Circulating microRNAs in patients with aneurysmal dilatation of coronary arteries

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Abstract. To understand the mechanism underlying coronary artery abnormal dilatation (CAAD), the present study identified and compared the expression of circulating microRNAs (miRNAs) in three groups of patients. Group 1 included 20 patients with CAAD, Group 2 included 20 patients with angiographically confirmed coronary artery disease (CAD), and Group 3 included 20 patients with normal coronary arteries (control). miRNAs were isolated from plasma samples and were profiled using PCR arrays and miRCURY LNA Serum/Plasma Focus PCR Panels. The present study demonstrated that the plasma miRNA levels were significantly different in Group 1 compared with in Group 2 and Group 3 (fold change >2 and P<0.05). The comparison of Group 1 with Group 3 identified 21 significantly upregulated and two downregulated miRNAs in patients with CAAD compared with in the control group. Moreover, six upregulated and two downregulated miRNAs were identified in patients with CAD compared with in the controls. The third comparison revealed four upregulated and three downregulated miRNAs in Group 1, when compared with patients with CAD. In conclusion, the present study identified a specific signature of plasma miRNAs, which were upregulated and downregulated in patients with CAAD compared with in patients with CAD and control individuals.

Introduction

The coronary artery abnormal dilatation (CAAD) is an uncommon cardiovascular disorder with an incidence ranging from 1.2 to 4.9% of patients undergoing coronary angiography (1). This pathology has been described interchangeably using the two terms: coronary artery aneurysm (CAA) and coronary artery ectasia (CAE) (2). However, these synonymously used terms refer to two different phenotypes. CAA is defined as a focal dilatation of the arteries with a diameter of 1.5 times the adjacent normal coronary artery, whereas CAE describes similar but more diffuse lesions (3). According to the anatomical shape of the dilated segment, CAA is classified into the fusiform type if the longitudinal diameter exceeds the transverse diameter or the saccular type in the reverse case (4). The presence of CAAD is associated with poor long-term outcomes, regardless of concomitant atherosclerotic coronary disease (5-7).

Clinical presentation includes asymptomatic cases and stable angina or acute coronary syndromes (8,9). Abnormal dilation of coronary arteries is attributed to atherosclerosis in 50% of cases, while the origin of 20-30% are considered inflammatory or congenital (10). Pathogenesis is multifactorial and can be influenced by many environmental and heritable risk factors. Understanding the underlying molecular and cellular mechanisms may contribute to diagnosing and preventing cardiovascular events. According to a growing body of literature, microRNAs (miRNAs) have been implicated in regulating human physiological processes, including gene expression in the cardiovascular system (11). MiRNAs could have a crucial role in physiological processes and disease development, including atherosclerosis, coronary artery disease (CAD), myocardial infarction (MI), heart failure (HF), and cardiac arrhythmias (12-14). MiRNAs are short (19-25 nucleotides), single-stranded, noncoding RNAs that function posttranscriptional regulation, attained by RNA degradation and translation silencing. MiRNAs regulate gene expression by binding to specific sites in the mRNA 3' untranslated region (15). A single miRNA downregulates numerous target genes, modulating complex physiological processes. MiRNAs are involved in cardiovascular remodeling, which results in cardiovascular diseases such as CAD, abdominal aortic aneurysm (AAA), and HF (16). Our understanding the roles of serum miRNAs in patients with CAAD is still not comprehensive; however, essential knowledge came from the studying selected groups

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of individuals with Kawasaki disease (KD) (17-19) and AAA (20,21).

Several studies have found that the miRNA profiles of serum exosomes or coronary artery tissues, including miR-23a, miR-27b, miR-223, and miR-145, are associated with acute KD, providing insight into the molecular mechanisms of the development of cardiovascular lesions (17-19). For example, increased levels of miR-23a contribute to cardiomyocyte apoptosis and may promote inflammatory responses by blocking macrophage autophagy activity (22). miR-145 is highly expressed in vascular smooth muscle cells (VSMCs), mediating phenotype by altering to proliferating neointimal cells (23). It is speculated that miR-145 modulates the generation of myofibroblasts from VSMC, which are involved in arterial wall destruction in acute KD (24). Interestingly, other studies suggest that noninflammatory vascular injury in animal models or human diseases characterized by chronic vascular inflammation, are associated with low levels of miR-145 (25,26). The expression of tissue miR-145 was downregulated at the site of experimental injury-induced lesions in animal models and human AAA (27). Similarly, plasma or serum levels of miR-145 in patients with stable CAD were lower than those in healthy controls (28). Interestingly, miR-223 is highly expressed in blood cells, such as neutrophils, eosinophils, monocytes, and platelets, and can then enter VSMCs to regulate their functions and atherogenesis via its target genes (29,30).

Our study aimed to identify the circulating miRNA signature in CAAD patients and explore its potential as a novel biomarker for these diseases.

Materials and methods

Study design and patient selection. A total of two hundred patients undergoing coronary angiography for angina symptoms were enrolled in the general cohort between March 2019 and March 2020, including 26 consecutive patients with CAAD. Due to technical exclusion (hemolysis), 20 patients meeting the criteria of CAE or CAA were included in the study cohort as Group 1. Moreover, two groups of 20 patients matched Group 1 in terms of sex and age from the overall cohort (Group 2 and Group 3) (Fig. 1). Patients with angiographically documented CAD were included in Group 2 (n=20), while patients with angiographic exclusion of coronary stenosis >50% were enrolled as control patients in Group 3 (n=20). Group 1 and 2 included patients aged 53-77 years, and Group 3 aged 51-78 years. The mean age was 66.1±7.2, 66.9±7.4, 65.1±7.4 for Groups 1, 2 and 3, respectively.

All patients with chest pain or discomfort were qualified for coronary angiography, according to the European Society of Cardiology (ESC) guidelines (31). CAE and CAA were defined as a diffuse or focal dilatation of the coronary artery with a diameter of 1.5 times the adjacent normal segment. The group also included patients with associated stenosis of the coronary arteries. The angiographic criteria for CAD were: coronary artery stenosis >90% or intermediate stenosis (50-90%) with documented ischemia or hemodynamically significant, defined as either fractional flow reserve (FFR) ≤0.80 or an instantaneous wave-free ratio (iFR) ≤0.89. All patients in the control group presented with normal ECG and echocardiography and had no evidence of ischemia during noninvasive stress tests. The exclusion criteria were as follows: i) Acute coronary syndrome; ii) elevated troponin I (TNI) or creatine kinase (CK-MB) levels; iii) history of severe hepatic and renal dysfunction; iv) leukemia, leukopenia, thrombocytopenia, or ongoing inflammatory and malignant diseases; v) systemic diseases of connective tissue; vi) interferon treatment; and vii) no informed consent.

The Institutional Review Board (or Ethics Committee) of Poznan University of Medical Sciences (protocol code, 985/18; date of approval, October 11, 2018) approved the protocols, and the study was conducted following the Declaration of Helsinki. We obtained written informed consent from each individual.

Sample collection and miRNA isolation. EDTA-blood samples (10 ml) were collected from all patients on the first day after the cardiac catheterization procedure and were processed within 30 min's of collection. Samples were centrifuged at 1300 g for 15 min at room temperature. The supernatant was transferred to RNase-free tubes and then stored at -80˚C.

The miRNAs were isolated from individual 200 μl frozen plasmas using the miRNeasy Serum/Plasma Advanced Kit (cat. no. 217204, Qiagen, Dusseldorf, Germany) according to the manufacturer's instructions. To correct sample-to-sample variation, a synthetic set of spike-ins UniSp2, UniSp4 and UniSp5 was applied [RNA Spike-In Kit, for reverse transcription (RT), cat. no. 339390, Qiagen]. Three of the RNA spike-in templates (UniSp2, UniSp4, and UniSp5) were premixed in one vial, each at a different concentration in 100-fold increments of UniSp2 (2 fmol/μl), UniSp4 (0.02 fmol/μl) and UniSp5 (0.00002 fmol/μl). Before starting the RNA isolation procedure, 1 μl of this RNA spike-in mix per RNA prep was aliquoted into 60 μl RPL lysis buffer. Approximate RNA quantity and quality were estimated using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham MA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Four microliters of each total RNA was reverse transcribed with a miRCURY LNA RT Kit (cat. no. 339340, Qiagen). The UniSp6 RNA spike-in from the miRCURY LNA RT Kit and cel-miR-39-3p RNA from the miRCURY LNA RT Kit were used as controls. RT temperature protocol: reverse transcription step 60 min at 42˚C, inactivation of reaction 5 min at 95˚C. All reverse transcribed samples were verified using the miRCURY LNA miRNA QC PCR Panel (cat. no. 339331, Qiagen). This panel contains eight samples per 96 well plate, controls for RNA isolation quality, and monitors cDNA synthesis in the reverse-transcription experiment. The QC panel contains primers for hsa-miR-103a-3p, mmu-miR-191-5p, hsa-miR-451a, hsa-miR-23a-3p, UniSp6, UniSp2, UniSp4, UniSp5, miR-39-3p, UniSp3, hsa-miR-124-3p and hsa-miR-30c-5p. Quantitative qPCR using the miRCURY LNA miRNA QC PCR Panel (cat. no. 339331, Qiagen), 0.8 μl of RT reaction product per sample, and the miRCURY LNA SYBR Green PCR Kit (cat. no. 339345, 339346, 339347, Qiagen) was used to control samples on a LightCycler 480 (Roche, Basel, Switzerland). According to manufacturer instruction we applied following cycling conditions: denaturation 2 min 95˚C, cycling: 50 cycles as follows 10 sec at 95˚C, 69 sec at 56˚C. Also according to the manufacturer

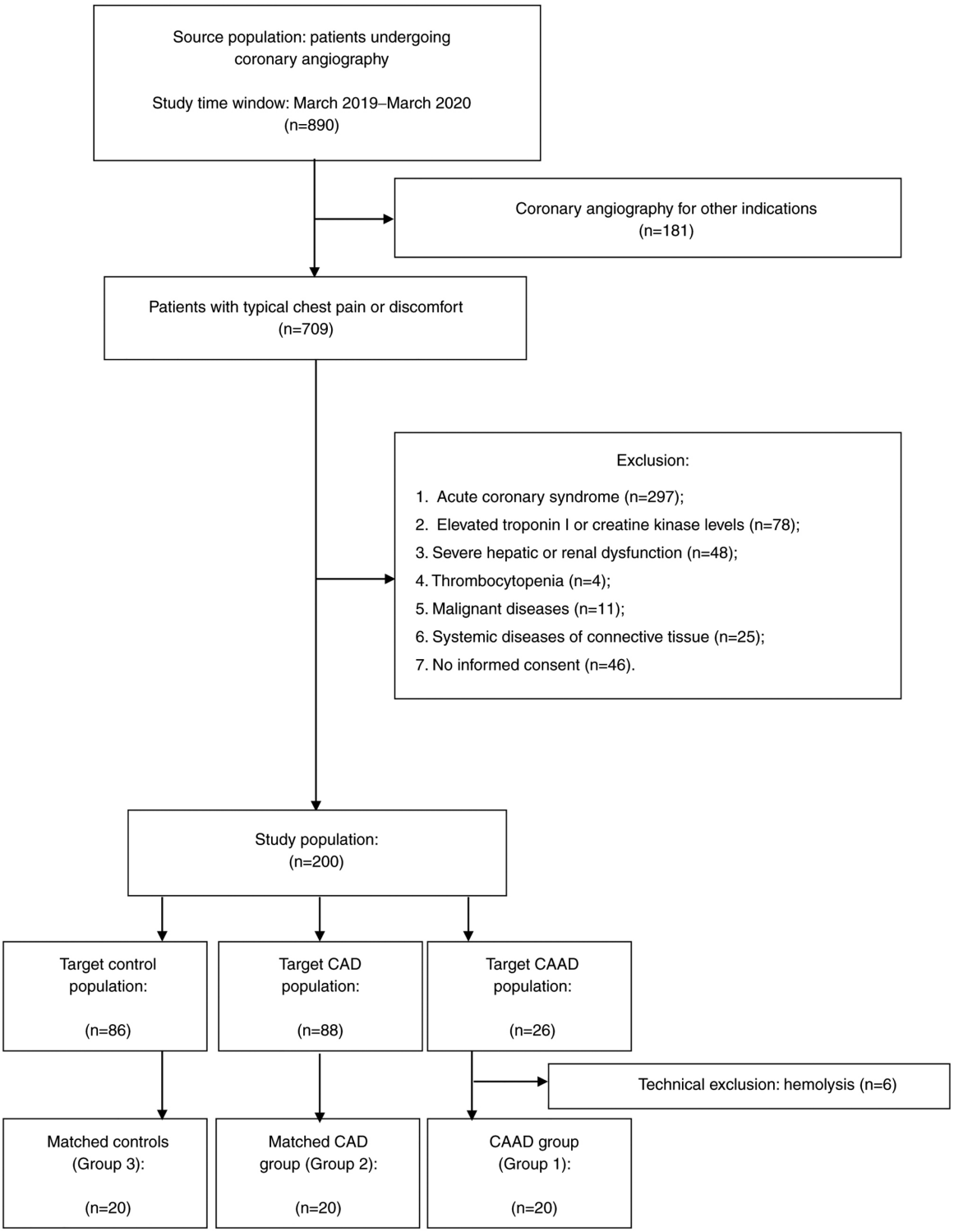

Figure 1. Flowchart of the study population. Inclusion and exclusion criteria for the study cohort and selection process for CAAD group (Group 1), CAD group (Group 2), and control group (Group 3). CAAD, coronary artery abnormal dilatation; CAD, coronary artery disease.

instructions 'miRCURY LNA miRNA QC PCR Panel Handbook,' we used samples only when they passed the following conditions: i) UniSp2, 4, and 5 showed consistent values across the sample (<2-3 Cq) and ΔCq=5-7 between spike-Ins. cDNA synthesis was evaluated using UniSp6 and cel-39-3p. Samples passed quantification when their Cq values were consistent across the sample set with low variance (<1-2 Cq). ii) Samples passed the qPCR reaction after evaluation by UniSp3, which showed consistent values across the dataset (<2 Cq across the dataset). Evaluation of hemolysis miR-451 and 2. iii) Hemolysis was evaluated by measuring miR-23a and miR-451 expression. The plasma sample quality was sufficient if ΔCq (miR-23a-miR-451) was lower than 7.

A total of 20 μl cDNA product of the miRCURY LNA RT Kit was applied for each qPCR. qPCR was performed using a miRCURY LNA SYBR Green PCR Kit (cat. no. 339345,

Table I. Baseline clinical characteristics.

Baseline data	Group 1 (n=20)	Group 2 (n=20)	Group 3 (n=20)	P-value		
				Group 1 vs. Group 2	Group 1 vs. Group 3	Group 2 vs. Group 3
Sex, male, n (%)	15 (75.0)	15 (75.0)	15 (75.0)	0.5	0.7	0.3
Age, years, mean ± SD	66.1±7.2	66.9±7.4	65.1±7.4	0.8	0.8	0.6
BMI, kg/m^2, mean ± SD	31.9±4.7	28.6±5.1	28.0±4.1	0.04	0.002	0.4
Previous MI, n (%)	5 (25.0)	7 (35.0)	0	0.3	0.01	<0.001
Previous PCI, n (%)	8 (40.0)	8 (40.0)	0	0.9	0.001	<0.001
Previous CABG, n (%)	2 (10.0)	3 (15.0)	0	0.3	0.3	0.06
Hypertension, n (%)	18 (90.0)	18 (90.0)	13 (65)	0.3	0.04	0.01
Heart failure, n (%)	11 (55.0)	12 (60.0)	9 (45.0)	0.2	0.9	0.2
Median LVEF, % (Q1-Q3)	60.0 (42.5-60.0)	52.5 (45.0-55.0)	60.0 (53.7-61.2)	0.5	0.1	0.01
Diabetes mellitus, n (%)	8 (40.0)	6 (30.0)	1 (5.0)	0.2	0.02	0.02
Hiperlipidemia, n (%)	17 (85.0)	16 (80.0)	13 (65.0)	0.3	0.1	0.6
Cigarette smoking, n (%)	8 (40.0)	8 (40.0)	8 (40.0)	0.8	0.9	0.9
Aortic aneurysm, n (%)	1 (5.0)	2 (10.0)	1 (5.0)	0.7	0.7	0.5
CKD, n (%)	12 (60.0)	13 (65.0)	13 (65.0)	0.2	0.2	1
Drug administration						
Statin, n (%)	16 (80.0)	17 (85.0)	17 (85.0)	0.3	0.9	0.3
CCB, n (%)	7 (35.0)	5 (25.0)	7 (35.0)	0.5	0.9	0.6
Beta-blocker, n (%)	18 (90.0)	18 (90.0)	15 (75.0)	0.3	0.08	0.02
Aspirin, n (%)	16 (80.0)	17 (85.0)	10 (50.0)	0.3	0.02	0.003
Clopidogrel, n (%)	8 (40.0)	14 (70.0)	0	0.03	0.001	<0.001
ACEI/ARB, n (%)	16 (80.0)	17 (85.0)	14 (70.0)	0.3	0.3	0.052
Laboratory tests						
LDL cholesterol, mmol/l	2.3±1.3	2.4±1.2	2.6±1.2	0.7	0.4	0.6
Creatinine, mmol/l	90.7±25.8	91.9±26.1	93.6±26.7	0.3	0.2	0.5
GFR, ml/min	74.0±14.5	73.2±14.2	73.2±14.5	0.4	0.6	0.6

ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; BMI, body mass index; CABG, coronary artery bypass graft; CCB, calcium channel blocker; CKD, chronic kidney disease; LDL, low-density lipoprotein; LVEF, left ventricle ejection fraction; MI, myocardial infarction; PCI, percutaneous coronary intervention; SD, standard deviation; Q, quartile.

339346, 339347, Qiagen) on miRCURY LNA Serum/Plasma Focus PCR Panels (cat. no. YAHS-106YF-8, Qiagen).

Statistical analysis. All continuous variables were presented as means with standard deviation for normal distribution or medians (upper and lower quartile) for non-normal distribution. The normality of the distribution of variables was tested using the Kolmogorov-Smirnov test. Categorical variables were presented as counts and percentages or frequencies. The significance of differences between the mean values of the continuous data consistent with the normal distribution was assessed using one-way ANOVA and Tukey's Test. Mann-Whitney and Kruskal-Wallis tests with Benjamini-Hochberg post-hoc analysis were used to compare the continuous data inconsistent with the normal distribution. Categorical variables were compared using the χ^2 test.

Analysis of array data was performed with GENEGLOBE online software (https://geneglobe.qiagen.com/pl/analyze). The global mean was used as a reference for the PCR-array analysis. The p values were calculated based on Student's t-test of the replicate 2(-ΔCq) values for each miRNA in the control and treatment groups (32). The P-value calculation used in this analysis is based on a parametric, two-sample equal variance, unpaired, two-tailed analysis. The alpha level was set a priori at 0.05.

We performed an additional multivariate analysis to evaluate the impact of selected comorbidities and cardiovascular risk factors on the obtained miRNA results. Due to the limited size of the group, it was possible to include up to three variables in one analysis. Two logistic regression models were built to consider the essential variables. All miRNAs, which expression was significantly different (P<0.05) in CAAD group than in the other groups, were included in the multivariate analysis. The results were obtained separately to compare Group 1 vs. Group 2 and Group 1 vs. Group 3. We used PQStat Software (PQStat v.1.8.0.476, Poland) for statistical analysis.

Table II. Angiographic characteristics of Group 1 (CAAD group; n=20).

Baseline data	Group 1, n (%)
CAE	15 (75.0)
CAA	4 (20.0)
Both	1 (5.0)
Number of vessels involved	
1	14 (70.0)
2	5 (25.0)
3	1 (5.0)
Vessel localization	
LM	0
RCA	10 (50.0)
LAD	9 (45.0)
LCx	8 (40.0)
Concomitant CAD	11 (55.0)

CAA, coronary artery ectasia; CAAD, coronary artery abnormal dilatation; CAD, coronary artery disease; CAE, coronary artery aneurysm; LAD, left artery descending; LCx, left circumflex artery; LM, left main; RCA, right coronary artery.

Table III. Deregulated miRNAs in the circulation of Group 1 compared with Group 3.

miRNA ID	Fold change	P-value
hsa-miR-210-3p	6.44	0.000314
hsa-miR-326	5.22	0.020272
hsa-miR-142-5p	4.79	0.020595
hsa-miR-19a-3p	4.76	0.002408
hsa-miR-19b-3p	4.35	0.012555
hsa-miR-339-5p	4.03	0.019595
hsa-miR-874-3p	3.79	0.034911
hsa-miR-497-5p	3.30	0.005334
hsa-miR-425-3p	3.30	0.023547
hsa-miR-20a-5p	2.85	0.017399
hsa-miR-106b-5p	2.85	0.003684
hsa-miR-378a-3p	2.74	0.007929
hsa-miR-532-5p	2.72	0.008666
hsa-miR-502-3p	2.61	0.001956
hsa-miR-20b-5p	2.54	0.028569
hsa-miR-328-3p	2.47	0.019208
hsa-miR-107	2.37	0.005988
hsa-miR-130b-3p	2.31	0.026696
hsa-miR-103a-3p	2.21	0.013745
hsa-miR-93-3p	2.10	0.021729
hsa-miR-339-3p	2.09	0.024420
hsa-miR-23a-3p	-2.36	0.001293
hsa-miR-125b-5p	*-2.55*	0.001279

Results

Clinical characteristics of the study population. The clinical characteristics of the study populations are summarized in Table I. Patients with CAAD had a significantly higher BMI than the patients with CAD and the control group. There were no significant differences in other cardiovascular risk factors between Group 1 and Group 2. The control patients had no history of cardiovascular events and were characterized by a significantly lower risk of hypertension and diabetes. Despite a comparable frequency of HF in all groups, left ventricular ejection fraction (LVEF) was significantly higher in the control patients than in those with CAD. Moreover, a significantly lower percentage of patients in Group 3 were treated with aspirin, and none of them had indications for dual antiplatelet therapy (DAPT). Clopidogrel has been significantly more often used in patients with CAD.

The angiographic characteristics of Group 1 are presented in Table II. CAE was diagnosed in the majority of patients (75%). CAAD, both diffuse and focal, often involves only one vessel in the right coronary artery (RCA). CAD was angiographically documented in 55% of patients.

Expression profile of miRNAs in the plasma of Group 1 vs. Group 2 and Group 3. We analyzed the miRNA expression profiles in the plasma of all patients from the study cohort a using miRNA PCR array and compared the results among the three studied groups. Due to the presence of hemolysis in the 3 tested samples, 57 patients were finally included in molecular analysis, 19 from Group 1, 18 from Group 2 and 20 from Group 3.

The levels of circulating miRNAs differed profoundly between Group 1 and Group 3, as illustrated in the heat map diagram shown in Fig. 2. We identified twenty-three significantly deregulated miRNAs (fold change >2 and P<0.05, see P-values in Table III). Twenty-one were upregulated, and two were downregulated in patients with aneurysmal coronary artery dilatation compared to the control group (Table III).

A comparison of circulating miRNAs, characterized by the greatest fold change in Group 1 vs. Group 3, is shown in Fig. 3. We selected: hsa-miR-210-3p, hsa-miR-326, hsa-miR-142-5p, hsa-miR-19a-3p, hsa-miR-19b-3p, hsa-miR-339-5p, hsa-miR-874-3p and hsa-miR-425-3p as potential markers.

Moreover, we compared the miRNA expression profiles in the plasma of Group 2 with Group 3 and identified eight significantly deregulated miRNAs (fold change >2 and P<0.05 see P-values in Table IV). Six were upregulated, and two were downregulated in patients with CAD compared to controls (Table IV).

A comparison of circulating miRNAs characterized by the greatest fold change in Group 3 vs. Group 2 is shown in Fig. 4. We selected: hsa-miR-885-5p, hsa-miR-133a-3p, hsa-miR-483-5p, hsa-miR-425-3p, hsa-miR-328-3p, hsa-miR-191-5p, hsa-miR-145-5p and hsa-miR-125b-5p as potential markers.

Comparing the miRNA expression profiles in the plasma of Group 2 and Group 1 identified seven significantly deregulated miRNAs (fold change >2 and P<0.05 see P-values in Table V). Three of these miRNAs were upregulated, and four were downregulated in Group 2 compared to Group 1 (Table V).

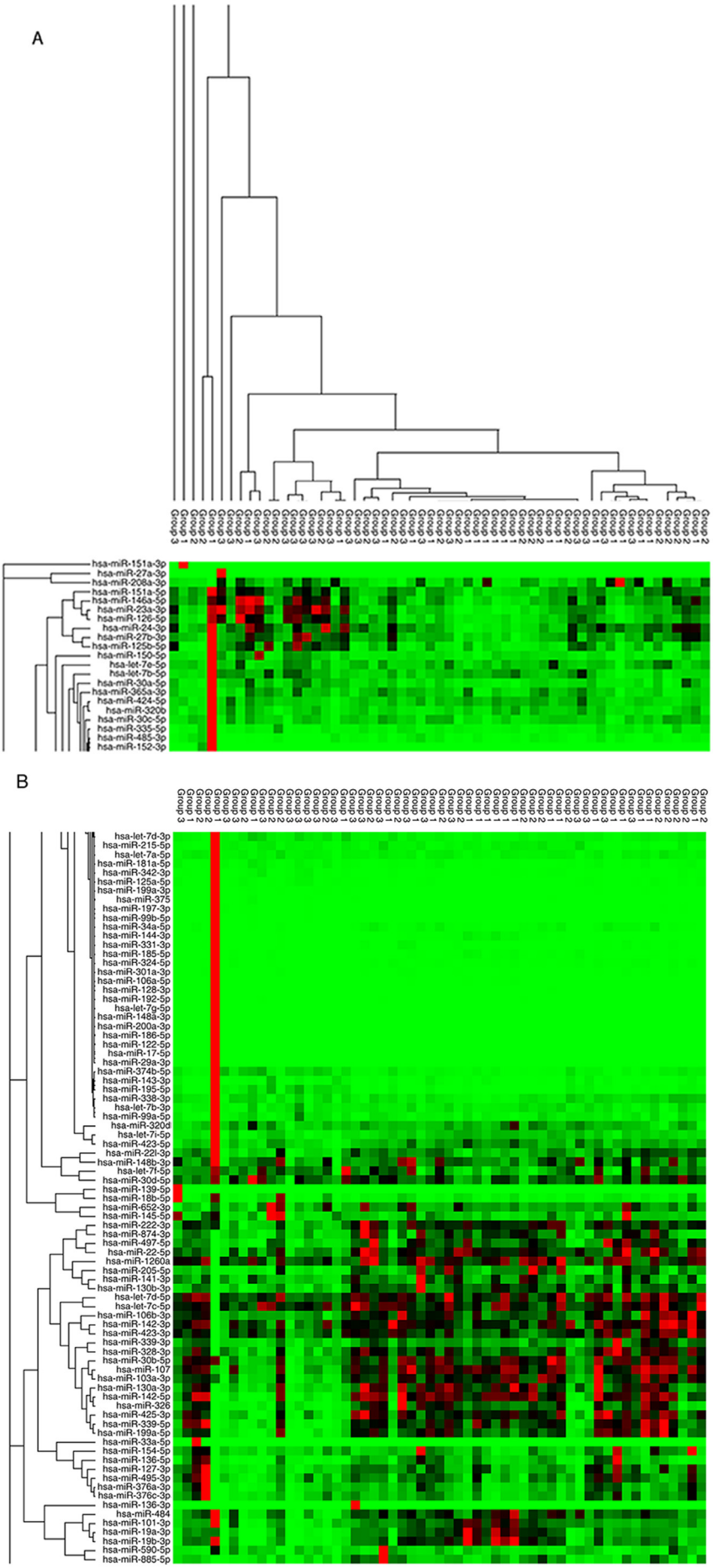

Figure 2. Continued.

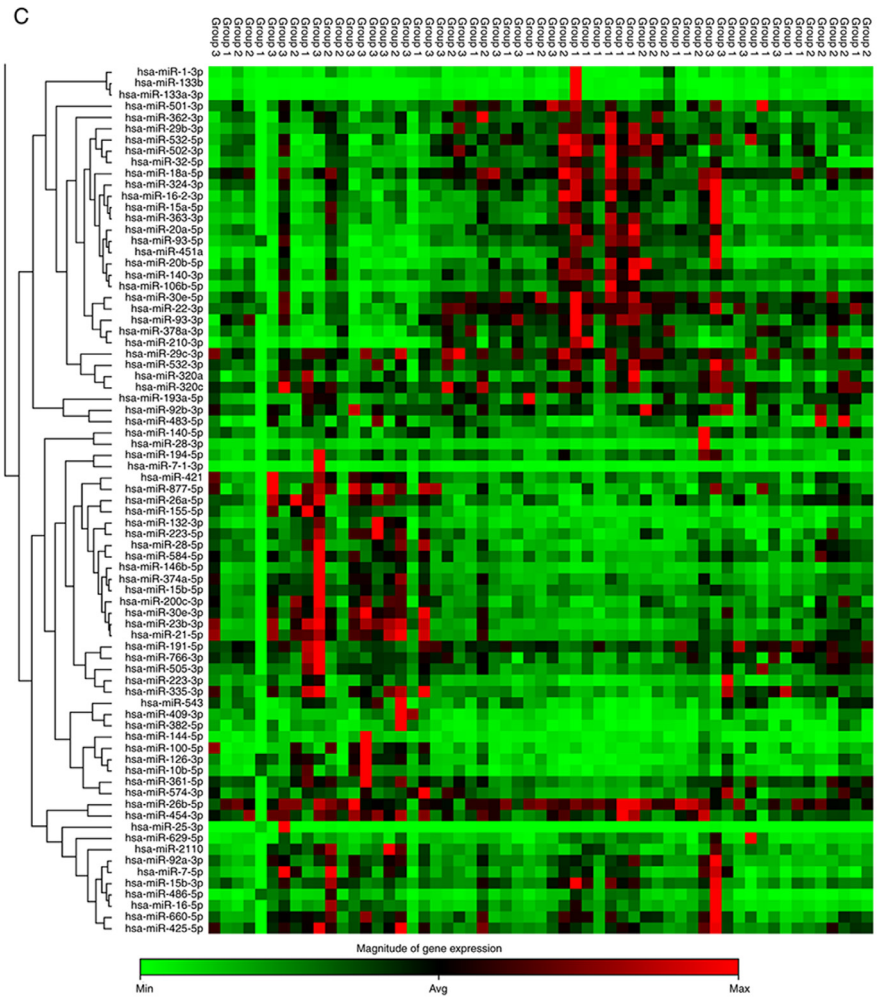

Figure 2. (A-C) Profile of circulating miRNAs in CAAD group (Group 1) (n=19), CAD group (Group 2) (n=18) and control group (Group 3) (n=20). (B) is a continuation of (A), and (C) is a continuation of (B). A heat map diagram is shown clustering the differentially expressed miRNAs. Color intensity is scaled within each row, such that the highest expression value corresponds to bright red and the lowest to bright green. CAAD, coronary artery abnormal dilatation; CAD, coronary artery disease.

Table IV. Deregulated miRNAs in the circulation of Group 2 compared with Group 3.

miRNA	Fold change	P-value
hsa-miR-885-5p	3.22	0.024577
hsa-miR-133a-3p	3.07	0.024260
hsa-miR-483-5p	2.77	0.002122
hsa-miR-425-3p	2.61	0.042032
hsa-miR-328-3p	2.42	0.012738
hsa-miR-191-5p	2.21	0.049314
hsa-miR-145-5p	-2.15	0.013063
hsa-miR-125b-5p	-2.32	0.007553

Table V. Deregulated miRNAs in the circulation of Group 2 compared with Group 1.

miRNA ID	Fold change	P-value
hsa-miR-223-5p	2.35	0.009035
hsa-miR-483-5p	2.06	0.015292
hsa-miR-375	2.04	0.026221
hsa-miR-16-2-3p	-2.08	0.017763
hsa-miR-210-3p	-2.22	0.036750
hsa-miR-652-3p	-2.25	0.005766
hsa-miR-18b-5p	-2.50	0.034395

A comparison of circulating miRNAs characterized by the greatest fold change in Group 3 vs. Group 2 is shown in Fig. 5. We selected: hsa-miR-885-5p, hsa-miR-133a-3p, hsa-miR-483-5p, hsa-miR-425-3p, hsa-miR-328-3p, hsa-miR-191-5p, hsa-miR-145-5p and hsa-miR-125b-5p as potential markers.

Multivariate analysis of miRNA expression profile in Group 1 (n=19) compared to Group 3 (n=20). In the first logistic regression model, the obtained results of miRNA expression were divided according to the presence of arterial hypertension, diabetes, and HF, which allowed the selection of eight miRNAs that were independent risk factors for coronary aneurysms compared to the control group (Table VI).

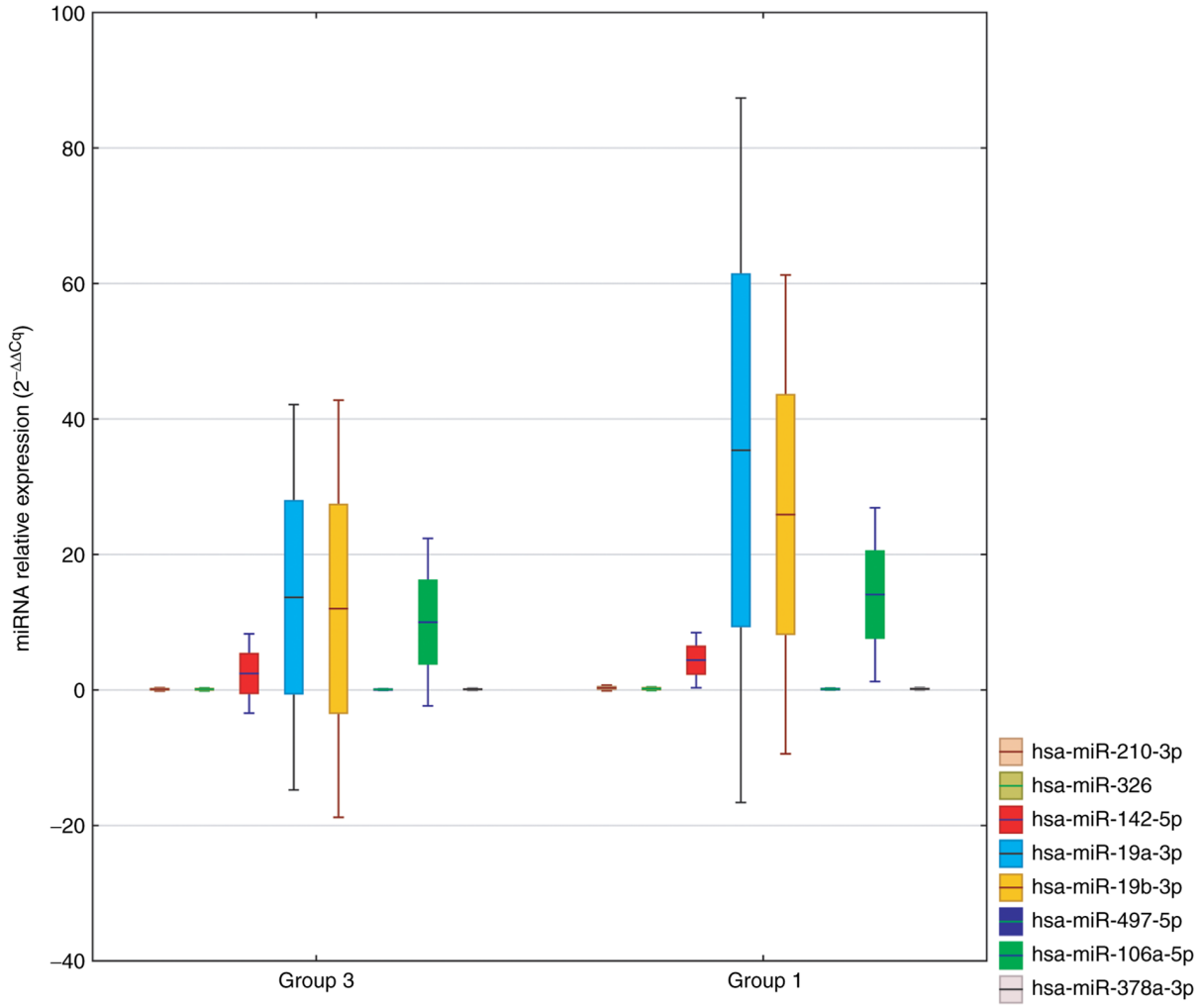

Figure 3. Profile of circulating miRNAs in CAAD group (Group 1) (n=19) vs. control group (Group 3) (n=20). Lines signify means of the normalized miRNA expression ($2^{-\Delta\Delta Cq}$), rectangles represent standard deviation and whiskers represent double standard deviation. All differences are significant. There are P-values for hsa-miR-210-3p, hsa-miR-326, hsa-miR-142-5p, hsa-miR-19a-3p, hsa-miR-19b-3p, hsa-miR-339-5p, hsa-miR-874-3p and hsa-miR-425-3p in Table III. CAAD, coronary artery abnormal dilatation; CAD, coronary artery disease.

Similarly, in the second regression model, which included smoking, renal failure, and dyslipidemia, a profile of eight miRNAs were identified. The upregulation of aforementioned miRNAs was significantly correlated with the risk of CAAD (Table VII). In addition, in a separate regression model, miRNA expression results were randomized on the basis of the use of antiplatelet drugs. The results are presented in Table VIII. In multivariate analysis, miRNA expression did not differ between Groups 1 and 2.

Discussion

The present study demonstrates that the plasma miRNAs detected in the blood were different in patients with CAAD than in other groups. We identified a specific signature of plasma miRNAs that were upregulated and downregulated in Group 1 compared to Group 2 and Group 3. In addition, we selected potential miRNA markers for further validation in a larger independent patient cohort.

Upon detailed analysis, we noticed that miRNA-210-3p was significantly upregulated in Group 1 vs. Group 2 and Group 3. In contrast, no differences in the expression of this miRNA were found between Groups 2 and 3. miR-210 represents prominent hypoxia-inducible miRs, also known as hypoxemic, which are expressed in a wide range of primary and transformed cells (33). Moreover, Fasanaro *et al* first reported that hypoxia-driven miR-210 supporting the angiogenic response in endothelial cells (34). The effect mainly resulted from the downregulation of EFNA3, an ephrin family member involved in vascular development (34). The proangiogenic effect of miR-210 was evaluated in MI, which was proved by the improvement of endothelial cell survival after the delivery of miR-210 in the heart (35). The involvement of miR-210 in the regulation of pathophysiological angiogenesis has also been demonstrated in renal ischemia/reperfusion (I/R) injury, indicating that miR-210 induction is necessary to drive the expression of VEGF and VEGFR2 in endothelial cells (36). The higher expression of miR-210-3p in Group 1 compared to Groups 2 and 3 remains challenging to explain. It would seem that the most severe ischemia occurs among CAD patients in Group 2, and thus the level of miR-210-3p expression should be the highest in this group. However, it is important to note that as many as 55% of patients with CAAD have been diagnosed with CAD. Furthermore, coronary dilatation can

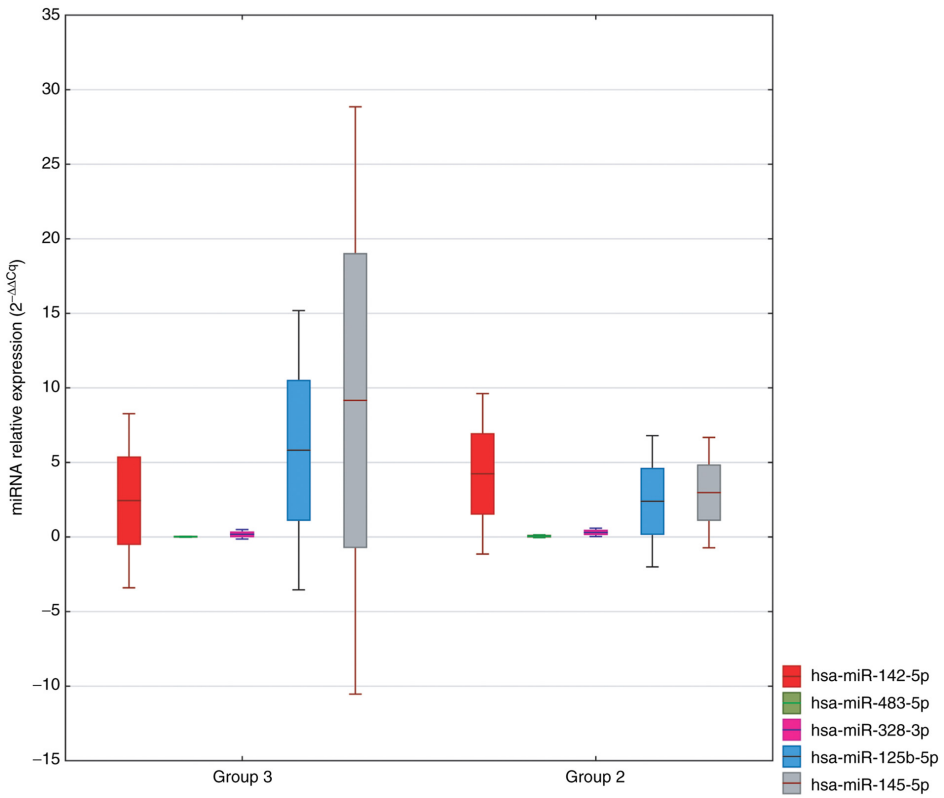

Figure 4. Profile of circulating miRNAs of control group (Group 3) (n=20) vs. CAD group (Group 2) (n=18). Lines signify means of the normalized miRNA expression ($2^{-\Delta\Delta Cq}$), rectangles represent standard deviation and whiskers represent double standard deviation. All differences are significant. There are P-values for hsa-miR-885-5p, hsa-miR-133a-3p, hsa-miR-483-5p, hsa-miR-425-3p, hsa-miR-328-3p, hsa-miR-191-5p, hsa-miR-145-5p and hsa-miR-125b-5p in Table IV. CAAD, coronary artery abnormal dilatation; CAD, coronary artery disease.

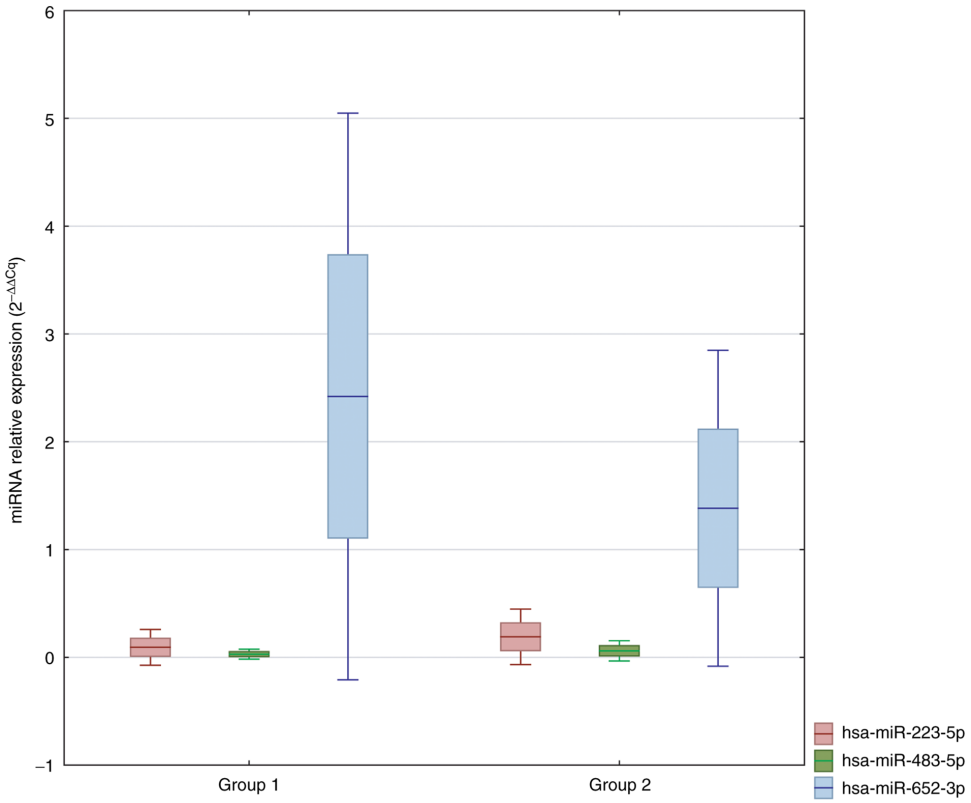

Figure 5. Profile of circulating miRNAs in CAAD group (Group 1) (n=19) vs. CAD group (Group 2) (n=18). Lines signify means of the normalized miRNA expression ($2^{-\Delta\Delta Cq}$), rectangles represent standard deviation and whiskers represent double standard deviation. All differences are significant. There are P-values for hsa-miR-885-5p, hsa-miR-133a-3p, hsa-miR-483-5p, hsa-miR-425-3p, hsa-miR-328-3p, hsa-miR-191-5p, hsa-miR-145-5p and hsa-miR-125b-5p in Table V. CAAD, coronary artery abnormal dilatation; CAD, coronary artery disease.

Table VI. Multivariate analysis of miRNA expression profile in Group 1 compared to Group 3, adjusted for heart failure, diabetes and hypertension.

miRNA	OR[a] [95% CI]	P-value
hsa-miR-103a-3p	2.6 [1.3, 3.1]	0.033
hsa-miR-20a-5p	3.2 [1.1, 5.2]	0.049
hsa-miR-107	1.5 [1.1, 2.6]	0.036
hsa-miR-19a-3p	2.7 [1.6, 3.8]	0.009
hsa-miR-19b-3p	1.4 [1.03, 3.8]	0.011
hsa-miR-106b-5p	1.6 [1.1, 2.4]	0.032
hsa-miR-142-5p	2.8 [1.3, 3.9]	0.031
hsa-miR-16-2-3p	9.0 [1.1, 2.9]	0.048

[a]In the correction of heart failure, diabetes, and hypertension.

Table VII. Multivariate analysis of miRNA expression profile in Group 1 compared to Group 3, adjusted for smoking, renal failure, and dyslipidemia.

miRNA	OR[a] [95% CI]	P-value
hsa-miR-103a-3p	9.1 [1.1, 14.1]	0.022
hsa-miR-20a-3p	5.5 [2.8, 7.2]	0.042
hsa-miR-107	3.2 [2.1, 4.3]	0.024
hsa-miR-19a-3p	6.8 [4.2, 8.2]	0.004
hsa-miR-19b-3p	1.2 [1.0, 1.9]	0.004
hsa-miR-106b-5p	4.2 [2.5, 7.2]	0.022
hsa-miR-425-3p	2.6 [1.1, 4.2]	0.048
hsa-miR-339-5p	1.2 [1.0, 2.1]	0.038

[a]In the correction of smoking, renal failure, and dyslipidemia.

Table VIII. Multivariate analysis of miRNA expression profile in Group 1 compared to Group 3, adjusted for the use of antiplatelet drugs.

miRNA	OR[a] [95% CI]	P-value
hsa-miR-103a-3p	2.7 [1.2, 3.8]	0.036
hsa-miR-107	1.05 [1.0, 2.18]	0.047
hsa-miR-19a-3p	2.02 [1.8, 3.6]	0.012
hsa-miR-19b-3p	8.4 [6.2, 10.1]	0.014
hsa-miR-106b-5p	1.5 [1.1, 2.2]	0.042
hsa-miR-142-5p	7.4 [3.2, 8.6]	0.029

[a]In the correction of use of antiplatelet drugs.

cause a turbulent and stagnant flow, possibly increasing the risk of thrombus formation and peripheral microembolization, which can also be a source of ischemia. The influence of miR-210-3p on the angiogenesis process also seems to be important. Ischemia, which arises from significant coronary stenosis, activates angiogenesis processes that lead to vessel remodeling and branching of new blood vessels. Angiogenesis requires many molecular mechanisms regulated by pro- and antiangiogenic factors (37,38). The imbalance between them can lead to thinning of the vessel walls and subsequent coronary aneurysm formation.

In addition, hsa-miR-328-3p and hsa-miR-425-3p were upregulated, and hsa-miR-125b-5p was downregulated in both Group 1 and Group 2 compared to Group 3. Data on the involvement of the above miRNAs in the pathogenesis of CAAD and CAD are minimal. Qin *et al* showed that miR-328-3p protects vascular endothelial cells against ox-LDL-induced injury by targeting FOXO4 (39). Its expression was downregulated in oxidized low-density lipoprotein (ox-LDL) treated human umbilical vein endothelial cells (HUVECs). Overexpression of miR-328-3p attenuated ox-LDL-induced inhibition of cell viability, migration, and invasion and stimulation of apoptosis, autophagy and inflammation in HUVECs. Moreover, numerous studies have shown that miR-328-3p and miR-425-3p may play a crucial role in malignant tumor progression in osteosarcoma, glioblastoma, and bladder cancer (40-44). miR-125b-5p was downregulated in the aneurysmal tissue of AAA, specifically in the FDG-uptake site, compared with a negative zone in the same aneurysm and with no FDG uptake aneurysms (45). Furthermore, it was inversely correlated with the expression of some of their potential gene targets at the positive uptake site, most notably matrix metalloproteinase 13. Increase of miR-133 in Group 2 has been found to regulate endothelial function and angiogenesis, vascular smooth muscle cell differentiation, apoptosis, and cardiac myocyte differentiation, and repress cardiac hypertrophy. Its role in these processes may be explained by the regulation of targets such as FSCN1, LASP1, purine nucleoside phosphorylase (PNP), and transgelin 2 (TAGLN2) (46). High expression of miR-483-5p in Group 2, has been found to inhibit angiogenesis by targeting serum response factor (SRF), providing a clue for combating angiogenesis in CAD patients (47). In conclusion, our findings correlated with the observed upregulation of plasma miR-483-5p and may be favorable for CAD patients protection against adverse effects caused by plaque rupture (48). Our results also revealed downregulated miR-145-3p in the patients with CAD compared to the control group. These findings are in line with previous reports, which showed that chronic vascular inflammation is associated with the level of miR-145. The authors postulated that miR-145 is a negative regulator of TGF-b signaling and thus may contribute to the downregulation of inflammation in the arterial wall (22,29,48). Nevertheless, the mechanism of miR-145 reduction in diseases associated with chronic inflammation is still unclear and requires further studies on animal models. Due to the varied etiology of CAAD, the level of miR-145-3p in Group 1 did not differ significantly from that in the other groups.

miR-145 expression levels strongly correlate with miR-223, suggesting that a shared mechanism may regulate these miRNAs (29,30). In our study, miR-223 expression was downregulated in Group 1 compared to Group 2. We know from previous studies that the level of miR-223 in the blood serum of

KD was higher in patients with vascular injury than in patients without vascular complications, and it was reduced after immunoglobulin treatment (17). One of the target genes for miR-223 is STAT1 mRNA at the 3-UTR. JAK/STAT signaling is one of the most important regulatory pathways in many inflammatory processes. It initiates innate and acquired immunity and mediates various cytokine signaling pathways (49,50). Dysfunction in the regulation of miRNA-223 and related target genes in immune and myocardial cells is believed to contribute to the development of heart disease, including CAD and HF. These data may indicate the most advanced inflammatory process and subsequent vessel damage in patients with atherosclerosis.

Reduced expression of miR-23a-3p in CAAD group compared to control is also worth discussing. This miRNA belongs to the miR-23/27/24 cluster members on chromosome 19 (19p13.13) and plays a role in cell cycle control, proliferation, differentiation, and apoptosis (51,52). It is involved in the angiogenesis process by regulating the growth of cardiomyocytes, inducing the proliferation of VSMCs, and inhibiting VSMC apoptosis by targeting the BCL2L11 (BIM) gene (51). Several lines of evidence revealed that miR-23a-3p was downregulated in the walls of large AAA, supporting the hypothesis that the downregulation of miR-23a-3p can contribute to AAA development and progression (53). The above data may suggest common pathogenesis of AAA and CAAD.

Multivariate analysis revealed that the several miRNAs with higher expression in Group 1 than in Group 3. One of these miRNAs is hsa-miR-103a-3p, whose expression is altered in the state of inflammation, immune disorders, and cancer (54-57). Li *et al* reported that miR-103a-3p is involved in septic injury. Its overexpression reduced lipopolysaccharide (LPS)-induced inflammation (23). Moreover, studies revealed that miR-103a-3p is proinflammatory also through the renal pathway. Increased miR-103a-3β expression enhances angiotensin II-induced renal inflammation and injury. Researchers have also found the increased levels of type I and IV collagen protein and mRNA in the kidneys (58). In addition, Jiao *et al* presented *in vitro* and in a murine model that have showed that miR-103a-3p could also be involved in AAA by targeting ADAM10 (59). Plana *et al* revealed the overexpression of miR-103a-3p in AAA tissue compared to healthy tissues (25). Moreover, the studies showed that the overexpression of miR-103a-3p inhibited high mobility group Box 1 (HMGB1) expression. HMGB1 is a universal, nonhistone DNA-binding protein and a common inflammatory regulator that sensitizes many inflammation-related signaling pathways, leading to the production of proinflammatory cytokines (60).

Similar results were obtained for hsa-miR-107, hsa-miR-19a-3p and hsa-miR-19b-3p. Interestingly, abnormal expression of miR-107-5p, member 3 of the solute carrier 24 family (SLC24A3), an integral membrane protein 2C (ITM2C), was identified in acute aortic dissection (AD) (61). miR-107-5p expression was higher in AD samples in comparison with normal aortic samples. In turn, the expression of ITM2C in AD tissue was lower than that in normal aortic samples. Moreover, miR-107-5p inhibited the progression of acute AD by targeting ITM2C.

The miR-19a-3p family is one of the most important factors regulating heart disease and cancer development, including the extensive invasion of malignancies (62). The expression level of miR-19a-3p is also upregulated after MI. For example, miR-19 inhibited apoptosis and promoted cardiomyocyte proliferation. In addition, the upregulation of miR-19 reduces the formation of endothelial cells and regulates the expression level of cyclin D1 and fibroblast growth factor receptor 2, blocking the cell cycle (62). Moreover, an essential role of miR-19a-3p in the regulation of angiogenesis has been demonstrated. It has also been shown that the inhibition of miR-19a-3p promotes angiogenesis in mice with MI (63).

Circulating miRNAs are influenced by many factors, most of them uncontrolled (12,13). The idea is to look for the most stable markers and signature of the patient's condition. In our study, the prevalence of almost all risk factors for ischemic disease and comorbidities was comparable in Groups 1 and 2. Antiplatelet therapy affects the expression of some miRNAs, such as miR-19b, miR-191, miR-223. However, due to the disease profile, the frequency of its use was significantly lower in the control group.

The current outcomes of miRNA profile in serum are uncertain in clinical diagnosis of CAAD, but we believe there is nonetheless a regular distribution of miRNA expression in a large number of patients' serum. Statistic randomness of miRNA in a serum profile is a kind of order that emerges only in a large number of repeats. On the other hand, our results, like many other earlier studies of miRNA serum profile, suggest that miRNA profiles are not statistically 'haphazard' (15). The precision of CAAD diagnostics upon miRNA profiles will probably increase in the future by applying new models for meta-analysis incorporating several studies on CAAD patients. The number of sufficient samples, cannot be predicted to estimate probability since the number of factors determining miRNA profiles is unknown.

Our study identified miRNAs as potential biomarkers of CAAD. However, the relationship of miRNAs with the process of atherosclerosis and other aneurysms has not been demonstrated. Regardless, the relationship of miRNAs with the angiogenesis process and cancer pathogenesis remains interesting. Undoubtedly, the obtained results require further validation.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the NCBI Gene Expression Omnibus repository and are accessible through GEO Series accession number

GSE198885 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE198885).

Authors' contributions

SI, TL and AA designed the current study and wrote the manuscript. AC, AR, KM and MW collected clinical samples and prepared them for further analyses. TL and SI conducted the experiments and statistical analyses. PJ, MG and ML made substantial contribiutions to conception and design, and revised this manuscript.SI and TL confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Ethics Committee of Poznan University of Medical Sciences (protocol code, 985/18; date of approval, October 11, 2018). Written informed consent was obtained from all subjects involved in the study. Patient clinical data remains anonymous and does not identify the patient. The consent form has been approved by the Institutional Ethics Committee of Poznan University of Medical Sciences.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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