Circulating microRNAs in suspected stable coronary artery disease: A coronary computed tomography angiography study

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Short Title: Circulating microRNAs as biomarkers of stable CAD

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ABSTRACT

Objectives: To explore the diagnostic performance of circulating microRNAs (miRNAs) as biomarkers in patients with suspected stable coronary artery disease (CAD).

Methods: Plasma samples were collected from 237 consecutive patients referred for coronary computed tomography angiography (CCTA). Presence, extension and severity of coronary stenosis were evaluated using the indexes: presence of diameter stenosis $\geq 50\%$, segment involvement score (SIS), segment stenosis score (SSS) and 3-vessel plaque score. A panel of 10 miRNAs previously associated with CAD was analyzed using RT-qPCR. Multivariate analyses were used to analyze the associations between biomarkers and indexes. Discrimination was evaluated using the Area Under the ROC curve (AUC). Decision trees were generated using Chi-squared Automatic Interaction Detector (CHAID) prediction models.

Results: After comprehensive adjustment including cardiovascular risk factors, medication use, confounding factors and protein-based biomarkers (hs-TnT and hs-CRP), several circulating miRNAs were inversely associated with coronary atherosclerosis extension (SIS and 3-vessel plaque score) and severity (SSS). In the whole population, circulating miRNAs showed a poor discrimination value for all indexes (AUC = 0.539-0.644) and did not increase the discrimination capacity of a clinical model of coronary stenosis presence, extension and severity based on conventional cardiovascular risk factors. Conversely, the inclusion of circulating miRNAs in decision trees produces models that improve the classification of cases and controls in specific patient subgroups.

Conclusions: This study identifies a group of circulating miRNAs that failed to improve the discrimination capacity of cardiovascular risk factors but that has the potential to define specific subpopulations of patients with suspected stable CAD.

Keywords: Biomarker; microRNA; Coronary artery disease; Coronary atherosclerosis; Coronary heart disease

GRAPHICAL ABSTRACT

INTRODUCTION

Coronary artery disease (CAD) constitutes a leading cause of morbidity and mortality worldwide [1]. Despite the enormous social and economic consequences, the assessment of coronary atherosclerosis remains challenging. Advances in coronary computed tomographic angiography (CCTA) technology have provided useful diagnostic and prognostic tools to assess the presence and degree of CAD. In fact, recent guidelines on stable CAD [2, 3] and non-ST segment elevation myocardial infarction [4] endorse the use of CCTA in suspected stable CAD patients with low to intermediate likelihood of disease. However, the need for specialized centers and trained personnel limit their feasibility in large-scale population screenings. The development of serological diagnostic tests could assist in clinical management and reduce costs for healthcare systems. Unfortunately, the routine use of biomarkers to detect stable CAD in the absence of an acute event is currently not recommended by clinical practice guidelines [3, 5].

In the last decade, the circulating noncoding transcriptome has emerged as a novel opportunity for the identification of clinical indicators [6, 7]. Among the noncoding RNA (ncRNA) superfamily, circulating microRNAs (miRNAs) have gained great attention as potential biomarkers of cardiovascular disease, and more specifically CAD [8, 9]. Despite promising due to their biochemical properties [10], miRNAs have failed to reach the clinic as biomarkers of atherosclerotic cardiovascular disease. The reproducibility of results between independent clinical investigations is limited due to the differences in the characteristics of the study populations, the variability in the experimental designs and the lack of standardization of the methodology [11, 12]. Furthermore, previous studies were generally based on case-control approaches using selected patients and healthy controls with an inadequate consideration of comorbidities, medication use and confounding factors [8, 13]. Similarly, the evaluation of the incremental diagnostic value of circulating miRNAs in conjunction with full clinical assessment and the comparison of their diagnostic properties to contemporaneous tests, e.g., high-sensitive troponins or CRP, are key steps to obtain convincing evidence for their clinical applicability [11].

In this scenario, our group has recently described a profile of extracellular miRNAs secreted by human coronary smooth muscle cells (SMC) that is altered after exposure to atherogenic

conditions and differs in human atherosclerotic coronary plaques compared to non-atherosclerotic areas [14]. Of note, both *in vitro* and *ex vivo* findings were reproducible in the plasma of patients with atherosclerotic-related conditions [14]. Since a biomarker of stable CAD should reflect the pathologically relevant processes occurring in the coronary artery wall, we hypothesized that the circulating signature of vascular miRNAs could constitute a blood-borne test of coronary atherosclerosis. Therefore, the aim of the current study was to evaluate the diagnostic performance of our panel of circulating miRNAs, in addition to the existing tests hs-TnT and hs-CRP, as biomarkers of epicardial coronary atherosclerosis assessed by CCTA in a real-world clinical practice setting using a cohort of patients with suspected stable CAD.

MATERIAL AND METHODS

Study population

Prospective, observational and single-center study. The study included patients with suspected stable CAD referred for a nonurgent CCTA in the Hospital de la Santa Creu i Sant Pau (Barcelona, Spain). Patients were clinically stable and none suffered from acute coronary syndrome of any presentation, including unstable angina, NSTEMI or STEMI. According to our previous data [14] accepting an alpha value of 0.05 and a beta value of 0.1, and considering that the common standard deviation (SD) was 0.45, 48 patients were necessary in both study groups to recognize as statistically significant a difference greater than or equal to 0.3. Since the evaluation of coronary atherosclerosis was performed after blood collection, the sample size used was higher to ensure the necessary number of patients in each study group. Ultimately, a total of 237 consecutive patients who accepted to participate were included in the study. Patients were excluded from this study due to contraindications to CCTA imaging or inconclusive CCTA studies (N=37). Information relative to demographic characteristics, conventional cardiovascular risk factors, clinical history and medication use was abstracted from the electronic medical record. The study protocol was approved by The Ethical Committee of Clinical Investigation (CEIC) in the Hospital de la Santa Creu i Sant Pau. The

study was conducted following the WMA Declaration of Helsinki. All subjects gave their written informed consent before participating in the study.

Coronary computed tomography angiography

All CCTA exams were performed using a 256-row CT system (iCT Philips Healthcare). The study was prospectively triggered between 75-81% of the RR interval if heart rate was lower than 65 beats per minute (bpm) and retrospectively gated if it was higher than 65 bpm. The tube voltage was from 100 to 120 kV (120 kV if body mass index was greater of 30 kg/m²). An iodinated contrast (Optiray 350, Mallinckrodt Medical Imaging) at a dose of 1 mL/kg was used followed by a saline flush of 40 mL, both injected at a rate of 6 mL/s through an 18-20 gauge catheter placed in an antecubital vein. A 16-coronary artery segment model according to the American Heart Association (modified 15-segment model, with segment 16 being the intermediate branch) [15] constituted the basis for visual/quantitative assessment of coronary artery lesions. In each coronary artery, coronary atherosclerosis was defined as any tissue structures > 1 mm² that existed either within the coronary artery lumen or adjacent to the coronary artery lumen that could be discriminated from surrounding pericardial tissue, epicardial fat, or the vessel lumen itself. Coronary atherosclerotic lesions were quantified for stenosis by visual estimation. Luminal diameter stenosis severity was graded as none (0% luminal stenosis), very mild (1–29% luminal stenosis), mild (30–49% luminal stenosis), moderate (50–69% luminal stenosis), obstructive (70–99% luminal stenosis) and totally occluded (100% luminal stenosis). To determine the extension and severity of coronary atherosclerosis, we calculated the segment involvement score (SIS) and segment stenosis score (SSS), respectively. These parameters were prospective and previously validated in patients with suspected stable CAD [16]. The SIS score, ranging from 0 to 16, was calculated as the total number of individual coronary artery segments exhibiting plaque (irrespective of its degree of luminal stenosis). The SSS score was calculated in each individual coronary segment as 0 points if luminal stenosis was 0 to 29%, 1 point if it was 30 to 49%; 2 points for 50 to 69%, and 3 points for stenosis equal to or greater than 70%. Then, the individual segment scores (from 0 to 3) of all 16 segments were summed to yield a total score ranging from 0 to 48. Finally, a 3-vessel plaque score was calculated as 0 or 1 based on the coexisting

presence of any plaque in the left anterior descending, left circumflex and right coronary arteries, irrespective of severity. All datasets were analyzed on an off-line workstation by a level-3 cardiac computed tomography reader (DV and RL) blinded to the study groups.

Blood collection

Blood collection and processing were performed in accordance with the Standard Operating Procedures for Serum and Plasma Collection: Early Detection Research Network Consensus Statement and Standard Operating Procedure Integration Working Group [17]. Blood samples were obtained in EDTA blood collection tubes (BD) by venipuncture after a night of fasting and before any contrast agent was administered and any interventional procedure was started. The tubes were immediately inverted 10 times to mix the anticoagulant additive with blood. The blood was processed within 2 hours of its extraction. To obtain plasma, blood samples were fractionated by centrifugation at 1,300 xg for 15 min at room temperature. After centrifugation, plasma supernatant was carefully aliquoted into 1.5 mL DNA LoBind tubes and stored at -80°C.

Protein-based biomarker concentration

hs-TnT and hs-CRP were compared against the diagnostic performance of our miRNA panel. hs-TnT concentrations were measured by electrochemiluminescence immunoassay using the hs-TnT assay on the Roche Cobas e601 analyzer (Roche Diagnostics). The hs-TnT assay has an analytic range from 3 to 10,000 ng/L. This assay showed no significant cross-reactivity with TnI. The assay had interrater coefficients of variation ranging from 1.2 to 3.7%. hs-CRP concentrations were determined using an immunoturbidimetry method on the Roche Cobas c501 analyzer (Roche Diagnostics). The hs-CRP assay has an analytic range from 0.3 to 350 mg/L. The assay had interrater coefficients of variation ranging from 1.2 to 3.6%.

Circulating microRNA expression

The panel of circulating miRNAs included the following candidates previously associated with CAD by our group in *in vitro*, *ex vivo* and patient-based approaches [14] and investigated as

biomarkers of CAD by independent groups [8, 9]: let-7g-5p, miR-15b-5p, miR-21-5p, miR-24-3p, miR-29b-3p, miR-130a-3p, miR-143-3p, miR-146a-5p, miR-222-3p and miR-663a (**Supplemental Table S1**).

miRNA profiling was conducted in the same laboratory and under the same conditions. Experienced staff blinded to clinical data performed all laboratory measurements. Total RNA was isolated from 150 μ L of frozen plasma samples using a miRNeasy Serum/Plasma Kit (Qiagen), according to the manufacturer's instructions. Briefly, 5 volumes of QIAzol Lysis Reagent were mixed with 1 volume of each plasma sample and incubated for 5 min at room temperature. For normalization, synthetic *Caenorhabditis elegans* miR-39-3p (cel-miR-39-3p), lacking sequence homology to human miRNAs, was added as an external reference miRNA (1.6×10^8 copies/ μ L). The mixture was supplemented with 1 μ g of MS2 carrier RNA (Roche), not containing miRNAs, to improve extracellular miRNA yield. All reagents were spiked into samples during RNA isolation after incubation with the denaturing solution. Subsequently, one volume of chloroform was added and after 3 minutes at room temperature, the mixture was centrifuged at 12,000 xg at 4°C for 15 min. The upper aqueous phase was transferred to a fresh reagent tube and 1.5 volumes of ethanol were added. Purification of RNA was performed with RNeasy MinElute spin columns according to the manufacturer's recommendations. RNA was eluted in 15 μ L of nuclease-free H₂O and stored in a -80°C freezer.

The overall amount of RNA that is present in plasma is very low; thus, RNA concentration cannot be accurately determined in these samples. Accordingly, the input RNA amount for subsequent analysis was based on starting volume rather than RNA quantity. A constant input amount was used for all samples. miRNA qPCR was performed according to the protocol for the miRCURY LNA Universal RT microRNA PCR System (Exiqon), which offers optimal sensitivity, specificity, accuracy and reproducibility [18]. Briefly, 2 μ L of RNA were reverse transcribed in 10 μ L reactions using the Universal cDNA synthesis kit II (Exiqon). The RT reaction was performed with the following conditions: incubation for 60 min at 42°C, heat-inactivation for 5 min at 95°C, and immediate cooling to 4°C. cDNA was stored at -20°C. For qPCR, cDNA was diluted to give a 50x dilution that was then combined with ExiLENT SYBR Green master mix (Exiqon). The reaction also contained 1:50 diluted ROX (Invitrogen). Each miRNA was quantified in 384-well Pick-&-Mix microRNA PCR Plates (Exiqon)

in 10 µl qPCR reactions. Negative controls excluding template from the RT reaction were also analyzed. qPCR was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems) with the following cycling conditions: 10 min at 95°C, 40 cycles of 10 sec at 95°C and 1 min at 60°C, followed by a melting curve analysis. The synthetic UniSp3 assay was analyzed as an interplate calibrator. SDS v2.3 software was used for both the determination of the quantification cycle (Cq) and for the melting curve analysis. The Cq was defined as the fractional cycle number at which the fluorescence exceeded a given threshold. The specificity of the PCR was corroborated by melting curve analysis. miRNAs were considered to be expressed when Cq values < 35 or were detected with at least 5 Cq below the negative control. Relative quantification was performed using the 2^{-dCq} method, where $dCq = Cq[\text{miRNA}] - Cq[\text{cel-miR-39-3p}]$. This approach has been previously used in the field of circulating ncRNAs as potential clinical biomarkers [19]. Expression levels were $\log_2$ -transformed. miR-663a expression levels were below the limit of detection in 84.5% of the samples. Therefore, it was analyzed as a categorical variable: detected vs nondetected.

Statistical analysis

Descriptive statistics were used to summarize the characteristics of the study population. Kolmogorov-Smirnov test was used to test normality. Data were presented as the **means $\pm$ SD** for continuous variables with normal distributions, medians (interquartile range) for continuous variables with skewed distributions and as frequencies (percentage) for categorical variables. Continuous variables were compared between groups using Student's t test or Mann–Whitney U test. Categorical variables were compared between groups using Fisher's exact test. **Spearman's rho coefficient was used to assess the correlation between continuous variables.** Logistic regression analyses were performed to examine in detail the association between potential biomarkers and the coronary atherosclerosis indexes. Coronary atherosclerosis indexes were entered as dependent variables and circulating miRNAs, hs-TnT and hs-CRP as independent variables (model 1). In model 2, conventional cardiovascular risk factors were also included as covariates. To establish whether the observed association could be influenced by potential confounding factors, model 2 was also adjusted for statin use and anti-platelet drugs (model 3), and peripheral artery disease and glomerular filtration (model

4). For circulating miRNAs, model 2 was also adjusted for hs-TnT and hs-CRP (model 5). The results were presented as odds ratio (OR) and 95% confidence intervals (CI). Receiver operating characteristic (ROC) curves were constructed for circulating miRNAs, hs-TnT and hs-CRP using the area under the ROC curve (AUC) as the global discrimination value measure. Their added discrimination capacity over a clinical model based on conventional cardiovascular risk factors was also evaluated. Patient profiles were explored through classification tree algorithms. We used Chi-square Automatic Interaction Detector (CHAID) analysis [20]. This statistical technique allows the detection of high-level interactions and combines variables and possible cutoffs that best discriminate between study groups. Conventional cardiovascular risk factors, circulating miRNAs, hs-TnT and hs-CRP were included in the classification-tree models. The statistical software package R (www.r-project.org) was used for statistical analyses. A P-value < 0.050 was considered statistically significant.

RESULTS

Clinical characteristics of the patients

The main demographic, clinical and pharmacological characteristics of the patients are summarized in **Table 1**. Mean age was 64.8 ± 12.9 years and males constituted 56.5% of the total population. Prevalence of conventional risk factors including hypertension, dyslipidemia, diabetes mellitus and smoking (active and former smoker) was 63.0%, 56.0%, 21.5% and 33.5%, respectively. Of the 200 patients included in the study, 28.0%, 33.5%, 37.0% and 33.5% showed presence of diameter stenosis $\geq 50\%$, SIS > 5, SSS > 5 and 3-vessel plaque score equal than 1, respectively.

Expression levels of circulating microRNAs and protein-based biomarkers according to coronary atherosclerosis indexes

Expression levels of the circulating miRNAs were compared for each coronary atherosclerosis index according to our study groups (**Figure 1**). As shown in **Figure 1A**, circulating levels of all miRNAs were similar according to the presence of diameter stenosis $\geq 50\%$ in the coronary artery

tree. Concerning SIS, miR-130a-3p and miR-143-3p were significantly lower in patients with SIS > 5 compared to patients with SIS ≤ 5 (**Figure 1B**). Lower levels of all miRNAs, except for miR-29b-3p (P-value = 0.058), were observed in patients with SSS > 5 when compared with patients with SSS ≤ 5 (**Figure 1C**). Decreased levels of miR-21-5p, miR-24-3p, miR-130a-3p, miR-143-3p, miR-146a-5p and miR-222-3p were observed in patients with a 3-vessel plaque score equal than 1 (**Figure 1D**). In all comparisons, miR-663a showed similar prevalence in both detected and nondetected samples (P-value from 0.304 to 0.842). Concerning protein-based biomarkers, hs-TnT concentrations were significantly higher in those patients with the presence of diameter stenosis ≥ 50%, SIS > 5, SSS > 5 and 3-vessel plaque score equal than 1. No differences in hs-CRP were observed for any comparison except for 3-vessel plaque score. No correlation was observed between hs-TnT concentrations and miRNA expression levels (**Supplemental Table S2**).

Association between circulating microRNAs and protein-based biomarkers with coronary atherosclerosis indexes

Multivariable logistic regression analysis was performed to assess potential confounding. As shown in **Table 2** and **Supplemental Table S3**, no association was observed between circulating miRNAs and the presence of diameter stenosis ≥ 50%. miR-143-3p was inversely associated with SIS in all logistic regression models. miR-130a-3p was also inversely associated with SIS in the unadjusted model; however, this association did not remain statistically significant in those models that included clinical data and protein-based biomarkers. let-7g-5p, miR-15b-5p, miR-21-5p, miR-24-3p, miR-130a-3p, miR-143-3p, miR-146a-5p and miR-222-3p were inversely associated with SSS after adjusting for all covariables. Circulating miR-130a-3p and miR-222-3p were inversely associated with 3-vessel plaque score in all models. hs-TnT was positively associated with all coronary atherosclerosis indexes in the unadjusted models. This association did not remain statistically significant after adjusting for conventional cardiovascular risk factors. No association was observed for miR-663a or hs-CRP and any coronary atherosclerosis index.

Diagnostic performance of circulating microRNAs and protein-based biomarkers in patients with suspected coronary artery disease

ROC curves and the AUC were used to assess the feasibility of using circulating miRNAs and protein-based biomarkers as diagnostic tools of coronary atherosclerosis. As shown in **Table 3**, all miRNAs exhibited a low discrimination value for the presence of diameter stenosis $\geq 50\%$, SIS, SSS and 3-vessel plaque score (AUC from 0.539 to 0.644). hs-TnT showed a slightly higher discrimination capacity compared to circulating miRNAs in all coronary atherosclerosis indexes. However, the discrimination capacity was also modest (AUC from 0.624 to 0.674). hs-CRP results suggested no discrimination ability (AUC from 0.413 to 0.478).

To further explore the possible role of circulating miRNAs as biomarkers of coronary atherosclerosis, we evaluated the additive effect of our candidates on the discrimination capacity of a clinical model based on conventional cardiovascular risk factors. To do that, we first analyzed the discrimination of our clinical model, and then, we added each miRNA or protein-based biomarker. As shown in **Table 4**, the clinical model resulted in an AUC from 0.793 to 0.841 depending on the coronary atherosclerosis index. The discriminatory power was not improved by adding circulating miRNAs or protein-based biomarkers.

Finally, we constructed descriptive decision tree models for each coronary atherosclerosis index using a CHAID algorithm (**Figure 2**). This analysis offers additional insights into the potential of circulating miRNAs as biomarkers compared to the evaluation of discrimination alone. It explores the effects of high-order interactions among the variables allowing the identification of subgroups according to the variables considered. CHAID combines variables and cutoff values that best discriminate between our study groups, here the conventional cardiovascular risk factors, miRNAs, hs-TnT and hs-CRP. The optimal combination of variables and cutoff values is determined through an exhaustive search by the CHAID algorithm. Sex was the variable that best discriminated between patients with the presence of diameter stenosis $< 50\%$ and $\geq 50\%$ (**Figure 2A**). For females, let-7g-5p was the predictor that best distinguished between both patient groups. Additionally, in female patients with let-7g-5p expression levels < 15.00 au, a miR-143-3p cutoff value of 12.00 au contributed to the classification of the patients. The prevalence of diameter stenosis $\geq 50\%$ was 0% among those

patients with miR-143-3p expression levels ≤ 12.00 au. This subgroup was not divided if we did not include miRNAs in the analysis (**Supplemental Figure S1**). Age was the most relevant predictor of SIS > 5 (**Figure 2B**). For patients older than 65 years, sex was the next most relevant predictor, since 65.6% of male patients who were > 65 years old have a SIS > 5 . In this subgroup, a miR-143-3p expression level < 12.00 au improved the classification by increasing the percentage of individuals with SIS > 5 to 94.4%. Age was again the most relevant predictor of SSS > 5 (**Figure 2C**). Similarly, we found that, in patients who were > 65 years old, sex was the most important factor to classify patients. In this subgroup, a miR-143-3p expression level ≤ 12.00 au improved the discrimination of SSS > 5 from 63.2% to 94.4%. For both SIS and SSS, these subgroups were not divided if we did not include circulating miRNAs in the analysis (**Supplemental Figure S1**). For 3-vessel plaque score, circulating miRNAs, hs-TnT or hs-CRP did not add value to the discriminative power of the decision tree (**Figure 2D**).

DISCUSSION

In the present study, we provide evidence for the potential use of a panel of circulating vascular miRNAs as a diagnostic tool in patients with suspected stable CAD. We report five major findings: i) patients with higher coronary atherosclerosis extension and severity show decreased circulating miRNA levels; ii) circulating miRNAs are inversely associated with coronary atherosclerosis indexes of extension and severity, even after adjusting for conventional cardiovascular risk factors, medication use, confounding variables and established clinical biomarkers; iii) circulating miRNAs show a poor discrimination capacity for coronary atherosclerosis presence, extension and severity; iv) there is no incremental benefit to combining any of the circulating miRNAs with a clinical model based on conventional cardiovascular risk factors with respect to discriminatory capacity for coronary atherosclerosis presence, extension and severity; v) circulating miRNAs, in combination with conventional cardiovascular risk factors, allow the classification of patients in specific subpopulations according to the presence, extension and severity of coronary atherosclerosis.

These results are consistent with previous findings from our group. We have demonstrated that the secretion of our miRNAs is decreased in an *in vitro* model of human coronary SMC exposed to atherogenic conditions and in an *ex vivo* model of human atherosclerotic coronary plaque [14]. Furthermore, we have reported reduced levels of circulating vascular miRNAs in different patient-based studies focused on atherosclerotic-related conditions [14]. Although comparison with other studies is limited, our data also confirm and extend previous results showing a reduced circulating expression level of vascular miRNAs in atherosclerotic coronary disease. Jia et al. [21] have recently proposed that plasma levels of miR-130a-3p are decreased in patients with coronary heart disease and may be an independent predictor for the disease. The smooth muscle-enriched miR-145-5p, localized in the same genomic cluster as miR-143-3p, was significantly lower in plasma from patients with stable CAD compared to healthy controls [22]. A study by Deng et al. [23] demonstrated that the expression levels of circulating miR-24-3p were significantly decreased in peripheral blood of patients with coronary heart disease compared to controls. It is worth mentioning that the inverse association between let-7g-5p and SSS, a powerful predictor of adverse outcomes [16, 24], is in line with a previous report that suggests the same association between serum levels of let-7g-5p and future fatal myocardial infarction in healthy individuals [25].

Circulating miRNAs failed to improve the AUC when added to a clinical model including conventional cardiovascular risk factors. These results show analogy with previous reports that demonstrated the limited usefulness of novel biomarkers for diagnosing stable CAD [26]. From a clinical point of view, miRNAs do not provide useful discriminatory information to make a decision in the context of stable CAD. These findings are in sharp contrast with our decision tree models. Here we suggest, for the first time, the potential of circulating miRNAs to identify specific subgroups of patients. Similar to proposed genetic risk scores and risk assessment tools [27, 28], circulating miRNAs lack biomarker value in the whole population, though such value may exist for selective subpopulations. For instance, according to our CHAID models, in the subgroup of male patients older than 65 years, miR-143-3p holds the potential to improve the diagnosis of those individuals with high coronary stenosis extension (SIS > 5) and severity (SSS > 5), and who are therefore at high risk for adverse events [16, 24]. The fact that circulating miRNAs added higher discriminative value to these

trees compared to other conventional cardiovascular risk factors, hs-TnT or hs-CRP underscore the potential diagnostic value of these novel biomarkers for clinical practice. Our results are particularly relevant in the current clinical context that promotes the incorporation of molecular profiling into decision-making to allow subgroups of patients to receive precision medical care [29, 30]. Coronary atherosclerosis is a complex pathology comprised of heterogeneous disease subtypes in whom occurrence and progression are highly variable. Additional phenotyping using auxiliary biomarkers, such as miRNAs, may lead to the identification of patient subgroups that could benefit from additional testing or be excluded from further unnecessary interventions [31]. Diagnostic models incorporating these novel data should be tested.

Previous evidence suggests that circulating miRNAs may function as hormone-like mediators [32]. Therefore, an important question relates to the biological relevance of the alterations observed in the circulating miRNA signature, and subsequently, the causal involvement in coronary atherosclerosis. All miRNAs were originally selected due to their role in vascular SMC physiology and pathophysiology [14]. Focusing on our best candidates, let-7g-5p has been described as a regulator of SMC autophagy and apoptosis [33]. The miR-143/145 cluster has been proposed as a master regulator of SMC differentiation [34] and phenotype [35]. The miRNAs analyzed are released by coronary SMC, the major cellular component of the human coronary arterial wall, and their profile is altered in atherogenic conditions [14]. The observation that circulating miRNAs are associated with coronary atherosclerosis extension and severity may indicate that they are functionally involved in atherosclerotic coronary disease. Our miRNA panel thus may have the potential to provide noninvasive insights into pathological processes of coronary atherosclerosis. Indirect evidence from studies on gene polymorphisms also suggest an association between miR-24-3p and miR-146a-5p with atherosclerotic cardiovascular disease [36]. Nevertheless, a “causal mediation” is merely hypothetical. First, our patient-based studies reflect associations. Second, despite the enormous research interest in miRNAs, the biology of this subclass of ncRNAs in the circulation is far from being completely understood. Target-tissue specificity, delivery pathways and how they mediate their biological effect on the recipient cell are still matters of investigation [37].

Strengths of our study are the random selection of the study participants, the control of potential covariates, the comparison of circulating miRNA data with established clinical tests, the number of subjects analyzed with CCTA and the use of four complementary indexes of coronary atherosclerosis presence, extension and severity. The study design avoided selection bias and ensured that our study population was representative of patients with suspected stable CAD in the hospital context. Patients initially suspected of having CAD constituted our nondiseased groups. Overall, the diagnostic performance of the circulating miRNA panel was evaluated in a real-life clinical scenario. Furthermore, CCTA is a well-established imaging technique that enables the anatomic visualization of coronary stenosis. Finally, we evaluated four indexes of coronary stenosis presence, extension and severity, which allowed us to provide a comprehensive picture of the atherosclerotic burden. Some limitations should be noted. First, although the diagnostic value of circulating miRNAs was tested in a relatively large cohort of patients and after sample size calculation, the current findings need to be validated in independent larger and multicentric studies. Second, generalization of results is limited to our study population characteristics (patients referred for CCTA). Third, the main objective of our study was to explore the biomarker potential of a miRNA panel previously described by our group, rather than uncover an exhaustive list of miRNAs. It should be expected that other miRNAs may have diagnostic value. Forth, miRNA secretion is presented in most, if not all, cell types. The possible noncoronary specificity should be considered. **Despite no correlation was observed between hs-TnT concentration and miRNA expression levels, a potential confounding of myocardial necrosis should not be discarded.** Fifth, we cannot exclude the impact on circulating miRNA levels caused by physiological and pathological conditions that were not recorded [38].

In conclusion, this study provides useful hypothesis-generating data. Despite their poor discriminatory capacity, circulating miRNAs emerge as an interesting tool to classify subpopulations of patients with suspected stable CAD according to the presence, extension and severity of coronary atherosclerosis.

DISCLOSURES

The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. Expression levels of circulating microRNAs and plasma concentration of high-sensitivity Troponin (hs-TnT) and high-sensitivity C-Reactive Protein (hs-CRP) according to the coronary atherosclerosis indexes: A) Presence of diameter stenosis $\geq 50\%$; B) Segment involvement score (SIS); C) Segment stenosis score (SSS); D) 3-vessel plaque score. Total RNA extraction was performed from 150 μL of plasma. Expression levels were quantified by RT-qPCR. Relative quantification was performed using cel-miR-39-3p as the external standard. The $2^{-\text{dCq}}$ method was used to analyze the expression levels, where $\text{dCq} = \text{Cq}_{\text{microRNA}} - \text{Cq}_{\text{cel-miR-39-3p}}$. microRNA levels were $\log_2$ -transformed to achieve a normal distribution. miRNA levels are expressed as arbitrary units. Differences between groups were analyzed using Student's t test for independent samples (for circulating microRNAs) and Mann–Whitney U test (for hs-TnT and hs-CRP). Data represent the means $\pm$ SD for circulating miRNAs and medians (IQR) for hs-TnT and hs-CRP. P-values describe the significance level of differences for each comparison.

Figure 2. Decision tree models for each coronary atherosclerosis index: A) Presence of diameter stenosis $\geq 50\%$; B) Segment involvement score (SIS); C) Segment stenosis score (SSS); D) 3-vessel plaque score. Decision tree models were calculated by Chi-square Automatic Interaction Detector (CHAID) analysis. CHAID analysis allows the detection of high-level interactions between the predictors and combines variables and possible cutoffs that best discriminate between study groups: cases and controls. The variables considered in the models were: age, sex, hypertension, dyslipidemia, diabetes mellitus, smoking, circulating microRNAs, hs-TnT and hs-CRP. The results are presented in decision trees with several splits based on the selected variables and cutoffs. Numbers indicate the frequencies (percentages) of cases and controls in each node. For presence of diameter stenosis $\geq 50\%$ SIS and SSS, the subgroups including circulating miRNAs were not further divided if they were not considered in the analysis. Total RNA extraction was performed from 150 μL of plasma. Expression levels were quantified by RT-qPCR. Relative quantification was performed using cel-miR-39-3p as the external standard. The $2^{-\text{dCq}}$ method was used to analyze the expression levels, where

$dCq = Cq_{\text{microRNA}} - Cq_{\text{cel-miR-39-3p}}$. microRNA levels were $\log_2$ -transformed to achieve a normal distribution. miRNA levels are expressed as arbitrary units.

TABLES

Table 1. Characteristics of the study population.

	N = 200
Age (years)	64.8 ± 12.9
Male N (%)	113 (56.5)
Arterial hypertension N (%)	126 (63.0)
Dyslipidemia N (%)	112 (56.0)
Diabetes mellitus N (%)	43 (21.5)
Active or former smoker N (%)	67 (33.5)
Peripheral artery disease N (%)	16 (8.0)
Glomerular filtration rate < 60 mL/min/1.73 m ² N (%)	23 (11.5)
Statins use N (%)	99 (49.5)
Antiplatelet therapy N (%)	83 (41.5)
Angiotensin-converting-enzyme inhibitors use N (%)	104 (52.0)
Beta blocker use N (%)	65 (32.5)
Diuretics use N (%)	51 (25.5)
High-sensitivity Troponin T, hs-TnT (ng/L)	11.5 (7.01-19.70)
High-sensitivity C Reactive Protein, hs-CRP (mg/L)	1.97 (0.97-4.21)

Coronary atherosclerosis

Presence of diameter stenosis ≥ 50% N (%)	56 (28.0)
Segment involvement score, SIS	3.0 (0.0-7.0)
Segment involvement score, SIS > 5 N (%)	67 (33.5)
Segment stenosis score, SSS	3.0 (0.0-9.0)
Segment stenosis score, SSS > 5 N (%)	74 (37.0)
3-vessel plaque score N (%)	67 (33.5)

Data are presented as frequencies (percentages) for categorical variables. Continuous variables with normal distribution are presented as mean ± standard deviation. Continuous parameters with skewed distributions as median (interquartile range).

Table 2. Logistic regression analysis of circulating microRNAs and protein-based biomarkers in patients with suspected stable CAD.

Presence of diameter stenosis $\geq$ 50%	Model 1		Model 2		Model 3		Model 4	
	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
let-7g-5p	0.83 (0.67-1.03)	0.098	0.82 (0.65-1.04)	0.100	0.83 (0.66-1.04)	0.107	0.83 (0.66-1.03)	0.085
miR-15b-5p	0.85 (0.65-1.12)	0.253	0.83 (0.62-1.12)	0.223	0.84 (0.63-1.12)	0.242	0.85 (0.65-1.12)	0.245
miR-21-5p	0.82 (0.59-1.16)	0.258	0.82 (0.57-1.18)	0.278	0.84 (0.58-1.19)	0.321	0.82 (0.58-1.16)	0.260
miR-24-3p	0.82 (0.59-1.14)	0.236	0.81 (0.57-1.15)	0.247	0.80 (0.57-1.14)	0.220	0.82 (0.59-1.13)	0.225
miR-29b-3p	0.89 (0.62-1.28)	0.532	0.89 (0.61-1.32)	0.592	0.86 (0.59-1.25)	0.426	0.88 (0.62-1.27)	0.502
miR-130a-3p	0.88 (0.63-1.23)	0.452	0.86 (0.60-1.22)	0.396	0.89 (0.62-1.26)	0.506	0.89 (0.64-1.23)	0.468
miR-143-3p	0.88 (0.67-1.16)	0.352	0.81 (0.60-1.11)	0.190	0.88 (0.66-1.18)	0.405	0.88 (0.67-1.16)	0.358
miR-146a-5p	0.83 (0.61-1.13)	0.245	0.81 (0.58-1.12)	0.196	0.83 (0.57-1.15)	0.259	0.83 (0.61-1.13)	0.238
miR-222-3p	0.77 (0.51-1.19)	0.240	0.73 (0.46-1.17)	0.190	0.73 (0.46-1.15)	0.171	0.77 (0.51-1.18)	0.236
miR-663a	0.70 (0.27-1.86)	0.474	0.78 (0.28-2.22)	0.643	0.48 (0.26-1.91)	0.483	0.83 (0.30-2.29)	0.717
hs-TnT	1.01 (0.99-1.04)	0.389	1.02 (0.99-1.05)	0.333	1.02 (0.99-1.06)	0.158	NA	
hs-CRP	0.98 (0.95-1.02)	0.366	1.00 (0.95-1.05)	0.940	0.99 (0.94-1.03)	0.985	NA	
Segment involvement score, SIS	Model 1		Model 2		Model 3		Model 4	
	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
let-7g-5p	0.82 (0.66-1.03)	0.089	0.84 (0.66-1.06)	0.141	0.83 (0.66-1.05)	0.125	0.82 (0.66-1.03)	0.090
miR-15b-5p	0.78 (0.57-1.03)	0.078	0.76 (0.56-1.04)	0.087	0.75 (0.55-1.03)	0.074	0.77 (0.58-1.04)	0.084
miR-21-5p	0.81 (0.57-1.15)	0.240	0.83 (0.57-1.20)	0.323	0.82 (0.57-1.19)	0.292	0.82 (0.58-1.17)	0.271
miR-24-3p	0.83 (0.59-1.16)	0.278	0.84 (0.59-1.20)	0.344	0.82 (0.58-1.17)	0.267	0.84 (0.60-1.17)	0.294
miR-29b-3p	0.78 (0.54-1.14)	0.202	0.79 (0.53-1.17)	0.238	0.76 (0.51-1.13)	0.178	0.78 (0.54-1.13)	0.190
miR-130a-3p	0.77 (0.55-1.08)	0.133	0.77 (0.54-1.10)	0.143	0.76 (0.53-1.09)	0.130	0.77 (0.55-1.09)	0.139
miR-143-3p	0.72 (0.54-0.97)	0.031*	0.68 (0.50-0.94)	0.019*	0.71 (0.52-0.97)	0.030*	0.72 (0.54-0.97)	0.030*
miR-146a-5p	0.82 (0.60-1.13)	0.225	0.82 (0.59-1.15)	0.248	0.81 (0.58-1.13)	0.209	0.83 (0.60-1.13)	0.236
miR-222-3p	0.72 (0.46-1.12)	0.139	0.70 (0.44-1.11)	0.131	0.68 (0.42-1.09)	0.111	0.72 (0.46-1.12)	0.147
miR-663a	0.80 (0.30-2.17)	0.665	0.79 (0.27-2.26)	0.654	0.74 (0.26-2.07)	0.560	0.85 (0.31-2.36)	0.854
hs-TnT	1.00 (0.97-1.03)	0.973	1.01 (0.99-1.04)	0.710	1.01 (0.97-1.04)	0.739	NA	
hs-CRP	0.98 (0.93-1.02)	0.288	0.99 (0.93-1.05)	0.719	0.98 (0.93-1.03)	0.445	NA	

Segment stenosis score, SSS	Model 1		Model 2		Model 3		Model 4	
	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
let-7g-5p	0.71 (0.56-0.89)	0.003*	0.69 (0.54-0.89)	0.004*	0.71 (0.56-0.91)	0.006*	0.71 (0.57-0.89)	0.003*
miR-15b-5p	0.66 (0.49-0.88)	0.005*	0.62 (0.45-0.85)	0.003*	0.64 (0.47-0.88)	0.005*	0.66 (0.49-0.89)	0.006*
miR-21-5p	0.63 (0.44-0.89)	0.010*	0.60 (0.41-0.88)	0.009*	0.62 (0.43-0.91)	0.014*	0.64 (0.45-0.91)	0.013*
miR-24-3p	0.65 (0.46-0.91)	0.012*	0.62 (0.43-0.89)	0.009*	0.62 (0.44-0.90)	0.010*	0.65 (0.47-0.92)	0.014*
miR-29b-3p	0.65 (0.45-0.94)	0.023*	0.61 (0.41-0.92)	0.018*	0.62 (0.42-0.93)	0.020*	0.65 (0.45-0.94)	0.023*
miR-130a-3p	0.67 (0.48-0.93)	0.018*	0.62 (0.43-0.90)	0.012*	0.65 (0.45-0.93)	0.020*	0.67 (0.48-0.94)	0.021*
miR-143-3p	0.65 (0.48-0.87)	0.004*	0.57 (0.41-0.80)	0.001*	0.63 (0.46-0.86)	0.004*	0.65 (0.48-0.87)	0.004*
miR-146a-5p	0.65 (0.47-0.89)	0.008*	0.61 (0.43-0.86)	0.005*	0.62 (0.44-0.87)	0.006*	0.65 (0.47-0.90)	0.009*
miR-222-3p	0.53 (0.34-0.83)	0.005*	0.47 (0.28-0.77)	0.003*	0.49 (0.30-0.79)	0.004*	0.54 (0.34-0.84)	0.007*
miR-663a	0.84 (0.32-2.00)	0.726	0.99 (0.35-2.77)	0.978	0.82 (0.30-2.24)	0.702	0.87 (0.33-2.30)	0.773
hs-TnT	1.00 (0.97-1.02)	0.738	1.00 (0.97-1.03)	0.938	1.00 (0.97-1.03)	0.980	NA	
hs-CRP	0.98 (0.94-1.02)	0.328	1.00 (0.96-1.04)	0.908	0.98 (0.94-1.03)	0.430	NA	
3-vessel plaque score	Model 1		Model 2		Model 3		Model 4	
	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
let-7g-5p	0.81 (0.65-1.02)	0.070	0.82 (0.65-1.03)	0.091	0.82 (0.65-1.03)	0.088	0.82 (0.65-1.02)	0.074
miR-15b-5p	0.78 (0.59-1.05)	0.101	0.78 (0.58-1.05)	0.103	0.77 (0.57-1.05)	0.099	0.79 (0.59-1.05)	0.109
miR-21-5p	0.73 (0.51-1.04)	0.080	0.73 (0.50-1.05)	0.092	0.73 (0.50-1.05)	0.093	0.74 (0.52-1.05)	0.092
miR-24-3p	0.73 (0.52-1.02)	0.068	0.73 (0.51-1.03)	0.073	0.72 (0.50-1.02)	0.065	0.73 (0.52-1.03)	0.073
miR-29b-3p	0.70 (0.48-1.02)	0.063	0.69 (0.47-1.02)	0.063	0.67 (0.46-1.00)	0.050	0.70 (0.48-1.02)	0.060
miR-130a-3p	0.69 (0.49-0.98)	0.037*	0.68 (0.47-0.97)	0.034*	0.67 (0.47-0.97)	0.034*	0.69 (0.49-0.98)	0.038*
miR-143-3p	0.75 (0.56-1.00)	0.051	0.71 (0.52-0.97)	0.032*	0.72 (0.53-0.99)	0.041*	0.75 (0.56-1.00)	0.052
miR-146a-5p	0.78 (0.57-1.07)	0.123	0.76 (0.55-1.06)	0.108	0.76 (0.55-1.07)	0.113	0.78 (0.57-1.08)	0.131
miR-222-3p	0.59 (0.38-0.92)	0.021*	0.56 (0.35-0.89)	0.015*	0.54 (0.34-0.88)	0.013*	0.59 (0.38-0.93)	0.023*
miR-663a	0.53 (0.19-1.43)	0.208	0.69 (0.24-1.96)	0.690	0.61 (0.22-1.71)	0.349	0.54 (0.19-1.49)	0.223
hs-TnT	1.00 (0.97-1.02)	0.839	1.00 (0.97-1.03)	0.960	1.00 (0.97-1.03)	0.899	NA	
hs-CRP	0.98 (0.94-1.02)	0.326	1.00 (0.95-1.05)	0.968	0.99 (0.94-1.03)	0.580	NA	

Model 1: Adjusted for age, sex, hypertension, dyslipidemia, diabetes mellitus and smoking; Model 2: Model 1 adjusted for use statin use and antiplatelet therapy; Model 3: Model 1 adjusted for peripheral artery disease and glomerular filtration rate < 60 mL/min/1.73 m²; Model 4: Model 1 adjusted for hs-CRP and hs-TnT. OR: Odd ratio; 95% CI: 95% confidence interval. hs-CRP: high-sensitivity C-Reactive Protein, hs-TnT: high-sensitivity Troponin T; NA: Not applicable. *: statistically significant.

Table 3. Discrimination performance of circulating microRNAs and protein-based biomarkers in patients with suspected CAD.

	Presence of diameter stenosis $\geq$ 50%			Segment involvement score			Segment stenosis score			3-vessel plaque score		
	AUC	95% CI	P-value	AUC	95% CI	P-value	AUC	95% CI	P-value	AUC	95% CI	P-value
let-7g-5p	0.560	(0.467-0.653)	0.189	0.563	(0.474-0.652)	0.148	0.602	(0.519-0.686)	0.016*	0.583	(0.497-0.669)	0.057
miR-15b-5p	0.552	(0.464-0.640)	0.254	0.575	(0.490-0.660)	0.084	0.605	(0.524-0.686)	0.013*	0.582	(0.499-0.666)	0.058
miR-21-5p	0.565	(0.477-0.654)	0.151	0.574	(0.487-0.661)	0.089	0.615	(0.533-0.696)	0.007*	0.600	(0.516-0.684)	0.021*
miR-24-3p	0.571	(0.481-0.662)	0.117	0.576	(0.489-0.663)	0.081	0.621	(0.539-0.703)	0.004*	0.612	(0.528-0.697)	0.010*
miR-29b-3p	0.539	(0.451-0.628)	0.388	0.563	(0.476-0.649)	0.149	0.594	(0.512-0.676)	0.026*	0.593	(0.509-0.676)	0.033*
miR-130a-3p	0.548	(0.460-0.637)	0.289	0.593	(0.509-0.678)	0.032*	0.616	(0.536-0.696)	0.006*	0.622	(0.539-0.704)	0.005*
miR-143-3p	0.571	(0.482-0.659)	0.121	0.626	(0.540-0.711)	0.004*	0.644	(0.564-0.723)	0.001*	0.618	(0.536-0.701)	0.006*
miR-146a-5p	0.575	(0.484-0.667)	0.099	0.581	(0.495-0.668)	0.061	0.628	(0.543-0.709)	0.003*	0.607	(0.524-0.691)	0.013*
miR-222-3p	0.568	(0.479-0.657)	0.135	0.583	(0.497-0.669)	0.056	0.625	(0.544-0.706)	0.003*	0.623	(0.539-0.706)	0.005*
hs-TnT	0.624	(0.542-0.707)	0.006*	0.674	(0.598-0.749)	<0.001*	0.653	(0.577-0.729)	<0.001*	0.674	(0.599-0.750)	<0.001*
hs-CRP	0.478	(0.387-0.568)	0.627	0.453	(0.367-0.538)	0.276	0.448	(0.365-0.532)	0.224	0.413	(0.330-0.496)	0.045*

AUC: Area Under the ROC curve; 95% CI: 95% confidence interval. hs-CRP: high-sensitivity C-Reactive Protein, hs-TnT: high-sensitivity Troponin T. *: statistically significant.

Table 4. Discrimination improvement of circulating microRNAs and protein-based biomarkers in patients with suspected CAD.

	Presence of diameter stenosis $\geq$ 50%	Segment involvement score	Segment stenosis score	3-vessel plaque score
	AUC (95% CI)	AUC (95% CI)	AUC (95% CI)	AUC (95% CI)
Clinical Model (CM)	0.793 (0.724-0.862)	0.841 (0.782-0.901)	0.828 (0.769-0.888)	0.839 (0.781-0.896)
CM + let-7g-5p	0.807 (0.741-0.874)	0.847 (0.788-0.906)	0.847 (0.791-0.904)	0.849 (0.793-0.905)
CM + miR-15b-5p	0.803 (0.736-0.871)	0.849 (0.790-0.908)	0.848 (0.792-0.904)	0.849 (0.792-0.905)
CM + miR-21-5p	0.802 (0.735-0.869)	0.846 (0.787-0.905)	0.845 (0.789-0.902)	0.850 (0.794-0.905)
CM + miR-24-3p	0.805 (0.738-0.872)	0.848 (0.789-0.907)	0.845 (0.788-0.902)	0.850 (0.794-0.906)
CM + miR-29b-3p	0.798 (0.730-0.866)	0.845 (0.786-0.904)	0.841 (0.783-0.899)	0.851 (0.795-0.907)
CM + miR-130a-3p	0.797 (0.729-0.865)	0.846 (0.787-0.904)	0.840 (0.782-0.897)	0.850 (0.794-0.906)
CM + miR-143-3p	0.796 (0.727-0.865)	0.849 (0.790-0.908)	0.846 (0.789-0.902)	0.850 (0.794-0.907)
CM + miR-146a-5p	0.805 (0.738-0.872)	0.847 (0.788-0.905)	0.846 (0.789-0.902)	0.850 (0.794-0.905)
CM + miR-222-3p	0.800 (0.733-0.868)	0.847 (0.788-0.905)	0.848 (0.791-0.904)	0.855 (0.800-0.909)
CM + hs-TnT	0.795 (0.726-0.865)	0.842 (0.782-0.901)	0.830 (0.770-0.889)	0.840 (0.782-0.897)
CM + hs-CRP	0.798 (0.730-0.866)	0.843 (0.784-0.901)	0.817 (0.757-0.878)	0.841 (0.784-0.898)

AUC: Area Under the ROC curve; 95% CI: 95% confidence interval. Clinical Model (CM): age, sex, hypertension, dyslipidemia, diabetes mellitus and smoking. hs-CRP: high-sensitivity C-Reactive Protein, hs-TnT: high-sensitivity Troponin T.

FIGURE 1

A. Presence of diameter stenosis $\geq 50\%$

B. Segment involvement score

C. Segment stenosis score

D. 3-vessel plaque score

FIGURE 2