

ORIGINAL ARTICLE

miR-27a/b Level is Associated with ABCA1 Expression and is a Potential PBMC-Based Biomarker for Coronary Artery Disease

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Abstract

Background: Coronary artery disease (CAD) is the most common form of cardiac disease with high morbidity and mortality rates.

Objective: In this study, we evaluated the expression of miR-27a and miR-27b as biomarkers in peripheral blood mononuclear cells (PBMCs) of patients with CAD and investigated its correlation with cholesterol-efflux transporter, ATP-binding cassette transporter A1 (ABCA1).

Methods: This study was performed on 54 men with CAD and 51 healthy, sex- and age-matched control participants. The expression of miR-27a/b and ABCA1 genes in PBMCs were measured by quantitative real-time polymerase chain reaction (qRT-PCR). The protein expression of ABCA1 was assessed by Western blotting. Concurrently, the specificity and sensitivity of miR-27a/b was evaluated through receiver operating characteristic (ROC) curve. The significance level adopted in the statistical analysis was 5%.

Results: We found that miR-27a and miR-27b expression were significantly increased, while both mRNA and protein expression of ABCA1 were markedly reduced in the PBMCs of CAD patients in comparison to non-CAD controls. miR-27a/27b expression was also shown to be inversely correlated with ABCA1. ROC analysis showed that the miR-27a had an area under the ROC curve (AUC) of about 92.6 (sensitivity 83.3% and specificity 86.6%) and miR-27b had an AUC of about 93.0 (sensitivity 86.6% and specificity 80.0 (%), suggesting the diagnostic potential of miR-27a/b in CAD patients.

Conclusion: Our data suggested a possible role of miR-27a/b in CAD pathogenesis. Additionally, we proposed that miR-27a/b expression in PBMCs may have potential clinical implications in the diagnosis of CAD patients, but further validations in large cohorts are required.

Keywords: Coronary artery disease; MicroRNAs; Biomarkers.

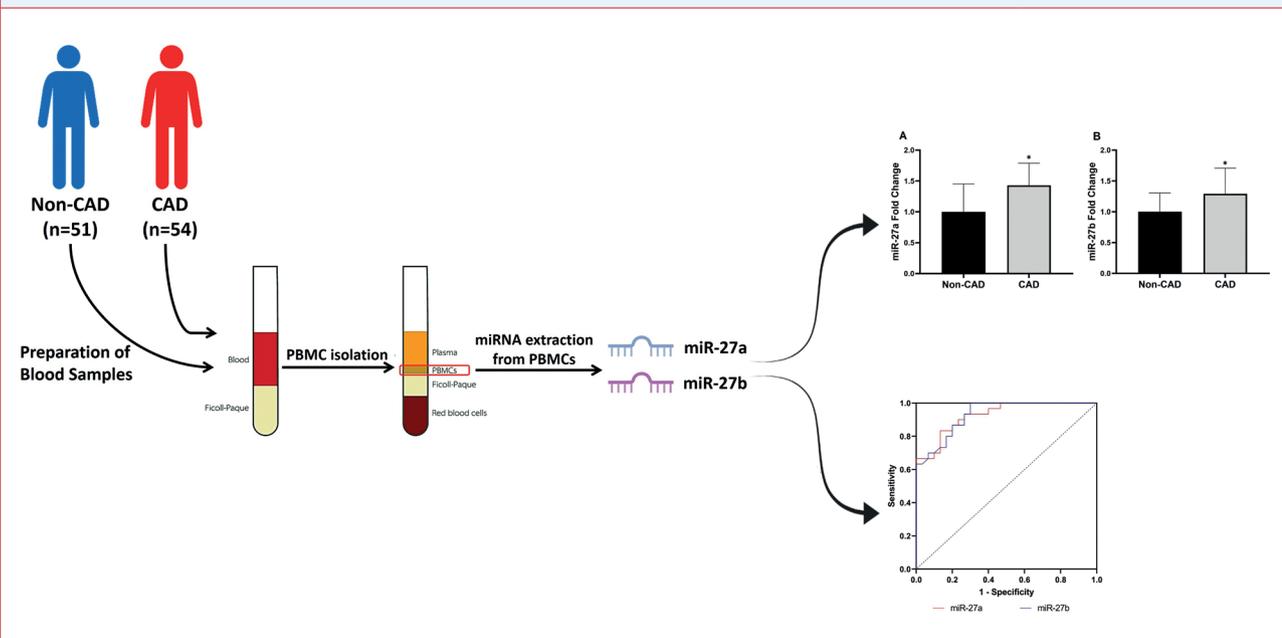
Introduction

Coronary artery disease (CAD) is a global health challenge associated with high morbidity and mortality rates.¹ CAD is usually caused by atherosclerotic plaque formation in coronary arteries,² and despite great improvements in diagnostic approaches, it is the major cause of death and disability among various types of cardiac disease. Therefore, innovative diagnostic and therapeutic methods are urgently required to reduce

the morbidity and mortality rates. Recently, the clinical potential of miRNAs as diagnostic, prognostic, and therapeutic tools has been documented in cardiovascular diseases, including CAD.³⁻⁵ miRNAs are short (21–23 non-coding RNAs) that bind to the 3' UTR region of the target mRNA molecules and regulate gene expression through inhibiting protein translation or targeting mRNAs for degradation.⁶ Interestingly, miRNAs have been found as potential regulators of cellular cholesterol status which is a major underlying risk factor in CAD.⁷ The miR-27

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Central Illustration: miR-27a/b Level is Associated with ABCA1 Expression and is a Potential PBMC-Based Biomarker for Coronary Artery Disease

Int J Cardiovasc Sci. 2023; 36:e20230016

CAD: coronary artery disease; PBMC: peripheral blood mononuclear cell.

gene family (miR-27a and miR-27b) is among the number of miRNAs implicated in the cholesterol metabolism regulation.⁸ miR-27 regulates several genes related to cholesterol metabolism, particularly ATP-binding cassette transporter A1 (ABCA1).⁹ ABCA1 is a transport protein that facilitates cholesterol efflux into ApoA1, thus reducing the risk of atherosclerosis.¹⁰ Accordingly, our computational analysis revealed that miR-27a/b targets ABCA1 with a high prediction score (Table 1).¹¹ miR-27 also plays critical roles in atherosclerosis by regulating several atherogenic promoting mechanisms, including inflammation,^{8,12} angiogenesis¹³ oxidative stress,¹² insulin resistance, and type 2 diabetes.^{14,15} Overexpression of miR-27 has also been suggested as a potential prognostic and diagnostic factor in patients with atherosclerosis.¹⁶ These findings demonstrated that miR-27 acts as an atherogenic promoting factor and might represent an ideal therapeutic target in atherosclerosis. However, the biological and clinical significance of miR-27 has not been investigated in CAD patients. Additionally, peripheral blood mononuclear cells (PBMCs) have been identified as one of the main mediators of inflammatory responses involved in CAD progression.¹⁷ Therefore, examining the expression of miRNAs in PBMCs might have greater diagnostic potential for CAD patients. Since the miR-27

expression has not been investigated in CAD patients, it prompted us to explore the miR-27a/b expression in PBMCs of CAD patients and to evaluate its correlation with ABCA1 and some anthropometric and biochemical parameters.

Materials and methods

Subject characteristics

The sample size calculation was performed based on a pilot study. Using error type 1 (α) of 0.05, statistical power of 80%, and a significance level of 5%, a minimum number of 30 individuals were required per group. This study included 105 male volunteers (aged 50–70 years) who underwent coronary angiography. According to the results of angiography, the subjects were divided into CAD group (n = 54) and non-CAD group (n = 51). Patients with $\geq 50\%$ stenosis in at least one major coronary artery were considered patients with CAD. Based on coronary angiography, CAD patients were divided into two subgroups, namely, double-vessel disease (2VD; n = 18) and triple-vessel disease (3VD; n = 36). Moreover, control subjects had normal coronary arteries (stenosis $< 30\%$). Patients with myocardial infarction (MI), diabetes, severe inflammation, and liver disorders were excluded

from our study (Figure 1). The study protocols received approval by the Ethics Committee of Tehran University of Medical Sciences (ECTUMS), and all patients signed informed consent before sampling.

Preparation of Blood Samples

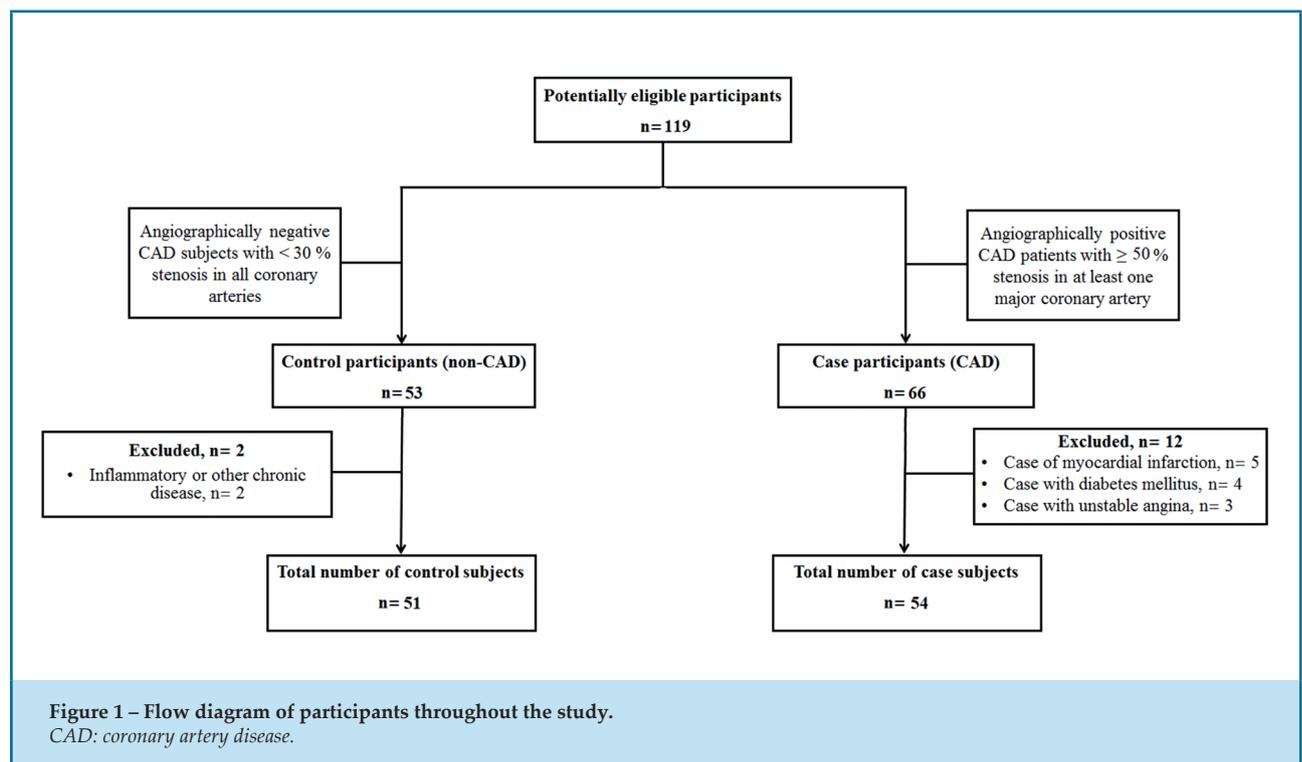
A volume of 12 ml of whole blood was taken from all participants after a 12-h overnight fast. Venous blood samples were collected into a tube with a serum separator as well as EDTA-containing tubes for biochemical analysis and PBMCs isolation. Sera were isolated by centrifugation at 4000 ×g for 12 min and then stored at -80°C. PBMCs from all EDTA-blood samples were isolated using the Ficoll-Hypaque (lymphocyte-H; Cedarlane, Canada) reagent according to the manufacturer's instructions and then stored at -80°C before RNA isolation.

Anthropometric features and Biochemical analysis

In serum, fasting blood sugar (FBS) and lipid profile were evaluated with commercially available kit (Pars Azmoon Inc., Tehran, Iran) using an autoanalyzer (Abbott, model Alcyon 300, USA). Besides, background information and anthropometric parameters of patients, such as age, systolic and diastolic blood pressures, BMI, and family history of CAD, were recorded.

RNA extraction, cDNA synthesis, and quantitative real-time PCR (qRT-PCR)

PBMCs total RNA (including miRNA) were isolated from all samples using the miRNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The concentrations and purity of isolated RNAs were examined by a NanoDrop ND-1000 spectrometer (Thermo Fischer Scientific, USA). Reverse transcription of ABCA1 mRNA and miR-27a/b was carried out using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Fermentas, USA) and miScript II RT Kit (Qiagen, Germany), respectively. qRT-PCR for miRNAs and ABCA1 was performed using miScript SYBR Green PCR Kit (Qiagen, Germany) and SYBR Premix Ex Taq II (Takara Biotechnology, Japan), respectively, in a Rotor-Gene Real-Time Detection System (QIAGEN). The sequences of the primers are as follows: ABCA1 5'-AACAGTTTGTGGCCCTTTTG-3' (forward) and 5'-TTCCAGGCTGGGGTACTT-3' (reverse), and β -actin 5'-TGGACTTCGAGCAAGAGATG-3' (forward) and 5'-GAAGGAAGGCTGGAAGAGTG-3' (reverse). The fold-change expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method, and *U6* snRNA and β -actin were used for normalization of miRNAs and mRNA expression as internal reference genes.



Protein extraction and Western blotting

PBMCs total proteins were isolated by RIPA lysis buffer, and the protein concentration of the cell lysate was measured by bicinchoninic acid (BCA) Protein Assay Kit according to the manufacturer's guidelines. The proteins (prepared in sample buffer) were loaded into the 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and separated. The protein gels were then moved to nitrocellulose membranes and blocked with a 5% solution of non-fat dry milk in Tris-buffered saline (TBS) for 2 h at room temperature. Subsequently, primary monoclonal antibodies specific to β -actin and ABCA1 (Santa Cruz Biotechnology, USA) are incubated overnight with the related membrane. After overnight incubation, the membranes were washed with TBS and exposed to the related horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, USA) in TBS at room temperature for 2 h. To visualize the protein bands, the enhanced chemiluminescence (ECL) detection kit (Thermo Fisher Scientific, USA) was used as instructed.

Computational analysis of miRNAs targeting ABCA1

The microRNA Data Integration Portal (mirDIP) was used to predict potential miRNAs to interact with the ABCA1.¹¹ mirDIP is a database that amalgamates miRNA-target interactions of 30 various prediction resources, including TargetScan, mirbase, RNA22, MirSNP, BiTargeting, CoMeTa, DIANA, BCmicrO, MirMAP, Cupid, MAMI, MirAncesTar, EIMMo3, miRTar2GO, miRDB, miRcode, MultiMiTar, microrna.org, Mirza-G, TargetSpy, mirCoX, RepTar, TargetRank, GenMir⁺, MirTar, RNAhybrid, PITA, PACCMIT, PicTar, and MBStar.¹¹ We found that miR-27a/b is among the ABCA1-targeting miRNAs that have the highest integrated scores and the highest number of the prediction resources. The integrated score is obtained from resource-specific measures of prediction confidence and is used for accurate prediction of miRNA-target interactions. Moreover, four different confidence classes, labeled as very high (top 1%), high (top 5%, excluding top 1%), medium (top 1/3, excluding top 5%), and low (bottom 2/3), have been offered by mirDIP to find out the classification of the predicted targets more intuitively.¹¹ The results of miR-27a/b-ABCA1 interactions are summarized in Table 1. Furthermore, using DIANA-TarBase v8, a database of experimentally validated miRNA-mRNAs interactions,

Table 1 – Computational analysis of miR-27a/b-ABCA1 interactions using mirDIP prediction tool

MicroRNA	Integrated score	Number of sources	Score class
hsa-miR-27a-3p	84.4%	21	Very high
hsa-miR-27b-3p	80.6%	19	Very high

we found that ABCA1 has been validated as a target of miR-27a through reporter gene assay.¹⁸

Statistical analysis

All the statistical analyses were performed using IBM SPSS Statistics, version 25.0 (SPSS Inc., Chicago, Illinois). Continuous variables were tested for normality by the Kolmogorov-Smirnov test. Data with normal distributions were reported as the mean \pm standard deviation (SD) and non-normally distributed variables were presented as median (25th–75th percentile). Categorical variables were analyzed by chi-square test and the results were shown as numbers and/or percentages. Independent Student's t-test was used to compare the means between two groups with normal distribution, while Mann-Whitney test was used when the assumption of normality was rejected. One-way ANOVA with Tukey's post-hoc test was conducted to assess the difference among multiple groups. The correlation between miR-27a/b and other variables was analyzed using Spearman correlation test. In addition, the ability of miR-27a/b to differentiate individuals with and without CAD condition was evaluated by receiver operating characteristic (ROC) curve analysis. Different measures of validity including specificity, sensitivity, and positive predictive value (PPV) and negative predictive value (NPV) were further evaluated according to the ROC curve. All P-values less than 0.05 were accepted as statistically significant.

Results

Baseline and clinical characteristics

The baseline and clinical features of CAD and control subjects are shown in Table 2. As reported in this table, no significant difference was found in age, BMI, blood pressure, triglyceride, and HDL-C between CAD and non-CAD groups, while a significant difference was

Table 2 – Anthropometrics and laboratory data of the study population

Characteristics	non-CAD (n = 51)	CAD (n = 54)	p-Value
Age (years)	55.30±8.453	58.47±5.218	0.086
BMI (kg/m ²)	26.15±3.670	27.27±3.469	0.229
SBP (mmHg)	121.18 (114.50–127.00)	118.00 (110.5–124.23)	0.200
DBP (mmHg)	78.43 (68.75–82.86)	74.21 (68.00–82.50)	0.629
Family history of CAD	Yes (41) No (10)	Yes (45) No (9)	0.559
TG (mg/dl)	116.00 (79.00–131.00)	131.00 (84.00–164.00)	0.368
TC (mg/dl)	138.70 (110.00–163.50)	156.70 (131.5–182.30)	0.047
LDL-C (mg/dl)	95.20 (89.75–132.50)	109.10 (79.50–111.30)	0.029
HDL-C (mg/dl)	41.13 (30.00–50.50)	36.93 (28.75–40.50)	0.244
Angiography			
2VD	-	18 (33.3%)	-
3VD	-	36 (66.6%)	-

Values given in mean ± SD and median, 25th, and 75th percentiles are given. CAD: coronary artery disease; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; FBS: fasting blood sugar; TG: triglycerides; TC: total cholesterol; LDL-C: low density lipoprotein-cholesterol; HDL-C: high density lipoprotein-cholesterol; VD: vessel disease.

observed in LDL-C ($p = 0.029$) and total cholesterol ($p = 0.047$) between the two groups. By coronary angiography, 18 CAD cases (33.3 %) had 2 VD and 36 cases (66.6 %) had 3 VD.

Expression of miR-27a/b mRNA in PBMCs of CAD and non-CAD groups

The analysis showed that the expression of miR-27a and miR-27b in PBMCs of CAD patients were significantly higher than non-CAD subjects ($p < 0.05$) (Figures 2A and 2B). As shown in Figures 2C and 2D, the expression of miR-27a and miR-27b in patients with 3-vessel CAD was higher compared to 2-vessel CAD patients, but no significant difference was found, probably due to the small sample size.

The mRNA and protein expression levels of ABCA1 in PBMCs of CAD and non-CAD groups

Given that ABCA1 is one of the main targets of miR-27a/b, we further analyzed the mRNA expression levels of ABCA1 in PBMCs of CAD and non-CAD groups by qRT-PCR. As shown in Figure 3A, the mRNA level of ABCA1 in PBMCs from the CAD group was significantly lower than the non-CAD group ($P < 0.01$). For further experimental validation, we performed Western blotting to assess the protein expression of ABCA1 in PBMCs of CAD and non-CAD groups. Western blot analysis also showed similar expression results (Figure 3B). Collectively, our results indicate that both mRNA and protein levels of ABCA1 in PBMCs from the CAD group were significantly lower than those in the non-CAD group.

Correlation analysis

We next analyzed the correlations of miR-27a/b with ABCA1 mRNA expression and some biochemical parameters. As illustrated in Figure 4, the expression of miR-27a/b had a significant negative correlation with the mRNA expression of ABCA1 in CAD patients. In addition, a significant positive correlation was found between miR-27a/b expression and LDL-C levels in the CAD group ($P < 0.05$).

Clinical application of miR-27a/b in the CAD diagnosis

As shown in Figure 5, ROC analysis was carried out to validate the ability of miR-27a/b as a diagnostic test. The area under the ROC curve (AUC) value determines the diagnostic potential of miR-27a/b to differentiate individuals with and without CAD conditions. An AUC of 1 indicates a perfect test, while an AUC of 0.5 indicates poor diagnostic performance. ROC analysis showed an AUC of about 92.6 (95% confidence interval [CI], 0.865–0.987) for miR-27a with a sensitivity of 83.3% and a specificity of 86.6% and an AUC of about 93.0 (95% CI, 0.871–0.988) for miR-27b with a sensitivity of 86.6% and a specificity of 80.0%. The relevant results from the ROC analysis are summarized in Table 3. Based on these results, miR-27a/b had marked sensitivity and specificity and may be useful markers in CAD patients.

Discussion

In this study, we evaluated the expression of miR-27a/b as a biomarker in PBMCs of patients with CAD

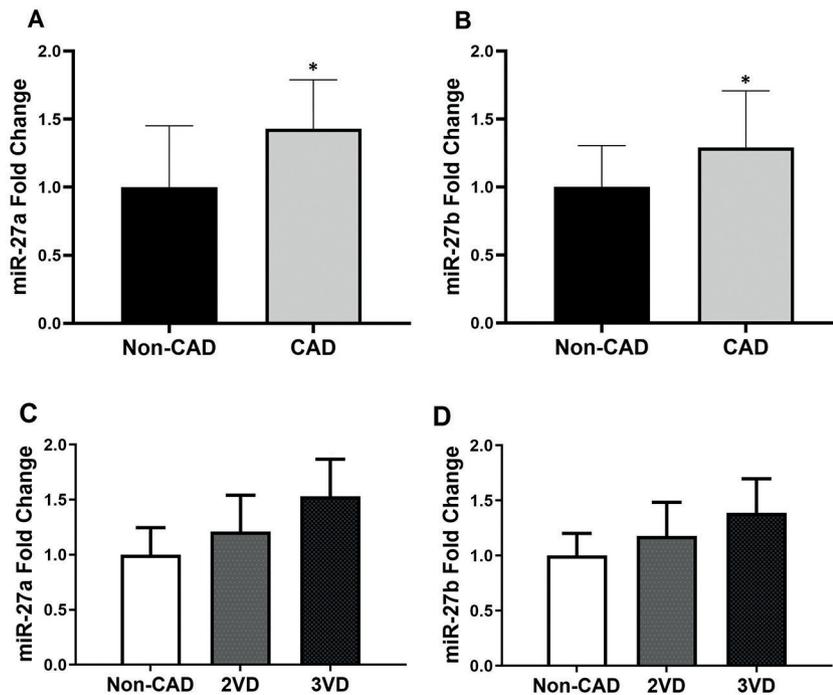


Figure 2 – Comparison of miR-27a (A) and miR-27b (B) levels between CAD (n = 54) and non-CAD groups (n = 51). Expression levels of miR-27a and miR-27b were higher in the CAD group than in the non-CAD group. The expression of miR-27a (C) and miR-27b (D) in 2VD and 3VD was compared to the non-CAD group. All data are presented as mean \pm SD (* p < 0.05). CAD: coronary artery disease; VD: vessel disease.

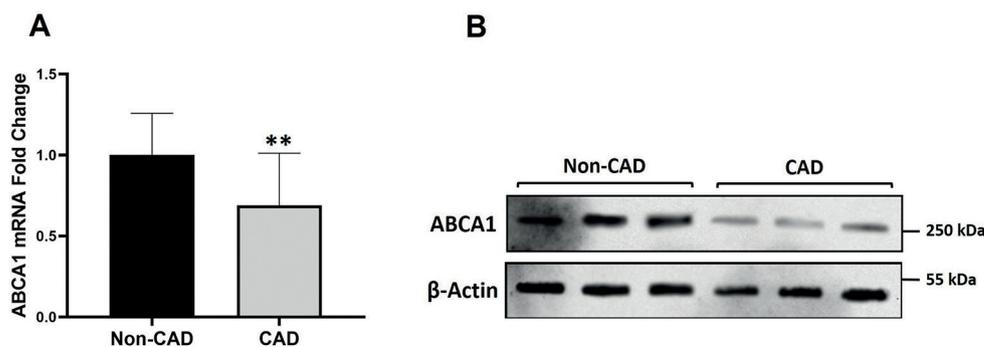
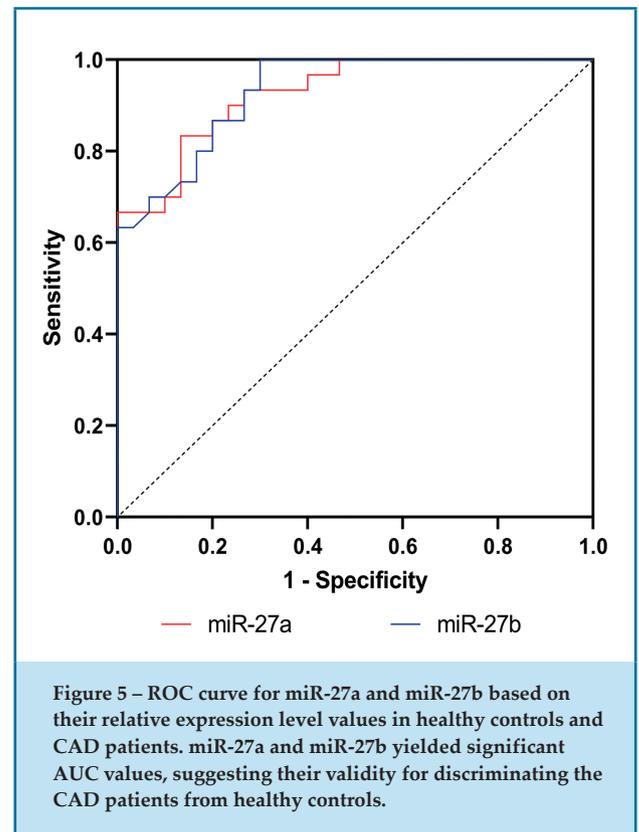
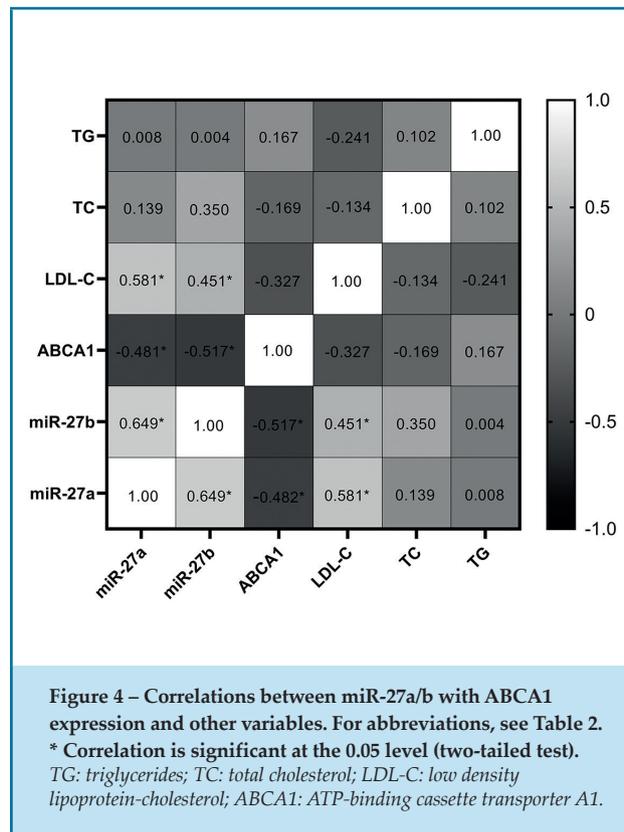


Figure 3 – The mRNA (A) and protein (B) expression levels of ABCA1 in PBMCs of CAD and non-CAD groups were assessed by qRT-PCR and Western blotting, respectively. The data are presented as mean \pm SD (** p < 0.01). CAD: coronary artery disease; ABCA1: ATP-binding cassette transporter A1.

and evaluated its association with ABCA1. We found that miR-27a/b levels were significantly higher in CAD subjects compared with the control group, and patients with 3-vessel CAD showed increased expression of miR-27a/b compared to patients with 2-vessel CAD. ROC analysis showed that miR-27a/b had high validity and may be useful markers in CAD patients. Moreover, we

found an inverse correlation between miR-27a/b and ABCA1 expression. CAD diagnosis and treatment is still a challenging problem faced by cardiologists. In the present years, great attempts have been made to identify innovative diagnostic or prognostic biomarkers for better clinical management of CAD.¹⁹ Recently, serum levels of miR-27a/b have been suggested as a potential



diagnostic or prognostic biomarker in cardiovascular diseases, including atherosclerosis and acute MI.^{16,20,21} In this study, we assessed the expression of miR-27a/b in PBMCs of CAD patients. Human PBMCs, consisting mostly of lymphocytes, monocytes, and macrophages, play a significant role in CAD development via the induction of several inflammatory responses. Importantly, miRNAs expression in PBMCs has been suggested to be related to PBMCs-induced inflammatory responses.¹⁷ Accordingly, Tian et al. demonstrated that overexpression of miR-155 in CD14⁺ monocytes of patients with CAD was associated with increased expression of pro-atherogenic cytokines, including interleukin 6 (IL-6) and tumor necrosis factor- α (TNF- α).²² Upregulation of miR-155 in macrophages of atherosclerotic plaques has also been shown to exert pro-inflammatory effects through suppressing B-cell leukemia/lymphoma 6 (Bcl6), an inhibitor of inflammatory NF- κ B signaling pathway.²³ MCP-1-targeting miRNA, miR-22, has been shown to be downregulated in PBMCs of CAD patients, thus contributing to CAD risk.^{5,24} Additionally, it has been suggested that PBMCs-related miRNAs represent an ideal biomarker for cardiovascular diseases. Meanwhile, PBMCs have their own specific miRNAs expression pattern, which might be different from that of serum or plasma. In

this line, Marketou et al. observed higher levels of miR-26b, miR-499, and miR-208b in PBMCs of hypertensive patients with heart failure with preserved ejection fraction (HFpEF) compared to patients without HFpEF.²⁵ miRNA-548 family signature in PBMCs has also been found as a potential biomarker of heart failure.²⁶ Overexpression of miR-140-3p, miR-2861, and miR-720 in monocytes of patients with acute coronary syndrome (ACS) have been described as potential diagnostic biomarkers in ACS.²⁷ The clinical significance of miRNA expression in PBMCs of patients with CAD has also been reported in several studies. According to Hoekstra et al., a cluster of three miRNAs, namely, miR-370, miR-134, and miR-198, has the ability to be a diagnostic biomarker for high-risk CAD patients.²⁸ Dong et al. observed upregulation of lipometabolism-related miRNAs, including miR-122, miR-103a, and miR-24, in PBMCs from CAD patients.²⁹ They also suggested that the evaluation of lipometabolism-related miRNAs in PBMCs might have better diagnostic accuracy compared with serum or plasma.²⁹ Similarly, in our study, we found increased expression of miR-27a/b as lipid metabolism-regulating miRNAs in PBMCs from CAD patients. Overexpression of miR-27a/b in PBMCs of CAD patients might reflect the involvement of miR-27a/b in the regulation of PBMCs-induced inflammatory responses.

Table 3 – Diagnostic performance of miR-27a and miR-27b for discriminating CAD patients from non-CAD controls.

Variable	AUC	Sensitivity	Specificity	95% CI		PPV	NPV	p-Value
				Lower	Upper			
miR-27a	92.6	83.3	86.6	0.865	0.987	85.5	83.4	<0.001
miR-27b	93.0	86.6	80.0	0.871	0.988	81.1	85.0	<0.001

AUC: area under the curve; CI: confidence interval; PPV: positive predictive value; NPV: negative predictive value.

On the contrary, due to high sensitivity and specificity of miR-27a/b, it can be proposed as a potential marker for early CAD diagnosis. Moreover, we found that patients with 3-vessel CAD have higher miR-27a/b expression compared with 2-vessel CAD patients, suggesting that miR-27a/b levels might be associated with the severity of the disease. However, our study sample size was not large enough to absolutely confirm the relationship between the miR-27a/b and the severity of CAD. Therefore, to obtain significant differences in miR-27a/b expression between 3-vessel CAD and 2-vessel CAD groups, further study with a larger sample size is required. Our data also indicated that miR-27a/b expression in PBMCs of CAD patients was positively correlated with LDL-C, suggesting that miR-27a/b might be a possible risk factor for CAD.

Due to the role of ABCA1 in reducing the risk of atherosclerosis by facilitating cholesterol efflux into lipid-free apolipoproteins, miRNA regulation of the ABCA1 gene might play a critical role in atherosclerosis, thereby controlling CAD condition.^{10,30} Interestingly, miR-27a/b has been found as an ABCA1 targeting miRNA. Accordingly, Zhang et al. indicated that miR-27 regulates the cholesterol homeostasis by directly targeting ABCA1 in THP-1 macrophages.⁹ miR-27-mediated inhibition of ABCA1 has also been reported in Huh-7.5 hepatocarcinoma cells.²¹ We next evaluated the correlation between miR-27a/b and ABCA1 in PBMCs of CAD patients. We found that both the mRNA and protein levels of ABCA1 in PBMCs of CAD group were significantly lower than those of the non-CAD group. Furthermore, we showed that ABCA1 mRNA expression had a significant inverse correlation with miR-27a/b expression. Therefore, it can be concluded that reduced levels of ABCA1 in PBMCs of CAD patients might be due to miR-27-mediated targeting of ABCA1. However, further in vitro experiments should be conducted to definitely elucidate the ABCA1-inhibiting functions of miR-27a/b in CAD patients.

Conclusion

Taken together, our findings provide the evidence that increased expression of miR-27a/b was associated with reduced levels of ABCA1 in PBMCs of CAD patients, suggesting a possible role for miR-27a/b in CAD pathogenesis. Our data also suggested miR-27a and miR-27b as possible PBMC miRNA biomarkers for CAD and highlighted the need for further studies to validate the diagnostic and therapeutic potencies of miR-27a/b in PBMCs of CAD patients.

Author Contributions

Conception and design of the research: Mirzavi F, Ebrahimi S, Alipoor B; acquisition of data and writing of the manuscript: Mirzavi F, Ebrahimi S; analysis and interpretation of the data: Rajabian A; statistical analysis: Hosseini H; obtaining financing: Alipoor B; critical revision of the manuscript for intellectual content: Rajabian A, Hosseini H, Alipoor B. Mirzavi F and Ebrahimi S have contributed equally in this study.

Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

Sources of Funding

This study was funded by Tehran University of Medical Sciences [grant number 30832].

Study Association

This study is not associated with any thesis or dissertation work.

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee

of the Ethics Committee of Tehran University of Medical Sciences under the protocol number IR.TUMS.MEDICINE.REC.1394.30832. All the procedures in

this study were in accordance with the 1975 Helsinki Declaration, updated in 2013. Informed consent was obtained from all participants included in the study.

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