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Circulating Microvesicles Enriched in miR–126–5p and miR–223–3p: Potential Biomarkers in Acute Coronary Syndrome

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Abstract: Background. The molecular mechanisms underlying acute coronary syndrome (ACS) have been extensively investigated, with a particular focus on the role of circulating microvesicles (MVs) as carriers of regulatory elements that influence hemodynamic changes and coronary flow. Endothelial and platelet dysfunction during ACS alters MVs composition, impacting clinical outcomes. This study explores the levels of miR–126–5p and miR–223–3p in circulating MVs and their association with the Thrombolysis in Myocardial Infarction (TIMI) coronary flow classification scale, proposing their potential as biomarkers. **Methods.** Bioinformatic tools identified miRNAs linked to ACS. Plasma MVs were isolated from ACS patients and healthy controls through high-speed centrifugation. miRNA levels were quantified using quantitative reverse transcription polymerase chain reaction (qRT-PCR) and compared across TIMI 0 and TIMI 3 groups. Diagnostic efficacy was assessed via receiver operating characteristic (ROC) curve analysis. **Results.** The bioinformatic analysis identified miR–126 and miR–223 present in ACS. miR–126–5p and miR–223–3p were significantly reduced in MVs from TIMI 0 patients compared to TIMI 3. ROC analysis showed high diagnostic accuracy for miR–126–5p (AUC = 0.918; 95% CI: 0.818–1.00; $p = 0.001$) and miR–223–3p (AUC = 1.00; 95% CI: 1.00–1.00; $p < 0.001$). **Conclusion.** Reduced levels of miR–126–5p and miR–223–3p in circulating MVs are strongly associated with impaired coronary flow, positioning these miRNAs as potential biomarkers for ACS risk stratification and therapeutic targeting.

Keywords: miRNAs; microvesicles; acute coronary syndrome; TIMI; biomarkers

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1. Introduction

Acute coronary syndrome (ACS) encompasses a spectrum of clinical conditions caused by insufficient coronary blood flow, including acute myocardial infarction (AMI) and unstable angina (UA) [1]. ACS is a leading cause of morbidity and mortality worldwide, with cardiovascular diseases (CVDs) responsible for over 17.9 million deaths annually, according to the World Health Organization (2023) [2]. This high prevalence underscores the urgent need for improved diagnostic and prognostic tools to better understand ACS pathophysiology and enhance patient care. A key clinical tool for assessing ACS severity is the Thrombolysis in Myocardial Infarction (TIMI) coronary flow classification scale, which uses coronary angiography to categorize epicardial blood flow into four grades: TIMI 0 (no perfusion), TIMI 1 (minimal perfusion), TIMI 2 (reduced-speed perfusion), and TIMI 3 (normal perfusion) [3]. Stratification using the TIMI scale has been linked to clinical outcomes such as reinfarction [4], mortality [5,6], ventricular aneurysm formation [7], and arrhythmias [8]. Achieving TIMI 3 flow is a critical benchmark for therapeutic success [9,10]. Despite advances in reperfusion therapies and pharmacological treatments, ACS remains a clinical challenge due to its complex and multifactorial nature. The lack of reliable biomarkers to identify molecular and hemodynamic changes in ACS limits clinicians' ability to accurately predict outcomes [11]. Circulating microvesicles (MVs) have emerged as promising biomarkers and mediators in cardiovascular disease. MVs are extracellular vesicles released by platelets [12], endothelial cells [13], and leukocytes [14] in response to stress or activation. They carry bioactive molecules, including proteins, lipids, and microRNAs (miRNAs) [15]. miRNAs, small non-coding RNAs (21–25 nucleotides), regulate gene expression post-transcriptionally by targeting messenger RNA for degradation or translational repression, influencing up to 30% of human genes [16]. In ACS, miRNAs such as miR–26, miR–126, miR–133, miR–144, miR–208, miR–223, and miR–483 modulate platelet activity, oxidative stress, cardiac remodeling, and inflammation [17,18]. MVs have been implicated in endothelial dysfunction, platelet activation, and disrupted coronary blood flow [19–21]. These vesicles facilitate platelet–platelet and platelet–endothelial communication, regulating thrombotic processes [22–24]. Alterations in MV cargo, including miRNAs, during ACS reflect underlying pathophysiological processes. This study focuses on miR–126–5p and miR–223–3p levels in circulating MVs and examines their potential as biomarkers for ACS, particularly in relation to the TIMI flow scale.

2. Materials and Methods

2.1. Study Population

This cross-sectional study included, by convenience, 42 participants: 32 ACS patients and 10 healthy controls. Patients were recruited from the Hemodynamics Department of the National Institute of Cardiology (INC) Ignacio Chávez, Mexico City. The inclusion criteria were age from 40 to 75 years, diagnosis of UA, ST–segment elevation (STEMI) and non–ST–segment elevation (NSTEMI) AMI made by angiographic according to the criteria of the American College of Cardiology [25]. Patients were stratified according to the TIMI flow scale by an expert cardiologist from the Hemodynamics Department. This stratification considered the impact of biological and mechanistic factors associated with total reperfusion or lack of reperfusion. The healthy group comprised clinically healthy subjects 40 to 75 years old from the blood bank of the INC. Exclusion criteria included autoimmune, hepatic, renal, or oncological diseases. All participants provided informed consent, and the study was approved by the Research and Ethics Committees of INC with register number FIMICOR 18-1043.

2.2. Microvesicles Extraction

Blood samples were collected in sodium citrate (0.109 M, relation 1:9) tubes and centrifuged at 1500 × g for 15 min to obtain plasma. Plasma was further centrifuged at 13,000 × g for 2 min and stored

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at −80°C. MVs were isolated through high-speed centrifugation (20,000 × g for 90 min, 4°C) and resuspended in 100 µL of phosphate buffer, was added 400 µL of QIAzol Lysis Reagent and stored in aliquots at –20 °C, before RNA extraction.

2.3. RNA Extraction and Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

RNA was extracted from MVs using the Direct–zol™ RNA MiniPrep Kit (Zymo Research, CA, USA), according to the manufacturer's instructions, and was eluted in 25 µL of RNase–free water. RNA samples were stored at –80 °C until their processing. For the miRNA complementary DNA (cDNA), we employed the TaqMan© MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions. Retrotranscription was developed according to the manufacturer's instructions. The results were analyzed and the samples with amplification after 35 cycles were discarded. miRNA levels were quantified using TaqMan© miRNA Assays (Applied Biosystems, Foster City, CA, USA) for miR–126–5p, miR–223–3p, and miR–39–3p as control were employed in a Roche LightCycler® 480–II instrument (Roche Applied Sciences, Beijing, China), under standard amplification conditions. Relative expression was calculated using the delta-delta Ct method ($2^{-\Delta\Delta Ct}$).

2.4. Bioinformatic Analysis

Potential ACS-associated miRNAs were identified using miRbase (https://www.mirbase.org/), miRNet (https://www.mirnet.ca/), the Human microRNA Disease Database (HMDD, http://www.cuilab.cn/hmdd#fragment–1) and miRandola (http://mirandola.iit.cnr.it/index.php). miRNAs present in at least three databases with validated roles in cardiovascular diseases were selected. The analysis focused on identifying miRNAs associated with ACS in the bloodstream, particularly those with previously reported controversial level patterns. This approach aimed to investigate whether these miRNAs could indicate potential transport mechanism via MVs. The selection included miRNAs that had functional evidence and were validated in the literature for their roles in cardiovascular disease.

2.5. Statistical Analysis

Quantitative data are expressed as mean ± standard deviation (SD) or median (interquartile range), and the qualitative variables are presented as frequencies (*n*) and percentages (%). We analyzed the normal distribution of data by Shapiro-Wilk; group differences were analyzed using ANOVA with Dunnett's post hoc test. A receiver operator characteristic (ROC) curve analysis assessed diagnostic efficacy, and the area under the curve (AUC) with their 95% confidence intervals (CI) was calculated. Statistical significance was set at $p < 0.05$. Analyses were conducted using SPSS v23.0 Software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism Software 8.0.1 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. MVs miRNAs Associated with ACS

Considering the association with ACS, the analysis revealed that the MVs content of hsa-miR–126–5p and hsa-miR–223–3p were identified in four of the evaluated databases. Figure 1 is a schematic representation of the network obtained from the miRNet database, which highlights the miRNAs selected for this study.

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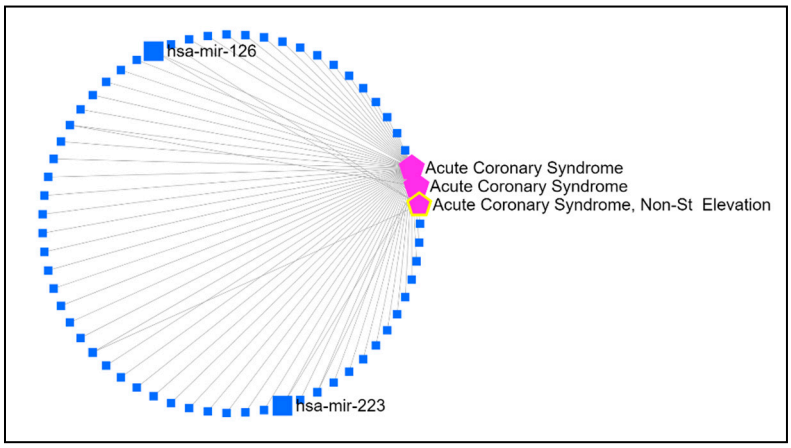

Figure 1. A schematic representation of the network from miRNAs associated with ACS, was developed using the miRNet database (https://www.mirnet.ca/miRNet/home.xhtml). The pink pentagon represents the disease (ACS); the square represents the miRNA of interest; the line represents the interaction between miRNAs and the disease.

3.2. Sociodemographic Data and Clinical Variables of Study Population

The clinical characteristics of the study population were obtained from clinical records and are presented in Table 1.

Table 1. Sociodemographic data and clinical variables of the patients according to their TIMI classification.

Clinical variable	TIMI 3 (*n* = 17)	TIMI 0 (*n* = 15)	*p* value
Age [years]	62.0 [57.0–66.0]	62.5 [55.0–65.7]	0.85
Male n (%)	9 (52.9)	9 (60.0)	0.24
BMI [kg/m2]	24.9 [22.2–30.1]	25.8 [23.7–31.0]	0.48
Laboratory data			
Glucose [mg/dL]	136.0 ± 47.0	112.9 ± 26.6	0.78
Triglyceride [mg/dL]	125.0 [96.1–183.1]	162.2 [130.0–257.2]	0.17
Total cholesterol [mg/dL]	150.5 ± 47.3	156.4 ± 43.3	1.00
LDL [mg/dL]	84.3 ± 44.9	90.5 ± 32.5	0.72
HDL [mg/dL]	41.0 ± 12.5	34.1 ± 9.5	0.18
Platelets [x103/µL]	214.1 ± 69.9	210.5 ± 86.3	0.91
MPV [fL]	9.2 ± 1.3	9.4 ± 1.4	0.74
Hemoglobin [g/dL]	14.9 [13.3–17.0]	15.0 [13.9–16.4]	0.87
Hematocrit [%]	41.9 ± 8.6	45.2 ± 6.3	0.31
McV [fL]	91.5 ± 7.1	91.3 ± 3.9	0.91
McH [pg]	32.1 ± 1.3	30.2 ± 1.7	0.08
CPK-MB [U/mL]	5.9 [3.2–54.1]	3.1 [2.6–103.3]	0.77
Troponin [ng/mL]	1.1 [0.3–37.4]	5.1 [0.6–16.3]	1.00
C-reactive protein [mg/dL]	8.3 [1.4–63.9]	4.0 [0.6–21.5]	0.39
Diabetes *n* (%)	6 (35.3)	2 (13.3)	0.20

Data are represented by mean ± SD or median (interquartile range), or n and (%) according to data type and its distribution (Shapiro-Wilk test). Mann–Whitney U test or Chi-square test with the SPSS v23.0 program. *p < 0.05 was considered statistically significant. ACS, acute coronary syndrome; BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein; MPV, mean platelet volume; McV, mean corpuscular volume; McH, mean corpuscular hemoglobin; CPK–MB, creatine phosphokinase–mb; TIMI, Thrombolysis in Myocardial Infarction; %, percentage; µL, microliter; fl, phentoliter; g/dL, gram/deciliter; kg/m2,

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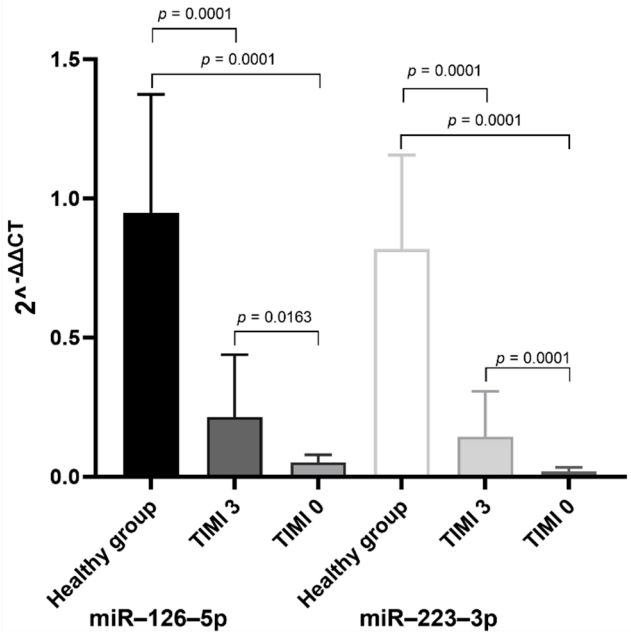
kilogram/square metre; mg/dL, milli-gram/deciliter; ng/mL, nano-gram/mililiter; pg, pico-gram; U/mL, units/mililiter.

3.3. miR–126–5p and miR–223–3p Levels

The 2^-ΔΔCT (Figure 2) of each analyzed sample represents the relative level of each of the miRNAs evaluated. miR–126–5p and miR–223–3p levels were significantly lower in TIMI 0 and TIMI 3 patients compared with healthy control. In TIMI 0 patients, miRNA–126–5p and miRNA–223–3p levels were 4.2 and 7.0 times lower, respectively, when compared with TIMI 3 patients.



miRNAs (2^-ΔΔCT)	*TIMI 3 (n = 17)*	*TIMI 0 (n = 15)*	*Healthy group (n = 10)*	*p value*
miR–126–5p	0.21 ± 0.22	0.05 ± 0.27	0.95 ± 0.42	0.0001¥ 0.0163*
miR–223–3p	0.14 ± 0.16	0.02 ± 0.01	0.82 ± 0.33	0.0001¥ 0.0001*

Figure 2. Levels of miR–126–5p and miR–223–3p in the study population. Data represent the mean ± SD. Differences in each miRNA between groups were compared by one-way ANOVA with Dunnet as a post hoc test with the SPSS v23.0 program. A p value < 0.05 was considered statistically significant. ¥ healthy group vs TIMI 3 and TIMI 0; * TIMI 3 vs TIMI 0;. miRNA, Small noncoding RNAs; 2^-ΔΔCT, delta-delta Ct method.

3.4. Diagnostic Value of miR–126–5p and miR–223–3p Levels

ROC analysis demonstrated high diagnostic potential, with AUC values of 0.918 for miR–126–5p 95% CI = 0.818–1.00, $p = 0.001$, while for miR–223–3p, the AUC was 1.00 (95% CI = 1.00–1.00, $p < 0.001$), Figure 3.

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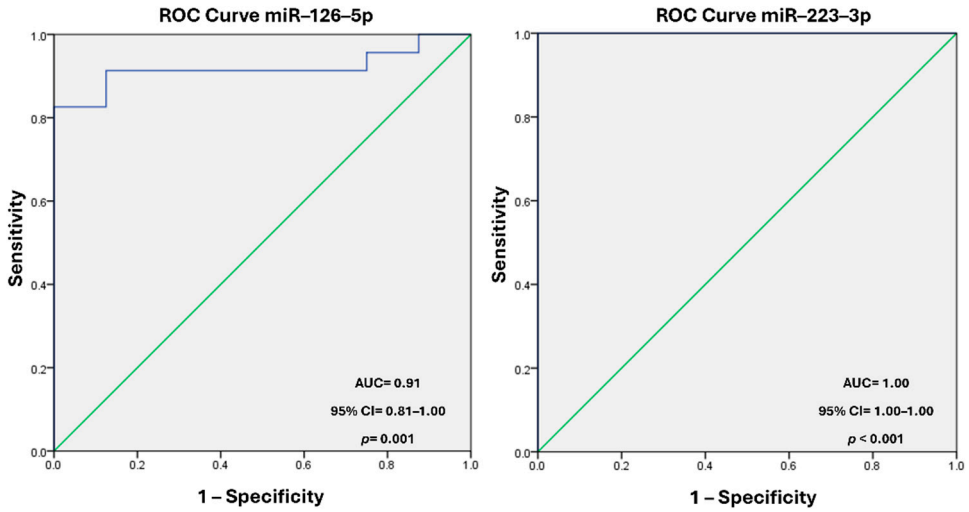

Figure 3. ROC Curve of miR–126–5p and miR–223–3p levels in the study population. SPSS v23.0 program. A *p* value < 0.05 was considered statistically significant. AUC, area under the curve; CI, confidence interval; ROC, receiver operator characteristic.

4. Discussion

The development of innovative strategies for diagnosing and stratifying the risk of ACS remains a critical area for both basic and clinical research. In this study, we analyzed a cohort of ACS patients and investigated the levels of miRNAs (miR–126–5p and miR–223–3p) present in circulating plasma MVs as potential mechanisms associated with ACS pathophysiology. Notably, both miRNAs are known to be present in circulating MVs [26,27].

miRNAs play a role in phenotypic changes influenced by environmental factors, including DNA methylation, histone modification, and chromatin remodeling [28]. The epigenetic involvement of miRNAs and their distribution to critical sites for coronary ischemic diseases underscore their role in regulating platelet activity and individual susceptibility to coronary conditions. This makes them valuable biomarkers for predicting coronary disease risk [29].

By studying miRNAs within MVs, we focused on two key mechanisms directly impacting the physiological environment. Recent reviews highlight the significance of MVs in various biological processes [30], reinforcing the importance of analyzing miRNAs within these vesicles. Bioinformatics tools offer a powerful methodology within omics sciences, facilitating the interpretation of clinical and molecular data [31–33]. By integrating bioinformatic analyses with molecular studies, we identified miRNAs consistently associated with ACS, providing robust evidence of their biomarker potential.

miR–223, known for its anti-thrombotic properties, is highly prevalent in platelets and their derived MVs. It regulates platelet activity in atherosclerotic progression by inhibiting platelet activation, aggregation, and granule secretion [34–37]. miR–223 is thus a promising marker of platelet activation in CVDs [38]. Similarly, miR–126 plays a vital role in maintaining endothelial integrity, vascular repair, and platelet activation/aggregation [34,39]. It serves as a potential biomarker for endothelial homeostasis in CVDs and ACS risk stratification [34,40].

Our study underscores the diagnostic and prognostic potential of miR–126–5p and miR–223–3p in ACS. Both miRNAs were significantly reduced in TIMI 0 and TIMI 3 patients. Their levels reflect the physiological and regulatory mechanisms underlying atherosclerosis progression and platelet activity, with their absence strongly correlating with impaired blood flow. In TIMI 0 patients, the reduction of these miRNAs is associated with coronary flow impairment and endothelial dysfunction, hallmarks of ACS pathophysiology. Additionally, we observed statistically significant differences in miR–126–5p and miR–223–3p levels in ACS patients compared to healthy individuals.

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ACS patients exhibited lower miRNA levels, with a more pronounced decrease in miR–223–3p expression relative to miR–126–5p.

In the literature, only a few studies have explored the levels of these miRNAs in ACS, with contradictory findings. For instance, Massodi et al. analyzed the expression of several platelet-derived miRNAs, including miR–223 and miR–126, in ACS patients but found no statistically significant differences compared to control subjects [41]. In contrast, Gager et al., in a prospective observational study, reported a marginal increase in miR–223 levels in plasma from ACS patients, particularly when comparing those with non-ST-segment elevation ACS (NSTE-ACS) to STEMI patients [42].

Another study observed elevated miR–126 levels in plasma during the acute phase of AMI patients compared to controls, followed by a significant decrease after a follow-up period [43]. miR–126 has been linked to modifications in platelet reactivity [44] and monocyte–platelet aggregates (MPAs) [45]. Ling et al. further reported that exosomal and serum miR–126 levels were significantly higher in patients with AMI and UA compared to controls [46].

Becker et al. evaluated miR–126 in relation to platelet reactivity in blood platelet in NSTE-ACS patients and suggested that miR–126 could serve as a marker for changes in platelet activity [44]. Stojkovic et al. found an association between platelet miR–126 and MPAs in ACS patients undergoing antiplatelet therapy [45]. Lastly, Szelenberger et al. identified significantly elevated levels of miR–223–3p in blood platelet in ACS patients compared to controls, but no notable differences were observed in miR–126–3p levels between the groups [47]. Currently, no studies have specifically examined the relationship between miRNAs in MVs and the TIMI flow scale. However, Jasen et al. analyzed miRNA expression (miR–126) in circulating MVs but in patients with coronary artery disease (CAD), they found lower levels of miR–126 in CAD patients *vs* no CAD group [26]. On the other hand, Zhang et al. investigated the association between circulating miR–660–5p and the no-reflow phenomenon in STEMI patients. In their study, NRP was defined as a TIMI flow grade lower than 2 or 3 [48].

The originality of our study lies in two key aspects: (1) it evaluates the miRNA profiles derived from circulating MVs in patients with ACS, in contrast to prior studies that focused on platelets, and (2) it includes a stratified analysis based on the TIMI flow scale.

This approach reflects a specific pathophysiological condition and minimizes bias by avoiding comparisons with a control group of healthy individuals. Instead, both groups in our study were under the same treatment and pathological conditions. Our findings have important clinical implications. First, the association of miR–126–5p and miR–223–3p with TIMI flow grades highlights their potential utility in stratifying ACS severity and guiding therapeutic decisions. Second, the high diagnostic accuracy of these miRNAs, demonstrated through ROC curve analyses, underscores their potential as biomarkers for distinguishing ACS patients from healthy individuals. These findings suggest that miR–126–5p and miR–223–3p could complement existing diagnostic tools, providing a non-invasive method to assess disease progression and optimize patient management. Similarly, studies by Masoodi et al. [41], Ling et al. [46], and Gager et al. [42] have proposed miRNAs as innovative biomarkers with significant diagnostic value.

We propose that miRNA levels reflect the dynamic processes occurring during platelet activation, a hallmark of ACS [49]. Platelet activation plays a pivotal role in thrombus formation following plaque rupture [50], and these miRNAs may offer valuable insights into cardiac blood flow dynamics after an ischemic event. Their study could pave the way for improved biomarker-based strategies to monitor and manage ACS patients effectively.

To the best of our knowledge, this is the first study to evaluate circulating MVs as carriers of miRNAs in ACS within the Mexican population. Previous studies have focused on European, Asian, or American populations, where genetic backgrounds may contribute to distinct clinical phenotypes and varying susceptibilities to diseases [51–53].

Our study highlights several key findings. Despite the small sample size, we detected statistically significant differences in MVs miRNA content, which suggests that our experimental

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approach could serve as a potential method for identifying ACS patients. Despite these promising results, the study has limitations. A future longitudinal study should explore changes in miRNA levels and their response to therapeutic interventions. Integrating our findings with additional mechanisms, such as genetic variants and epigenetic factors (e.g., DNA methylation), would provide a more comprehensive understanding. Furthermore, exploring other miRNAs involved in ACS pathophysiology would strengthen our conclusions.

5. Conclusion

In conclusion, miR–126–5p and miR–223–3p levels in circulating MVs provide valuable insights into the pathophysiology of ACS. Their diagnostic and prognostic utility warrants further investigation, with potential applications in precision medicine to improve risk stratification and therapeutic monitoring in ACS patients.

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Institutional Review Board Statement: This study was approved by the Ethical and Research Committees of INC with the number registered FIMICOR 18-1043 (03/22/2018). All participants provided informed consent for the collection of blood samples and participation in the study. Moreover, the study was performed according to the Helsinki Declaration.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data underlying this article will be shared on reasonable request to the corresponding author.

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Conflicts of Interest: The authors declare no conflicts of interest.

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