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Original Article

The Relationship Between miRNA-126 And Matrix Metalloproteinase-2 in Patients with Acute Coronary Syndrome

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ABSTRACT

Introduction: Acute coronary syndrome (ACS) represents a critical cardiovascular disease. The primary cause of ACS is typically a thrombus (blood clot) forming within a coronary artery at the site of a vulnerable atherosclerotic plaque. Both miRNA-126 (miR-126) and matrix metalloproteinase-2 (MMP-2) are involved in plaque instability and probably related to ACS.

Materials and Methods: Based on the results of angiography and electrocardiography, 46 individuals diagnosed with ACS and 46 patients with stable coronary artery disease (CAD) were enrolled in this study. Gene expression of serum miR-126 was measured using quantitative real-time polymerase chain reaction. Serum total MMP-2 was determined using enzyme-linked immunosorbent assay kit. MMP-2 enzymatic activity was measured through gelatin zymography. The correlation between miR-126 expression and MMP-2 levels was analyzed using the Pearson's bivariate correlation analysis.

Results: The miR-126 level in ACS patients was significantly lower compared to that in the stable CAD group, ($p<0.05$). In contrast, the serum concentration of MMP-2 was significantly higher in the ACS group (617.3 ng/ml) relative to the stable CAD group (477.1 ng/ml) ($p<0.05$). Similarrly, MMP-2 activity in serum was significantly higher in ACS patients (3700.1 ± 97 units) than in patients with stable CAD (1912.2 ± 31 unit) ($p<0.05$). A significant inverse correlation was observed between the expression of miR-126 and serum levels of MMP-2, ($r= -0.21$, $p<0.05$).

Conclusion: The findings of this study suggest that miR-126 and MMP-2 may contribute to ACS pathogenesis mechanisms related to coronary plaque instability.

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Introduction

Acute coronary syndrome (ACS) refers to a clinical condition that includes patients who have experienced myocardial infarction or unstable angina [1]. ACS is believed to occur due to thrombus formation following the atherosclerotic plaque rupture in the arterial wall, a process that results from local inflammation [2]. The markers for ACS in circulation have prognostic and diagnostic roles for this disease. MiRNAs (miRNAs) are a class of small, non-coding RNAs that regulate protein levels in post-transcription status [3]. These miRNAs participate in the pathogenesis of numerous diseases, including cardiovascular disorders [4]. Various tissue-specific miRNAs are differentially expressed -either upregulated or downregulated- in response to pathological stress associated with heart diseases [5]. Additionally, miRNAs have been detected in plasma and other body fluids, where they can be quantified, suggesting their potential utility as biomarkers for cardiovascular diseases [6]. Endothelial miRNAs play crucial roles in vascular development as well as in responses to hemodynamic stress and inflammation stimuli [7]. Among them, miRNA-126 is highly enriched in vascular endothelial cells [8]. MiRNA-126 regulates the endothelial expression of vascular cell adhesion molecule-1 [9]. According the study by Sun et al., miR-126 levels were not significantly altered in coronary artery disease (CAD) patients [10]. Nevertheless, the relationship between miRNA-126 and ACS has not yet been fully clarified. Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes capable of degrading components of the extracellular matrix (ECM) [11]. Bt breaking down ECM elements within the fibrous cap of atherosclerotic plaques, MMPs may contribute to plaque rupture [12]. Matrix metalloproteinase-2 (MMP-2) is involved in several physiological and cellular processes, including cell development, and functions as a proteolytic enzyme [13]. MMP-2 has been proposed as a potential marker for evaluating its associations with atherogenesis, tumor initiation, invasion, and metastasis [14]. Given that MMP-2 is a principal gelatinase in cardiac and vascular tissue, it was hypothesized that it may play a key role in plaque instability. Therefore, the aim of this study was to investigate the roles of serum miR-126 and MMP-2 as potential biomarkers for distinguishing ACS from stable CAD by comparing patients with ACS to those with stable CAD.

Materials and Methods

A total of 92 patients with complaints of chest pain referred for angiography to Chamran Hospital in Isfahan, Iran, were enrolled in this study. Based on angiographic findings, electrocardiography results, and clinical symptoms, the participants were divided into two groups: 46 patients with ACS and 46 patients with stable CAD [15].

Patients were excluded if they had severe heart failure, malignancy , liver dysfunction (ALT>2-time upper normal limit), renal failure (creatinine >2 mg/dl), major trauma, or recent surgery. Additionally, individuals taking medications

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other than statins and aspirin were excluded from the study. Coronary angiography was performed using either the radial or femoral artery approach. Significant CAD was defined as at least one major coronary artery having ≥70% or left main coronary artery ≥ 50% luminal diameter stenosis [16, 17]. All participants were informed about the objectives and procedures of the study, and written informed consent was obtained from each individual prior to enrollment. Venous blood samples were collected from all participants into serum separator tubes. After following the samples to clot at room temperature for 20 minutes, the tubes were centrifuged at 3000g for 10 minutes to separate the serum. The obtained serum samples were aliquoted and stored at -80 °C until further analysis.

Routine biochemical parameters were analyzed using an using automated analyzer (Hitachi 902, Kyoto, Japan) along with commercial assay kits (Pars-Azmoon, Tehran, Iran), according to the manufacturer's instructions. The following parameters were measured using the enzymatic endopoint method: Fast blood sugar (FBS), triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), hemoglobin A1C, C-reactive protein (CRP) was determined using the immunoturbidimetric method.

miRNA isolation and cDNA synthesis

Serum miRNAs were extracted using a miRNA Purification & Isolation Kit (MACHEREY-NAGEL, German) in accordance with the manufacturer's protocol. The extracted miRNAs, were immediately stored at -80 °C until further analysis. The concentration and purity of the isolated miRNAs were quantified using a NanoDrop 2000.

For polyadenylation and reverse transcription, the Mir-X miRNA First-Strand Synthesis Kit (Takara, Japan) was used following the the manufacturer's instructions. Briefly, 3.75 µl of total small RNA was mixed with 5 µl mRQ buffer and 1.25 µl enzyme mix. The reaction mixture was incubated at 37 °C for 60 minutes to allow polyadenylation and cDNA synthesis. Enzyme inactivation was subsequently performed at 85 °C for 5 minutes, resulting in the generation of the reverse transcription product [18].

miRNA-126 expression using quantitative reverse transcription polymerase chain reaction (qRT-PCR)

qRT-PCR was conducted to evaluate miR-126expression levels in all samples, as previously described [17]. Amplification was performed using an ABI StepOnePlus Real-time PCR system (Applied Biosystems, USA) and Real Q Plus 2x Master Mix Green, ROXTM kit (Ampliqon, A325402), according to the manufacturer's instructions. The primers for miR-126 were designed based on sequences obtained from the miRBase database (http: //mirna.sanger.ac.uk/). The forward and reverse primers of U6 snRNA, as internal control Gen, provided with the kit (Takara, Japan).

qPCR reactions were incubated at 95 °C for 15 min (1 cycle), followed by 15s (40 cycles) at 95 °C and 60 °C for 1 min (40 cycles). At the end of PCR cycles, the melting curve analysis was performed to validate the specificity of PCR product according to the MIQE guidelines [19].

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Each reaction was carried out in a total volume of 20 µl containing: 10 µl Master Mix Green, High ROXTM (Ampliqon), 0.5 µl forward primer, 0.5 µl reverse primer, 2 µl first-strand cDNA and nuclease-free H_2O to adjust the volume. Finally, the cycle threshold (Ct) obtained, and $2^{-\Delta Ct}$ formula was used as previously described [17] to calculate the relative amounts of miRNA and normalized to U6, where ΔCt = Ct mean of miR-126 – Ct mean of U6 snRNA (internal control). Group comparisons were based on the mean $2^{-\Delta Ct}$ ± standard error (SE) [20]. This method indicates the presence of each miRNA relative to the level of an internal control (U6 snRNA). Also, the $2^{-\Delta\Delta Ct}$ formula was used to calculate the relative expression of miRNA in ACS group compared to the stable CAD group [21].

Measurement of Serum MMP-2

Total MMP-2 levels were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) Kit (Hangzhou Eastbiopharm Co. Ltd, China) according to the manufacturer's instructions. The lower limit detection for MMP-2 was 3 ng/ml. The inter-assay coefficient of variation was<5%, indicating good reproducibility for MMP-2.

Assessment of MMP-2 activity by gelatin zymography

MMP-2 enzymatic activity was assessed by gelatin zymography as previously described by Frankowski et al. [22]. In brief, 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gelswere copolymerized with 1mg/ml of gelatin (Sigma, G-9382). Serum samples (20 µL) were mixed with 5 µL of loading buffer and loaded onto the gel wells, followed by electrophoresis. After electrophoresis, gels were washed in renaturing buffer (2.5% Triton X-100 in 50 mM Tris-HCl, pH 7.5) for 1 hour at room temperature to remove SDS and restore enzyme activity. The gels were then incubated in developing buffer (5 mM CaCl2, 3 mM NaN3 in 50 mM Tris-HCl, pH 7.5) for 42 hours at 37 °C. Subsequently, gels were stained with 0.5% (w/v) Coomassie Brilliant Blue R-250 (Bio-Rad) for 60 minutes and destained in a solution containing 30% methanol and 10% acetic acid until clear bands appeared against a dark background. The gels were photographed using a gel documentation system (Bio-Rad, USA). The gels were photographed using a gel documentation system (Bio-Rad, USA). Gelatinolytic activity was quantified by measuring the area of the destained bands (in pixels) using ImageJ software (NIH, USA).

Statistics analyses

Continuous variables were presented as Mean ± standard error for normally distributed data or as median values for non-normally distributed data. Categorical variables were presented as percentages.

Bivariate correlation analysis was perfrmed to assess the relations of miR-126 expression and serum levels of MMP-2, as well as biochemical parameters. Qualitative variables were compared using Fisher's exact test. Comparisons between two groups were performed using the Student's t-tests for Gaussian data or Mann–Whitney tests for non-Gaussian data. Receiver operating characteristic (ROC) curves were generated to evaluate the ability of miR-126 and MMP-2 to discriminate between ACS patients and stable

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CAD patients. A two-tailed P value < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS software version 22.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Demographics and characteristics of study population

The demographic and clinical characteristics of the study population were summarized in Table 1. There were no statistically significant differences between the ACS and stable CAD groups regarding age, sex distribution, body mass index, or blood pressure. Similarly, no significant differences were observed between the two groups in biochemical parameters, including: LDL cholesterol, TC, TG, creatinine, FBS and hemoglobin A1C. However, HDL-cholesterol levels were significantly lower in ACS group compared to the stable CAD group ($p<0.05$). In contrast, CRP levels were significantly higher in patients with ACS than in those with stable CAD group ($p<0.05$) (Table 1).

Expressions of miR-126 in serum samples were detected by real-time PCR

The median miR-126 expression level was significantly lower in ACS patients compared to stable CAD patients (1.03 vs. 1.97, $p<0.01$). Similarly, the mean of miR-126 expression level was significantly reduced in ACS patients compared with stable CAD patients (1.9 ± 0.28 vs. (4.29 ± 0.67, $p<0.01$) , (Fig. 1). Analyses in ΔΔCt method showed that the serum expression of miR-126 ($2^{-\Delta\Delta ct}$) decreased in patients with ACS, at 2.19 fold, compared to stable CAD.

The serum level of MMP-2 increased in patients with ACS

The medium serum MMP-2 level was significantly higher in ACS patients than in stable CAD patients (617.3 ng/ml vs. 477.1 ng/ml, $p<0.01$). Likewise, the mean serum MMP-2 concentration was significantly elevated in ACS patients compared to stable CAD patients (654.9 ± 30.95 ng/mL vs. 527.8 ± 23.8 ng/mL, $p < 0.01$) , (Fig. 2).

Activity of MMP-2 increased in patients with ACS

Gelatin zymography was to evaluate MMP-2 enzymatic activity in its different forms (pro-, active- and total MMP-2) in serum samples (Fig. 3). As shown in table 2, the activity of MMP-2 in all forms was significantly higher in the ACS group compared with the stable CAD group ($p<0.001$ for all comparisons). The discriminating value of serum miR-126 and MMP-2 for ACS receiver operating characteristic (ROC) curve analysis was performed to assess the ability of serum miR-126 and MMP-2 levels to discriminate between ACS and stable CAD patients. Diagnostic accuracy was measured using the area under the ROC curve, where an area under the ROC of 1.0 represents a perfect diagnostic test. The optional cutoff value was determined based on the point yielding, the maximum combined sensitivity and specificity. For the miR-126, the area under the ROC curve ($2^{-\Delta Ct}$) was 0.356 (95 %CI 0.243–0.469, $p< 0.01$), (Fig. 4a). For discriminating ACS from stable CAD the Cut-off point ($2^{-\Delta Ct}$) was 0.95 (sensitivity, 61.00%; specificity, 40.00%). For MMP-2 the area under the ROC curve was 0.674 (95%CI:

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0.563–0.784, P< 0.001) (Figure 4b). For discriminating ACS from stable CAD the Cut-off point was 528.3 ng/mL (sensitivity: 72%, specificity: 55%) for MMP-2.

The relationship between serum levels of miR-126, MMP-2

Pearson's correlation analysis was run to evaluate the relationship between serum miR-126 expression and serum MMP-2 levels. A significant negative correlation was observed between miR-126 and MMP-2 levels (r= -0.23, p = 0.001) (Fig. 5).

Discussion

Coronary heart disease is the most prevalent type of cardiovascular disease, characterized by the accumulation of a fatty, wax-like substance known as plaque within the coronary arteries [23, 24]. Two primary clinical forms of coronary heart disease are stable CAD and ACS, also referred to as unstable coronary artery disease. Distinguishing between these two conditions is extremely important in clinical practice.

Table 1. Demographic and clinical characteristics of the study population

Characteristics	Patients with ACS (n=46)	Patients with Stable CAD (n=46)	p-value
Age (yrs)	63.89 ± 1.69	61.78 ± 1.3	0.327
Male sex, n (%)	30/16	27/19	0.525
Blood pressure, mm Hg			
Systolic	133.8±3.1	128.7±2.01	0.17
Diastolic	81.65±1.34	79.35±0.77	0.141
Physical data			
Height, cm	172.7± 1.18	174.1± 0.78	0.334
Body weight, kg	72.4± 1.29	74.17.1± 1.12	0.312
Body mass index (kg/m^2)	24.5 ± 0.37	24.3 ± 0.33	0.719
Blood urea nitrogen, mg/dL	20.79 ± 0.39	21.45 ± 0.25	0.16
Serum creatinine, mg/dL	1.05 ± 0.032	1.01 ± 0.029	0.37
Hemoglobin A1c, %	6.08 ±0.092	6.27 ±0.059	0.089
Glucose (mmol/L)	6.66 ± 0.14	6.91 ± 0.1	0.08
Total cholesterol (mmol/L)	5.05 ± 0.11	5.03 ± 0.11	0.88
Low-density lipoprotein-cholesterol (mmol/L)	3.31 ± 0.097	3.15 ± 0.089	0.25
High-density lipoprotein-cholesterol (mmol/L)	0.93 ± 0.019	1.04 ± 0.016	<0.01
Triglycerides (mmol/L)	1.76 ± 0.07	1.8 ±0.05	0.75
C-reactive protein (mg/dL)	2.22 ± 0.06	1.67 ± 0.05	<0.001
Statins, n (%)	19 (41)	17 (37)	0.47
Aspirin, n (%)	20 (44)	18 (39)	0.69
Smoking, n (%)	18 (39)	16 (35)	0.67

Data are expressed as means ± SE. p-value <0.05 was considered significant.

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Table 2. Results of Gelatin zymography for acute coronary syndrome and stable coronary artery disease

Patients	Active MMP-2	Pro MMP-2	Total MMP-2
Acute coronary syndrome	2268 ± 72	1430.7 ± 12	**3700.1 ± 97**
Stable coronary artery disease	1314.3 ± 74	597.9 ± 27.5	**1912.2 ± 31**
P value	0.001	0.001	**0.001**

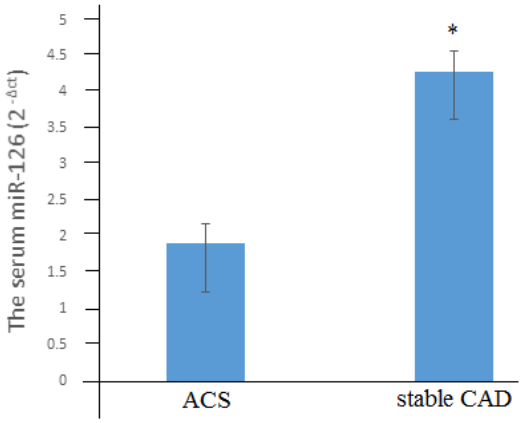

Fig. 1. miRNA-126 levels are reduced in patients with ACS. ACS= Acute coronary syndrome; CAD= Coronary artery disease.
* p-value= 0.001

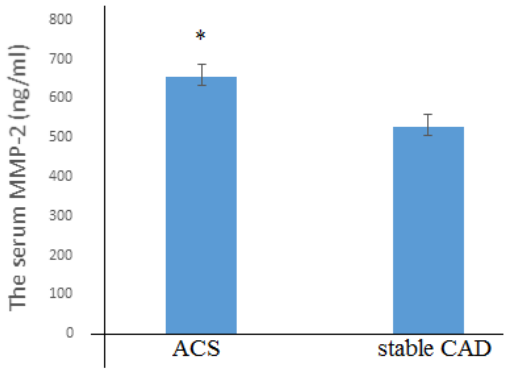

Fig. 2. The serum MMP-2 levels are increased in patients with ACS. MMP-2 = Matrix metalloproteinase-2; ACS= Acute coronary syndrome; stable CAD= Stable coronary artery disease
* p-value= 0.001

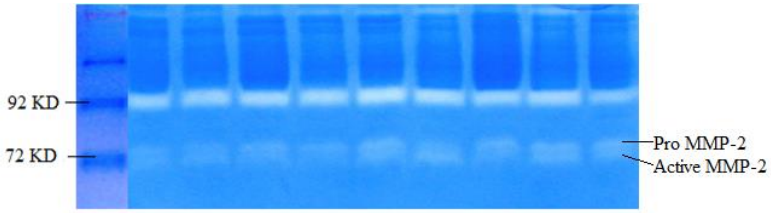

Fig. 3. The Gelatin zymography for MMP-2. The 62 kDa matrix metalloproteinase (MMP)-2 and 92 kDa MMP-9.

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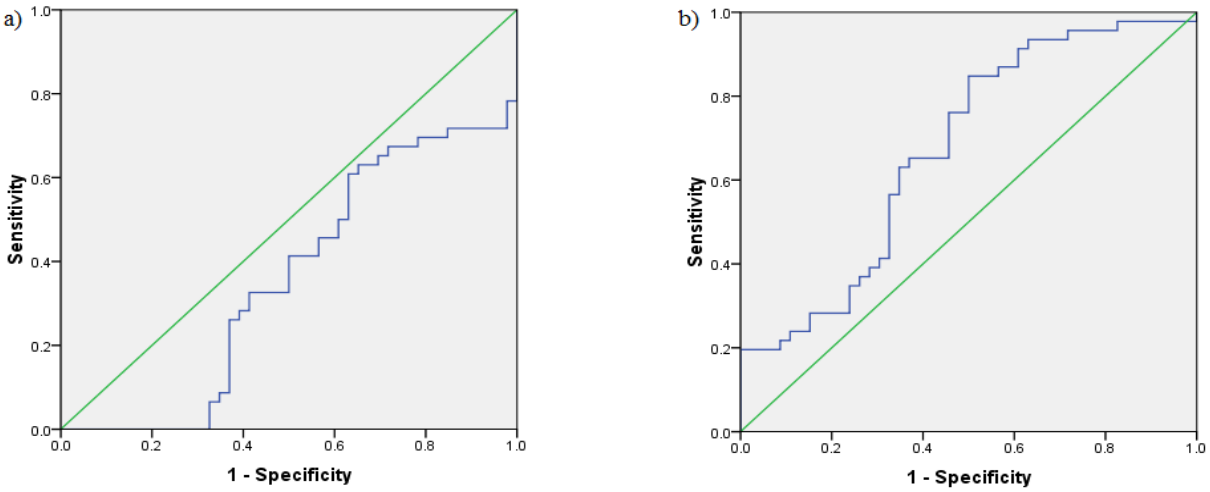

Fig. 4. Receiver operating characteristic (ROC) curve for discriminating of acute coronary syndrome (ACS) from stable coronary artery disease (CAD). a: ROC curve for serum miR-126 to discriminating of ACS from stable CAD. The area under the ROC curve was 0.356 (95 %CI 0.243–0.469, p< 0.01). b: ROC curve for serum matrix metalloproteinase-2 (MMP-2) to discriminating of ACS from stable CAD. The area under the ROC curve was 0.674 (95 %CI 0.563–0.784, p< 0.001).

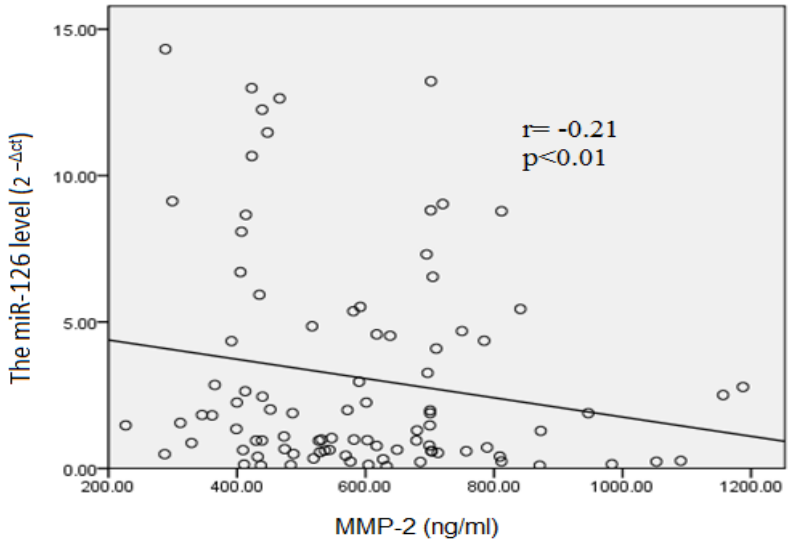

Fig. 5. The correlation between miR-126 and the circulating levels of matrix metalloproteinase-2 (MMP-2). Serum miR-126 level was negatively correlated with MMP-2 (r= -0.23, p < 0.01).

Biomarkers can be utilized to differentiate ACS from stable CAD. Therefore, the main objective of this study was introducing a biomarker for differentiation of ACS from stable CAD. miRNAs have attracted considerable attention as potential disease biomarkers. Although miRNAs are generally unstable RNA molecules, they remain stable in serum and plasma because they are located inside the mivesicles (exosomes). Furthermore, circulating miRNAs demonstrate resistance to RNase activity, as well as to

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extreme pH and temperature variations [25-27]. Consequently, miRNAs present in blood and other body fluids are stable and may serve as promising biomarkers for disease detection. The results of this study showed that serum miRNA-126 expression levels were significantly lower in patients with ACS compared to those with stable CAD. In contrast, serum total-MMP-2 levels and their zymographic activities were significantly higher in patients with ACS relative to stable CAD patients. Additionally, serum miRNA-126 levels showed a negative correlated with serum MMP-2 levels.

Previous studies have demonstrated that miR-126 is associated with cardiovascular diseases. The study by Meister et al. [28] showed that miRNA-126 is expressed in vascular endothelial cells and plays a role in involved in inflammatory processes. According to the study by Harris [29], overexpression of the precursor to miR-126 leads to increases levels of miR-126 and decreases vascular cell adhesion molecule-1 expression.

Additionally, decreasing endogenous miR-126 levels increases leukocyte adherence to endothelial cells. Therefore, miRNA-126 is capable of modulating adhesion molecule expression and may provide additional control of vascular inflammation.

Taken together, these findings suggest that miR-126 is associated with inflammation and atherosclerotic plaque instability. Given that the primary cause of ACS, are inflammation and atherosclerotic plaque instability, it is suggested that miR-126 related to this disease. However, these findings are consistent with the results of our study. MMPs are a family of endopeptidase that degrade a variety of substrates, including ECM proteins, proteinases, and their inhibitors. Gelatinase A, also known as type IV collagenase or MMP-2, has been identified in a wide range of normal and malignant cell types, including macrophages and fibroblasts [30, 31]. Connective tissue primarily consists of collagen and elastin. Gelatinases A and B can degrade the collagen peptide in ECM [32].

The low miR-126 expression has been correlated with tumor progression through the stimulation of angiogenesis and lymphangiogenesis. However, the exact mechanism underlying the miR-126 mediated the activation of angiogenesis and lymphangiogenesis has not yet been fully clarified. According to the study by Kjaer et al. [32], miR-126 is down-regulated under hypoxic conditions both *in vitro* and *in vivo,* and it may halt the hypoxia-induce neovascularization by suspending the cell cycle progression and inhibiting the expression of vascular endothelial growth factor and MMP-9. In addition, Khan et al. study [33] showed a relationship between MMP-2 and VEGF. However, these studies suggest that miR-126 through its interaction with MMP-2, and MMP-9, is associated with inflammation and the instability of atherosclerotic plaque.

In our study, miR-126 at a cutoff point of 0.95 yielded a sensitivity of 61% and a specificity of 40%. Similarly, MMP-2 at a cutoff of 528.3 ng/mL yielded a sensitivity of 72% and a specificity of 55%. In comparison, the

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biomarkers proposed by Darabi et al. [34] for discriminating ACS from stable CAD indicated that miR-21 at a cutoff of 0.73 achieved a sensitivity of 74% and a specificity of 70%. Furthermore, MMP-9 at a cutoff of 218.72 ng/ml yielded a sensitivity and specificity of 70% each. Therefore, the sensitivity and specificity reported in that study were higher than those observed in our study. However, we introduce down-regulateed miR-126 and increased MMP-2 levels as potential biomarkers for discriminating ACS from stable CAD.

Conclusion

In conclusion, patients with ACS exhibited significantly lower serum levels of miR-126 and higher serum levels of MMP-2 compared to stable CAD patients. Decreased serum miRNA-126 and increased MMP-2 levels were identified as independent discriminating factors for distinguishing ACS from stable CAD. Based on our findings, we suggest a novel and potential discriminating value of miRNA-126 and MMP-2 levels for distinguishing ACS from stable CAD among cardiovascular patients. If confirmed in larger studies, our study could have important clinical implications.

Ethical Considerations

The study protocol was approved by the Ethics Committee of Isfahan University of Medical Sciences (Approval No. 394943).

Funding Statement

The responsible author covered the experiment and other associated costs.

Conflict of Interest

The authors declared no conflict of interest.

Acknowledgment

We sincerely appreciate all the staff members and participants who contributed to this study. The study protocol was approved by the Ethics Committee of Isfahan University of Medical Sciences, Isfahan, Iran, in accordance with the Helsinki Declaration. This research was funded by grant Number 394943 from Isfahan University of Medical Sciences. This manuscript benefited from AI-assisted refinement and formatting support, including language polishing and abbreviation standardization.

Data Availability Statement

The data presented in this study are available on request from the corresponding author.

Authors' Contributions

AA analyzed and drafted the manuscript. FD and NS reviewed the study and edited the manuscript. AM and MA was the study supervisor, designed the study, advised on analyses and edited the manuscript. All authors read and approved the final manuscript.

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