the age in all study groups: r =-0.2884.

Discussion. Multiple studies have shown that miRNAs are expressed in vascular walls, and their dysregulation can cause vascular diseases. They are involved in the development of atherosclerosis through their target genes and are considered potential biomarkers for cardiovascular and cardiometabolic disorders and promising therapeutic targets [13]. Moreover, cardiovascular diseases are also associated with age due to the rise of oxidative stress, DNA damage and endothelial cell senescence. Additionally, an increase in reactive oxygen species (ROS) quantity can initiate the first steps of fibrotic diseases through fibroblast activation and collagen deposition, and contribute to the occurrence of atherosclerosis, diabetic vasculopathy, hypercholesterolemia, myocardial infarction and stroke [14].

In this study, we have investigated the differences in levels of miRNA-122, -29a, -27a, and markers of oxidative stress, together with lipid and liver biochemical parameters in patients with atherosclerosis aiming to detect the interrelating characteristics and define reliable signs of the occurrence of atherosclerosis. We have found that only miRNA-122 was notably upregulated in the patients with atherosclerosis, and negatively correlated with AGEs levels. Previous studies have indicated that miRNA-122 mediates inflammation, apoptosis, oxidative stress, and fibrosis in cardiovascular dysfunctions [15]. Meanwhile, it has been discovered that oxidative stress induced by H_2O_2 suppresses miRNA-122 expression in HUVECs. At the same time, miRNA-122 overexpression antagonizes H_2O_2 triggered oxidative stress injury by lowering the ROS and increasing superoxide dismutase levels [16]. Moreover, it has been shown that the increase in miRNA-122 induces endothelial cell apoptosis [1], which is consistent with our results.

Table 4

Odds ratios for the parameters which correlated with control/experiment variable

							95% CI for Exp(B)	
Parameter	**B**	**SE**	**Wald**	**df**	**Sig.**	**Exp(B)**	**Lower**	**Upper**
Sex	2.28	0.84	7.35	1.00	**0.01**	9.75	1.88	50.56
miRNA-122	14.72	8.28	3.16	1.00	0.08	2.48E+06	0.22	2.78E+13
NT-proBNP	0.01	0.00	3.95	1.00	**0.05**	1.01	1.00	1.02
TC	-0.02	0.01	4.58	1.00	**0.03**	0.98	0.96	1.00
TG	0.03	0.01	4.47	1.00	**0.03**	1.03	1.00	1.05
HDL-C	-0.18	0.07	6.36	1.00	**0.01**	0.83	0.72	0.96
LDL-C	-0.02	0.01	4.89	1.00	**0.03**	0.98	0.96	1.00
non-HDL-C	-0.02	0.01	3.35	1.00	0.07	0.98	0.96	1.00
TG/HDL-C	1.50	0.60	6.18	1.00	**0.01**	4.48	1.37	14.62
Bilirubin	-0.03	0.04	0.42	1.00	0.52	0.97	0.90	1.05
GSH/GSSG	4.74	2.56	3.43	1.00	0.06	114.38	0.76	17270.33
GSH/total glutathione	21.26	11.78	3.26	1.00	0.07	1.71E+09	0.16	1.83E+19
GSSG/total glutathione	-42.52	23.56	3.26	1.00	0.07	0.00	0.00	39.01

Note. The values marked in bold indicate significance (*p*-value 0.05).
B, values for the logistic regression equation for predicting the dependent variable from the independent variable (in log-odds units); SE, standard error; Wald and Sig., Wald chi-square value and 2-tailed p-value used in testing the null hypothesis that the coefficient (parameter) is 0; Exp(B), odds ratios for the predictors.

Additionally, in this study miRNA-27a and miRNA-29a had positive correlation with GSH, and miRNA-29a also negatively correlated with AGEs, which indicates their role in the antioxidant system. AGEs are the markers of redox system imbalance with oxidative stress prevalence. They accumulate during ageing in extracellular matrix proteins, exacerbating the development of pathological conditions such as neurodegenerative diseases, diabetes, or atherosclerosis [17]. The latter also has been confirmed in our study, as AGEs negatively correlate with the age of all groups of participants.

While other research demonstrates that miRNA-29 upregulation is associated with cardiovascular diseases [18], in our study cohort a non-significant increase in its level in the group 2 and a significant increase in group 1 has been detected. Consistent with our findings, miRNA-29 has been considered to be protective against the accumulation of oxidative stress, and its rise can limit cardiac fibrosis [19]. Indeed, many investigations suggest that aberrant expression of miRNA-27 is associated with atherosclerosis, as well as angiogenesis, adipogenesis, inflammation, lipid metabolism, oxidative stress, insulin resistance and type 2 diabetes [20].

Furthermore, significant differences between groups have been shown for other parameters. Specifically, NT-proBNP, TG, TG/HDL-C, GSH/GSSG, and GSH/total glutathione was elevated in atherosclerosis groups. At the same time, the levels of TG, HDL-C and bilirubin were reduced in the group with atherosclerosis compared to group 0. Higher TG/HDL ratio has been also claimed to relate to coronary atherosclerosis in previous studies. Other researchers revealed a positive correlation of TG/HDL ratio with global cardiac microcalcification – one of the markers of coronary atherosclerosis [3]. The metabolism of TG-rich lipoproteins is involved in vascular inflammation, and their high levels increase the catabolism of cardio-protective HDL lipoprotein, which is involved in reverse cholesterol transport [21]. Meanwhile, miRNAs regulate reverse cholesterol transport, HDL biogenesis and function, cellular cholesterol efflux, hepatic HDL-C uptake and bile acid synthesis and secretion [22]. The antiatherogenic effect of HDL has been also explained by the finding that HDL-enriched miRNAs were shown to influence the gene expression in recipient cells [23]. Another pathway of miRNAs action was described for miRNA-27a/b, which regulates cholesterol efflux in hepatocytes and macrophages via influencing the expression of member 1 of human transporter sub-family ABCA [24]. For example, subjects with hypercholesterolemia and dyslipidemia had differences in HDL-miRNA levels compared to the healthy group, which provides evidence for its involvement in the occurrence of atherosclerosis and cardiometabolic disorders [25]. Our study has revealed that the direct correlation of HDL with miRNA-29a levels is present only in female participants. At the same time, Zhang L et al. (2018) showed that treatment with miRNA-29a-3p modulates serum levels of TC, TG, LDL-C – the risk factors, and HDL-C – the protective factor, in an atherosclerotic model. In contrast, in the present study, we have not found any associations of LDL-C levels with investigated miRNAs [26].

It should be noted that the growth of GSH/GSSG and GSH/total glutathione ratios in the study group demonstrated in this study indicates the proper defense of the antioxidant system against high concentration of ROS, which contradicts numerous articles, where oxidative stress developed with a decrease in GSH and an increase in GSSG levels in the patients with cardiovascular diseases [27]. The results of the present study may suggest a novel pathway of how GSH form has antioxidative and antiatherogenic properties, which may be of benefit in remission of atherosclerosis. The reduced (GSH) and oxidized (GSSG) forms make a redox buffer, with the GSH quantity prevailing over the GSSG under physiological conditions [28]. miRNAs, mutations and long non-coding RNAs regulate the tissue-specific level of GSH. However, Zhang X et al. (2010) described that an increased GSH/GSSG ratio raised glutathione peroxidase 1, and reduced levels of ROS represented the reductive stress in myopathic hearts [29].

Thus, low concentration of GSH can result in diseases caused by oxidative stress, and, on the contrary, high average GSH concentration will lead to a prolonged state of reductive stress which may be one of the contributors to cardiovascular disease. Rajasekaran et al. confirmed that elevated levels of GSH, nicotinamide adenine dinucleotide phosphate and antioxidative pathway enzymes are associated with reductive stress, while declined oxidative stress biomarkers could be associated with protein aggregation cardiomyopathy and cardiac hypertrophy [30]. Such shifts towards the reductive state that induces the "reductive stress-related redox collapse" correlate with cytotoxicity, mitochondrial dysfunction, lipid damage, triacylglycerol deposition and cardiac ischemic injury.

It has been shown previously that the link between serum bilirubin and atherosclerosis has a dose-dependent inverse correlation with plaque calcifications and coronary artery atherosclerosis, which is consistent with our results. These results suggest that serum bilirubin may be a protective biomarker of coronary artery disease, as especially in slightly elevated concentrations it is more effective against LDL oxidation. Its action involves negative correlation with TC and LDL-C and a positive correlation with HDL-C. However, we could not find such an association in our study. Meanwhile, Vítek et al. reported that patients with a mild increase in serum bilirubin had lower incidence of carotid atherosclerosis [3]. Those findings were confirmed by Kundur et al. about the favorable influence of bilirubin on lipid metabolism, its anti-ageing effects, possible anti-thrombotic effects through suppression of platelet activation, and high immunosuppressive effects at almost all levels of the innate and adaptive immune response, as

well as anti-mutagenic effects [31]. Other hepatic biochemical markers in our study correlated with certain lipid parameters showing their involvement in lipid metabolism. In brief, ALT positively correlated with TG, TG/HDL, and AST, while the last one directly correlated with HL/HDL, LDL/HDL, and non-HDL/HDL. Determination of serum AST and ALT activity is the method most used for assessing hepatocellular damage. However, it is hard to evaluate AST and ALT serum levels to assess the tissue-specific disease, as they also occur in skeletal or heart muscles [32]. At the same time, measurement of ALT enzyme activity and the level of circulating miRNA-122 can indicate liver-specific diseases [33]. These two parameters had no intercorrelation in our study group, which may supposedly indicate the absence of severe liver damage.

Conclusion. The main benefit of our study is the complex investigation of differences in levels of miRNA-122, -29a, -27a and redox state together with lipid and liver biochemical parameters in patients with atherosclerosis. However, the limiting factor of our study is a relatively small cohort. So, a larger cohort of subjects is needed to verify and expand our findings.

Conflict of interests. Authors declare no conflict of interests.

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МікроРНК та показники окисного стресу як потенційні маркери в діагностиці хронічного коронарного синдрому

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Резюме. Атеросклероз є одним з найпоширеніших захворювань, що може спровокувати ішемічну хворобу серця, інсульт, захворювання периферичних артерій або ниркову патологію залежно від локалізації ураження артерій. Виявлення надійного біомаркера/набору біомаркерів є вкрай важливим для раннього виявлення як уже наявного атеросклерозу, так і прогресування уражень для своєчасної профілактики серцево-судинних захворювань та успішного лікування. Мікрорибонуклеїнові кислоти (мікроРНК) є малими некодуючими рибонуклеїновими кислотами. Вони контролюють функціонування клітин серцево-судинної системи та можуть надати додаткову інформацію для розуміння патогенетичних процесів, таких як запалення, атеросклеротичне ураження, фіброз тканин, гіпертрофія міокарда. Також у патогенезі численних захворювань важливу роль відіграють

маркери окисного стресу, дослідження яких є доцільним як імовірних маркерів атеросклеротичного ураження в цілому і безпосередньо наявної ішемічної хвороби серця.

Мета – проаналізувати значення мікроРНК і маркерів окисного стресу, як предикторів розвитку атеросклерозу, та взаємозв'язку між рівнем окисного стресу та мікроРНК у пацієнтів з ішемічною хворобою серця.

Матеріали та методи. 40 пацієнтів були розділені на контрольну групу, що складалася з 10 здорових суб'єктів (група 0), 4 пацієнти з нестенотичним ураженням вінцевих артерій (група 1) і 26 пацієнтів з багатосудинним атеросклеротичним ураженням (група 2). Проаналізовано біохімічні показники, що включали як стандартні параметри, так і рівні експресії мікроРНК та загальні маркери окисного стресу.

Результати. Групи були зіставні за віком, проте спостерігався нерівномірний розподіл щодо осіб чоловічої та жіночої статі поміж груп, тому всі параметри було проаналізовано окремо для жінок і суттєвої різниці не відзначено. Виявлено значні відмінності в рівнях мікроРНК-122, N-кінцевий пропептид натрійуретичного гормону (NT-proBNP), ліпідному профілю та маркерах окисного стресу між групою 0 та групами з атеросклеротичними ураженнями. Зокрема, рівень мікроРНК-122 був підвищений у цій групі пацієнтів разом з NT-proBNP, тригліцеридами, співвідношенням тригліцеридів/холестерину ліпопротеїдів високої щільності та маркерами окисного стресу. І навпаки, порівняно з групою 0, загальний холестерин, холестерин ліпопротеїдів високої щільності, білірубін та показники глутатіону були знижені у пацієнтів з ураженням вінцевих судин.

Висновки. В нашому дослідженні було продемонстровано, що мікроРНК, особливо мікроРНК-122, може бути прогностично новим маркером атеросклеротичного ураження вінцевих судин. Подальше вивчення некодуючих рибонуклеїнових кислот з більшою когортою пацієнтів є доцільними для підтвердження цих висновків, оскільки може виявити нові таргетні молекули як для діагностики, так і для лікування серцево-судинних захворювань.

Ключові слова: *мікроРНК-122, глутатіон, ліпопротеїди, біомаркери, атеросклероз, ішемічна хвороба серця.*

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