Research paper

Differential miRNAs in acute spontaneous coronary artery dissection: Pathophysiological insights from a potential biomarker

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ABSTRACT

Background: Spontaneous Coronary Artery Dissection (SCAD) is an important cause of acute coronary syndromes, particularly in young to middle-aged women. Differentiating acute SCAD from coronary atherothrombosis remains a major clinical challenge.

Methods: A case-control study was used to explore the usefulness of circulating miRNAs to discriminate both clinical entities. The profile of miRNAs was evaluated using an unbiased human RT-PCR platform and confirmed using individual primers. miRNAs were evaluated in plasma samples from acute SCAD and atherothrombotic acute myocardial infarction (AT-AMI) from two independent cohorts; discovery cohort (SCAD *n* = 15, AT-AMI *n* = 15), and validation cohort (SCAD *n* = 11, AT-AMI *n* = 41) with 9 healthy control subjects. Plasma levels of IL-8, TGFB1, TGBR1, Endothelin-1 and MMP2 were analysed by ELISA assays.

Findings: From 15 differentially expressed miRNAs detected in cohort 1, we confirmed in cohort 2 the differential expression of 4 miRNAs: miR-let-7f-5p, miR-146a-5p, miR-151a-3p and miR-223-5p, whose expression was higher in SCAD compared to AT-AMI. The combined expression of these 4 miRNAs showed the best predictive value to distinguish between both entities (AUC: 0.879, 95% CI 0.72−1.0) compared to individual miRNAs. Functional profiling of target genes identified an association with blood vessel biology, TGF-beta pathway and cytoskeletal traction force. ELISA assays showed high plasma levels of IL-8, TGFB1, TGFBR1, Endothelin-1 and MMP2 in SCAD patients compared to AT-AMI.

Interpretation: We present a novel signature of plasma miRNAs in patients with SCAD. miR-let-7f-5p, miR-146a-5p, miR-151a-3p and miR-223-5p discriminate SCAD from AT-AMI patients and also shed light on the pathological mechanisms underlying this condition.

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1. Introduction

Spontaneous coronary artery dissection (SCAD) is an important cause of acute coronary syndromes (ACS), occurring predominantly in young to middle-aged women. [1] It is characterized by the development of an intramural coronary haematoma leading to a compressive false lumen that develops in the absence of an atherosclerotic, traumatic or iatrogenic aetiology. The resulting compromise of the true lumen leads to progressive myocardial ischemia and acute myocardial infarction (AMI). [2] Accurate diagnosis of SCAD is critical as management differs in key aspects from conventional atherothrombotic ACS (AT-AMI). In particular, percutaneous coronary intervention (PCI) in SCAD is associated with poorer clinical and angiographic results, and a higher rate of procedural complications; whilst spontaneous healing of the dissected coronary segment occurs at follow-up in most SCAD patients treated conservatively. Therefore, contrary to guideline-based management for AT-AMI, a conservative approach to revascularisation where possible is the consensus optimal management for most SCAD patients. [1−5]

At present, there are no known diagnostic biomarkers with specificity for acute SCAD and invasive angiography remains the first line diagnostic tool despite a well-recognized increased risk of iatrogenic dissection. Although typical angiographic appearances are well described, ambiguous cases are common and whilst intracoronary imaging can be helpful, complications may arise even during invasive imaging. [6] Therefore, the identification of non-invasive biomarkers allowing an early and precise diagnosis is key to improving diagnostic accuracy, enhancing an appropriate management of the condition and ultimately constituting a novel form of non-invasive diagnosis.

MicroRNAs (miRNAs) are non-coding, short RNA molecules (19−25 nucleotides) that mediate post-transcriptional gene expression regulation by inhibiting mRNA translation or promoting its degradation. [7] miRNAs are an important form of intercellular communication [8] with relevant roles in physiological and pathological processes. Circulating miRNAs can be detected in bodily fluids, which makes them attractive targets as non-invasive diagnostic or prognostic biomarkers for different diseases. [9−11]

In this study we sought to determine the plasma miRNA profile of patients with acute SCAD compared with samples of AT-AMI patients and to assess their usefulness to discriminate both clinical entities. Additionally, this profile aids gaining insight into the pathological molecular mechanisms underlaying SCAD.

2. Methods

2.1. Study design

To explore the usefulness of circulating microRNAs to discriminate SCAD and AT-AMI patients, we analysed 2 independent cohorts of patients (cohort 1, discovery cohort; and cohort 2, validation cohort). The discovery cohort included n = 15 SCAD and n = 15 AT-AMI patients; the validation cohort included n = 11 SCAD and n = 41 AT-AMI patients. AT-AMI patients from validation cohort were separated in 2 subsets, AT-AMI subset 1 (19 males/7 females) and AT-AMI subset 2 (15 females). Differentially expressed miRNAs identified in the discovery cohort were analysed in the validation cohort with the AT-AMI subset 1. An unbiased human PCR panel that allows the evaluation of 179 miRNAs was used in this phase. Finally, validated

miRNA candidates were confirmed by individual miRNA PCR primer sets in the validation cohort with the AT-AMI subset 2. The SCAD group from the validation cohort was not divided further as the number of patients was already low.

2.2. Patients and sample collection

Patients presenting acutely with AMI (STEMI and NSTEMI) undergoing urgent coronary angiography were included. Diagnosis of STEMI and NSTEMI was performed according to recent guidelines based on clinical, electrocardiographic findings and data from cardiac biomarkers. [12] Patients with systemic inflammatory disease or immunosuppressive treatment were excluded. The diagnosis of SCAD by angiography was made according to well-established criteria. [1,3] All angiograms were reviewed by at least 2 experienced angiographers to determine the presence of SCAD versus an atherosclerotic culprit coronary lesion. The use of intracoronary imaging was recommended when deemed safe and feasible in cases with suspected SCAD but ambiguous angiographic findings. Patients with unclear culprit lesions or poor-quality angiograms were excluded. Left ventricular function was assessed by transthoracic echocardiography performed systematically before discharge. All patients otherwise received routine clinical care according to current best practice. [1,3,13,14]

Plasma samples from discovery cohort (SCAD n = 15 patients, AT-AMI n = 15) were from UK-SCAD. Samples from validation cohort (SCAD n = 11, AT-AMI n = 41) were from ESP-SCAD, as were plasma samples from 9 control subjects. Samples were collected before heparin administration in EDTA tubes during the 0−6 days following the acute event. Blood was processed no later than 24 h from collection, being kept at 4 °C until processing.

2.3. Sample size

Because the low prevalence of SCAD, the sample size in this study was not calculated using standard procedures. We included all samples available at the participating research centres.

2.4. Ethics

The UK SCAD study (UK-SCAD), (ISRCTN42661582) and the Sample and Data Collection for Cardiovascular Research Study were approved by the UK National Research Ethics Service (14/EM/0056 and 09/H0406/114) and the UK Health Research Authority. The Hospital Universitario Princesa SCAD (ESP-SCAD) sample study was approved by the institutional ethics committee for clinical research (PI-815). These approvals enabled prospective recruitment of patients presenting with SCAD and AT-AMI and collection of clinical, angiographic, imaging and biomarkers data. All patients gave written informed consent and study procedures were conducted in accordance with the Declaration of Helsinki.

2.5. RNA isolation, RT-PCR and analysis

Blood samples were centrifuged 20 min at 4 °C and 2000xg, aliquoted and stored at -80 °C for RNA isolation. All samples were tested for haemolysis at 414 nm absorbance in a NanoDrop One spectrophotometer (Thermo Scientific). RNA was extracted using miRNeasy Serum/Plasma Advanced Kit (Qiagen), following the manufacturer's

instructions and eluted using 20 μl of RNAse-free water. RNA samples were stored at -80 °C. Reverse transcription was performed with 2 μl of RNA using miRCURY LNA RT Kit (Qiagen) according to manufacturer's instructions in a final reaction volume of 20 μl. cDNA samples were stored at -20 °C. RT-PCR assays were performed using ready-to-use miRCURY LNA miRNA Focus PCR Panels and miRCURY LNA SYBR Green PCR Kit (Qiagen) following the manufacturer's instructions in a CFX384 PCR detection system (Bio-Rad).

UniSp3 RNA spike-in Cq values were used as inter-plate calibrators following the manufacturer's instructions. Then, data were analysed using the global mean normalization method. [15] Briefly, after exclusion of values above 36, Cq values were converted to relative quantities (RQ) and sample specific normalization factor (NF) was calculated as the geometric mean of the RQs of all expressed targets per sample. Normalized Relative Quantities (NRQ) were obtained by dividing the RQs by the sample specific NF.

2.6. Confirmatory RT-PCR using individual primers

miRCURY LNA miRNA PCR Assay primer kits (Qiagen) for miR-let-7f-5p, miR-146a-5p, miR-151a-3p and miR-223-5p were used to perform the individual PCR assays following the manufacturer's instructions in a CFX384 PCR detection system (Bio-Rad). To select the most stable miRNAs suitable for the normalization, NormFinder algorithm [16] was applied to miRNA expression data obtained from the miRNA PCR panels. The most stable miRNAs were miR-17-5p, miR-20-5p, miR-30e-5p, miR-106a-5p and miR-let-7i-5. After exclusion of values

above 36, Cq values were converted to relative quantities (RQ) and the normalization factor (NF) was calculated as the geometric mean of miR-17-5p, miR-20-5p, miR-30e-5p, miR-106a-5p and miR-let-7i-5. The Normalized Relative Quantities (NRQ) were obtained by dividing the RQs by the sample specific NF.

2.7. ELISA assays

Plasma levels of C-X-C Motif Chemokine Ligand 8 (CXCL8 also known as IL-8), Transforming Growth Factor beta1 (TGFB1), Transforming Growth Factor Beta Receptor 1 (TGFBR1), Endothelin-1 (EDN1) and Matrix Metalloproteinase-2 (MMP2) were analysed using a custom ELISA multiplex array (RayBiotech Life, GA, USA) according to manufacturer's instructions.

2.8. Statistical analysis

When continuous data did not fulfil normality criteria, a log transformation was applied; otherwise, a non-parametric test was used to detect differences between samples. Univariate analyses (pairwise) and multiple test corrected by false discovery rate (FDR) (adjusted p value <0.05) were used to identify those microRNAs differentially expressed in AT-AMI and SCAD patients from both cohorts. FDR was calculated by the Benjamini-Hochberg method. microRNAs differentially expressed in cohort 1 and cohort 2 were further analysed for differential expression depending on age, sex, diabetes mellitus, smoking condition and the haemolysis levels of plasma using a multiple linear regression analysis. When miRNA levels were not affected by any of these factors, univariate (pairwise) analysis followed by a correction for multiple comparison was applied. Selected microRNAs were also confirmed by individual RT-PCR in cohort 2. The results of the ROC show the confidence interval (CI) of 95% and the p-value of the hypothesis testing (H0: AUC = 0.5). Differences between AT-AMI and control subjects were analysed using a univariate test followed by a correction for multiple comparisons. Differences in cytokine plasma levels between SCAD and AT-AMI were analysed using two-tailed level of significance Mann-Whitney U test. Data were analysed using STATA (STATA 14.0 for MAC, StataCorp, College Stataion, Texas, USA) and R software (version 3.5.1 http://www.R-project.org (last accessed 9-20-2020); the R Foundation for Statistical Computing, Vienna, Austria) and graphs were performed with GraphPad Prism 8 (Prism 8 for macOS, GraphPad Software, LLC) and R software. For the functional profiling of the microRNA gene targes, data were analysed by Fisher exact test and Bonferroni correction, the complete *Homo sapiens* genome was used as reference list. Only those biological processes with a corrected *p* value <0.05 were considered statistically significant.

2.9. miRNA target identification and their association with biological processes

miRTarBase database was used to identify validated miRNA targets for those miRNAs differentially expressed between SCAD and AT-AMI (miR-let-7f-5p, miR-146a-5p, miR-151a-3p and miR-223-5p). Then, the association of functional target genes with biological processes was determined using the PANTHER Classification System with terms from Gene Ontology.

2.10. Role of the funding source

3. Results

3.1. Differences in circulating miRNAs profile among SCAD, AT-AMI patients and healthy controls

To validate our approach, we first compared plasma miRNA expression levels of AT-AMI patients from both cohorts (*n* = 41) and control subjects (n=9). This analysis showed 23 differentially expressed miRNAs in AT-AMI patients compared with control subjects (Supplementary Table 1), including let-7d-5p, miR-103a-3p, miR-106a-5p, miR-125b-5p, miR-191-5p, miR-22-3p, miR-26b-5p and miR-30c-5p (Fig. 1) previously associated to atherosclerotic acute myocardial infarction. [17-22] Confidence intervals (95%) of miRNAs in Fig. 1 are shown in Supplementary Table 2

### 3.2. SCAD and AT-AMI patients display specific signatures of plasma miRNAs

Next, we specifically analysed differences in the expression of circulating miRNAs in SCAD patients compared to AT-AMI patients. miRNAs expression was analysed in the two independent cohorts using an unbiased panel of 179 miRNAs. A total of 56 AT-AMI and 26 SCAD patients were studied. Discovery cohort consisted of 15 SCAD patients (14 females and 1 male) and 15 AT-AMI patients (all females), and validation cohort consisted of 11 SCAD patients (all females) and 41 AT-AMI patients (22 females and 19 males) (Fig. 2). The presence of diabetes mellitus (DM) was more prevalent in the AT-AMI group than in SCAD group (discovery cohort 13% vs 0%; validation cohort 44% vs 27%). The percentage of smokers was also higher in the group of patients with AT-AMI (discovery cohort: 47% vs 7%, validation cohort: 44% vs 27%). Regarding hypertension (HT), no statistically significant differences were observed in the discovery cohort. However, the percentage of AT-AMI patients with this comorbidity was higher in the validation cohort compared to SCAD patients. Dyslipidaemia (DL) was also higher in AT-AMI group in both cohorts (discovery cohort: 13% vs 7%, validation cohort: 56% vs 9%). All patients were taking acetylsalicylic acid (ASA) at sampling time.

Regarding the use of statins, no differences were detected in the discovery cohort, while in the validation cohort a higher percentage of AT-AMI patients were taking statins at the time of sampling (Table 1).

The analysis of discovery cohort showed a differential expression of 15 miRNAs with an FDR corrected *p* value <0.05 (Table 2). These miRNAs were then analysed in the validation cohort (AT-AMI subset 1). Our data showed a statistically significant (FDR corrected p value <0.05) difference in 4 out of 15 miRNAs: miR-let7f-5p, miR-146a-5p, miR-151a-3p and miR-223-5p (Supplementary Fig. 1), confidence intervals (95%) are shown in Supplementary Table 3. The presence of DM, HT, DL, smoking condition or the use of statins did not affect the expression of these miRNAs (data not shown). In order to confirm these results, we performed PCR assays with individual PCR primer sets in samples from the validation cohort using the AT-AMI subset 2 (*n* = 15, female = 15, male = 0, Fig. 2). Our data demonstrate that the circulating levels of miR-let7f-5p, miR-146a-5p, miR-151a-3p and miR-223-5p were higher in SCAD patients compared with AT-AMI patients (*p* < 0.05) (Fig. 3a), confidence intervals (95%) are shown in Supplementary Table 4. To evaluate whether the expression of miR-let-7f-5p, miR-146a-5p, miR-151a-3p or miR-223-5p could be useful to distinguish SCAD from AT-AMI patients, receiver operating characteristic (ROC) curves analysis was performed. Expression of miR-let-7f-5p showed an AUC of 0.8 (95%CI: 0.62−0.98, *p* = 0.004). The AUC values for miR-146a-5p were 0.83 (95%CI: 0.66−1, *p* = 0.0013). miR-151a-3 showed an AUC of 0.82 (95% CI: 0.64−1(*p* = 0.0016) while the AUC values for miR-223-5p were 0.80 (95% CI: 0.66−0.96, *p* = 0.0026) (Fig. 3b). The global evaluation of the 4 miRNAs showed a higher predictive value compared to each individual miRNA, showing an AUC of 0.88 (95% CI: 0.72−1, *p* = 0.0002) (Fig. 3c), specificity of 0.64 (95% CI: 0.51−0.77), sensitivity 0.72 (95% CI: 0.62−0.83), positive predictive value 0.68 (95% CI: 0.58−0.76) and negative predictive value of 0.83 (95% CI: 0.79−0.87).

3.3. Association of miRNA-target genes with blood vessel biology

To explore the possible functional implication of miR-let-7f-5p, miR-146a-5p, miR-151a-3p and miR-223-5p we determined the
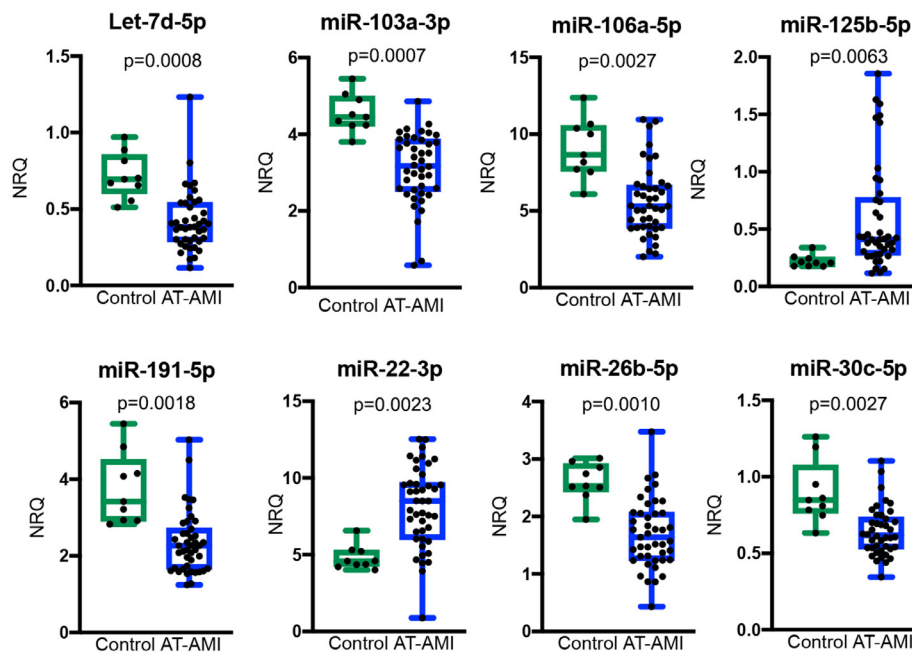
Fig. 1. Plasma miRNAs differentially expressed in control subjects compared to AT-AMI patients. Box and whiskers Min to Max plots showing plasma levels of microRNAs. Data correspond to the expression of miRNAs in AT-AMI n=41 and control subjects n=9. Expression of miRNAs was analysed by RT-PCR using a serum/plasma focus PCR panel. Data were normalized using the global mean, expression levels are shown as Normalized relative quantity (NRQ), as indicated under Methods. Differences among groups were analysed by univariate test followed by FDR multiple correction test.
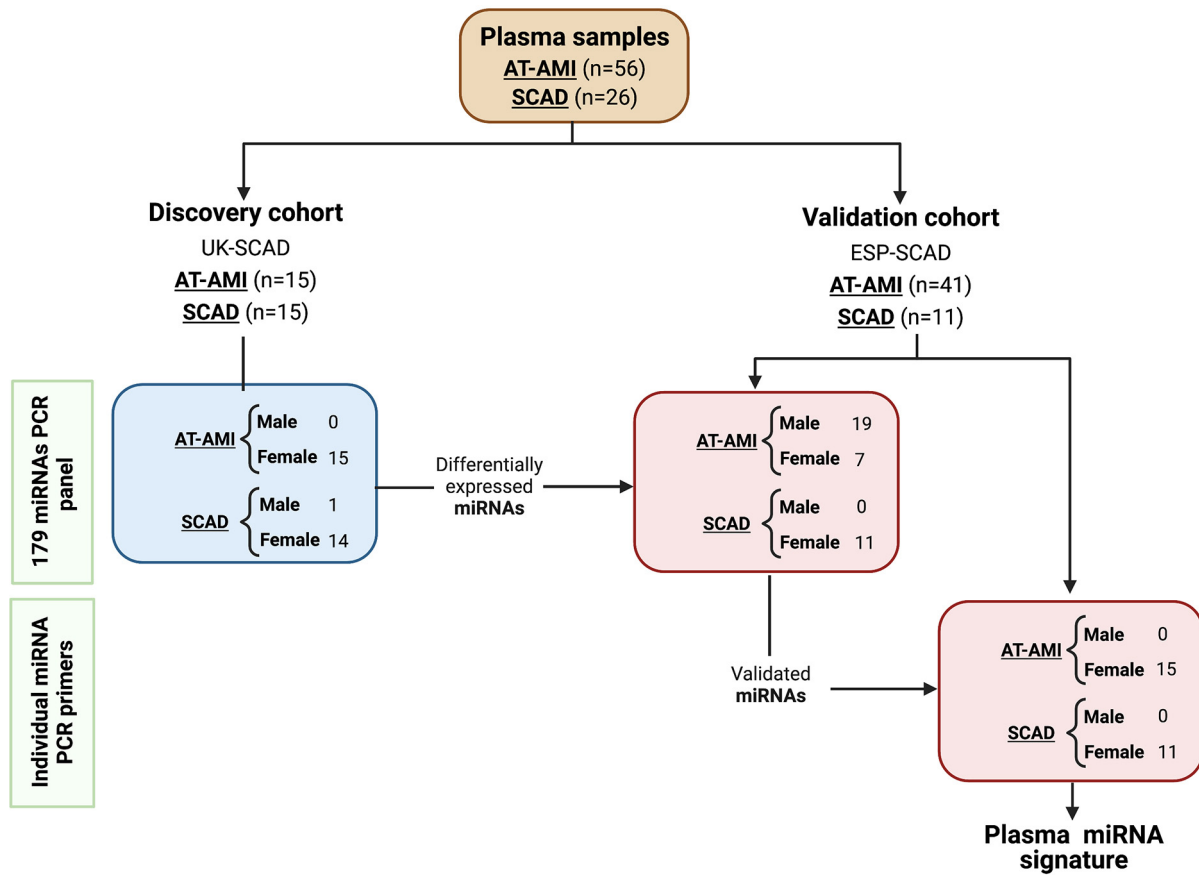
Fig. 2. Study design and cohorts. Plasma samples (AT-AMI *n* = 56, SCAD *n* = 26) were collected in both UK (AT-AMI *n* = 15, SCAD *n* = 15) and Spain (AT-AMI *n* = 41, SCAD *n* = 11), constituting the discovery and the validation cohorts, respectively. The number of males and females of each sample set is shown in the figure. Firstly, differences in the expression of plasma miRNAs between AT-AMI and SCAD patients from the discovery cohort were assessed performing RT-PCR assays using unbiased PCR panels of 179 miRNAs. Differentially expressed miRNAs identified in the discovery cohort were then analysed in 26 AT-AMI and 11 SCAD patients from the validation cohort using the same method. Lastly, resulting differentially expressed miRNAs were confirmed by performing PCR assays with individual PCR primer sets in 15 AT-AMI (all female) not included in the previous phase and 11 SCAD patients from the validation cohort. Created with BioRender.com

Table 1
Clinical and demographic variables.

	Cohort 1 (Discovery cohort)		Cohort 2 (Validation cohort)		
	SCAD (*n* = 15)	AT-AMI (*n* = 15)	SCAD (*n* = 11)	AT-AMI (*n* = 41)	CONTROL (*n* = 9)
Sex (F/M)	15/0	15/0	11/0	22/19	9/0
Age mean ± SD (years)	49.6 ± 6.5	52.0 ± 6.6	52.6 ± 12.1	61.4 ± 9.2	53 ± 5.2
STEMI	7	7	4	25	0
NSTEMI	8	8	7	16	0
SMOKING (NO/YES/EX)	10/1/4	8/7/0	8/3/0	16/18/7	6/3/0
HT (NO/YES)	12/3	11/4	8/3	15/26	0
DM (NO/YES)	15/0	13/2	8/3	23/18	0
DL (NO/YES)	14/1	13/2	10/1	18/23	0
BMI mean ± SD	29.1 ± 10.2	31.0 ± 6.2	25.7 ± 4.9	27.5 ± 5.4	26.3 ± 8.5
Use of statins	15	15	4	27	0
Use of ASA	15	15	11	40	0
Culprit vessel					N/A
24-LAD	7	3	5	8	
24-RCA	3	9	0	14	
24-CX	5	3	2	3	
Number of vessels affected					N/A
24-0	0	0	0	2	
24-1	14	5	8	21	
24-2	0	9	2	11	
24-3	1	1	1	7	
Coronary revascularization					N/A
24-Conservative	10	1	11	0	
24-PCI	5	13	0	37	
24-CABG	0	1	0	2	
LVEF (%) mean ± SD	55.6 ± 6.6	61.3 ± 4.7	54.3 ± 12.0	57.1 ± 9.5	N/A

HT hypertension, DM diabetes mellitus, DL dyslipidaemia, BMI body mass index, ASA acetylsalicylic acid, LAD left anterior descending artery, RCA right coronary artery, CX circumflex artery, PCI percutaneous coronary intervention, CABG coronary artery bypass graft, LVEF left ventricular ejection fraction. N/A: not applicable.

Table 2
DE miRNAs in SCAD vs AT-AMI patients in Discovery cohort.

miRNA ID	p value (FDR)	SCAD/AT-AMI
hsa_mir_2110	0.0233887517108984	UP
hsa_let_7d_3p	0.0233887517108984	UP
hsa_let_7f_5p	0.0346269703856619	UP
hsa_mir_92b_3p	0.0346269703856619	UP
hsa_mir_1	0.0371248112086971	UP
hsa_mir_320c	0.0371248112086971	UP
hsa_mir_146a_5p	0.0371248112086971	UP
hsa_mir_223_5p	0.0371248112086971	UP
hsa_mir_200c_3p	0.0371248112086971	UP
hsa_mir_151a_3p	0.0371248112086971	UP
hsa_mir_361_5p	0.0498538460706333	UP
hsa_mir_133a_3p	0.0498538460706333	UP
hsa_mir_421	0.0498538460706333	UP
hsa_mir_501_3p	0.0498538460706333	UP
hsa_mir_93_3p	0.0498538460706333	UP

target genes using miRtarBase (http://mirtarbase.cuhk.edu.cn/php/index.php, last accessed 10-4-20) a database of experimentally validated miRNA-target interactions, a total of 924 genes were identified as functional targets. We assessed whether these genes were

associated with biological processes according with terms of Gene Ontology. The functional profiling showed that the list of target genes was enriched (FDR corrected p value <0.05) in genes involved in blood vessel biology including blood vessel morphogenesis, circulatory system development, and the regulation of smooth muscle cell proliferation (Fig. 4a). Forty-eight genes were involved in blood vessel development among them Transforming Growth Factor beta-1 (TGFB1) and Endothelin-1 (EDN1) genes regulated by miR-146a-5p and miR-let-7f-5p, respectively (Fig. 4b). A statistically significant over-representation of target genes in TGFB1 signalling pathway was also identified (Fig. 4a). Target genes associated with blood vessel development/morphogenesis and TGF-beta signalling are shown in Fig. 4b. Among the target genes associated with these biological processes, we detect a group of genes associated to cytoskeletal traction forces (WASF2, RHOA, ROCK1, MYLK and MYH9, Fig. 4b).

Finally, we evaluated the plasma levels of most relevant molecules identified as targets: TGFB1, EDN1, C-X-C Motif Chemokine Ligand 8 (CXCL8 also known as IL-8), Transforming Growth Factor Beta Receptor 1 (TGFBR1), and Matrix Metalloproteinase-2 (MMP2). ELISA assays revealed statistically significant higher levels of these molecules in plasma samples from SCAD patients compared with AT-AMI patients (Fig. 4c, confidence intervals in Supplementary Table 5).
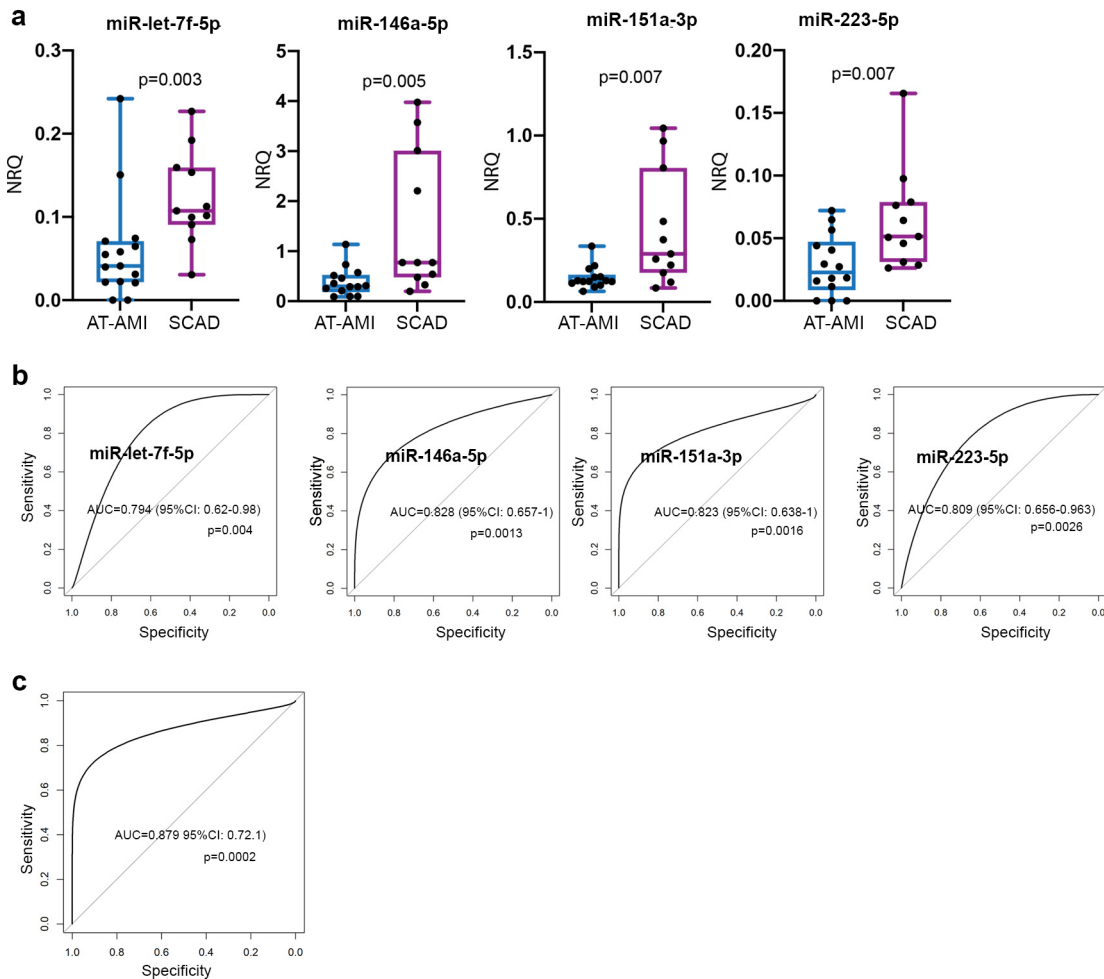
Fig. 3. Plasma microRNA signature and its predictive value to distinguish SCAD from AT-AMI patients. a, Box and whiskers Min to Max plots showing plasma levels of miR-let-7f-5p, miR-146a-5p, miR-151a-3p and miR-223-5p from SCAD (*n* = 11) and AT-AMI (*n* = 15) patients from validation cohort. Expression of miRNAs was analysed by RT-PCR using individual sets of primers. Data were normalized using the geometric mean of the five most stable miRNAs. Expression levels are shown as Normalized relative quantity (NRQ), as indicated under Methods. Differences among groups were analyzed by a Mann-Whitney test. **b**. Receiver operating characteristic (ROC) curves corresponding to miR-let-7f-5p, miR-146a-5p, miR-151a-3p and miR-223-5p expression levels from SCAD (*n* = 11) and AT-AMI (*n* = 15) patients from validation cohort. **c**. Receiver operating characteristic (ROC) curve corresponding to the concomitant evaluation of miR-let-7f-5p, miR-146a-5p, miR-151a-3p and miR-223-5p expression levels from SCAD (*n* = 11) and AT-AMI (*n* = 15) patients from validation cohort. b,c The bold line shows the ROC curve. The values inside the graph correspond to the area under the curve (AUC) and its 95% confidence interval; p value is also indicated.
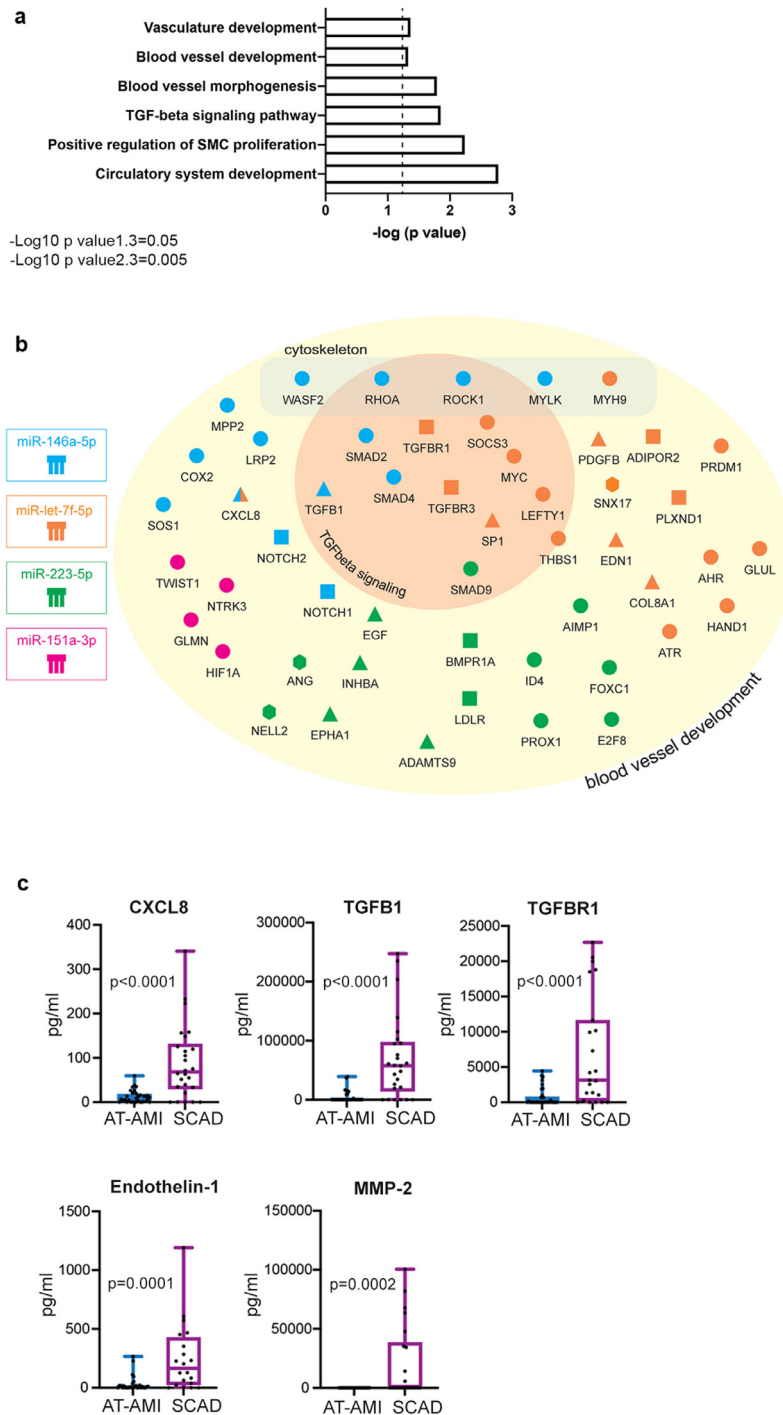
Fig. 4. Target genes of miR-let-7f-5p, miR146a-5p, mir-151a-3p and miR-223-5p are associated with blood vessel biology. a, Bar graph illustrating the significance (-log p value) of blood vessel biology-associated biological processes predicted by PANTHER Classification System. Functional miRNA targets were selected using miRTarBase database and analysed to obtain functional classifications with terms from Gene Ontology. Data were analysed by Fisher's test and Bonferroni correction. **b,** Functional miRNA targets associated with blood vessel biology, TGF-beta pathway and cytoskeleton are shown. Colours indicate each miRNA with its corresponding targets. SMC, smooth muscle cell. Triangles indicate cytokines and soluble factors; squares indicate plasma membrane receptor proteins; circles, intracellular proteins (cytoplasmic and/or nuclear); hexagons, proteins with extra- and intracellular function and/or presence. **c,** Box and whiskers Min to Max plots showing plasma levels of CXCL8, TGFB1, TGFBR1, Endothelin-1 and MMP2 in SCAD patients (*n* = 26) and AT-AMI patients (*n* = 34). Levels of these molecules were analysed using a custom multiplex ELISA assay. Differences between groups were evaluated using Mann-Whitney U test.

4. Discussion

To our knowledge, this is the first study that investigates the role of plasma miRNAs as potential biomarkers for acute SCAD. According to the data presented herein, acute SCAD has a specific miRNA signature that is different from that of athero-thrombotic acute myocardial infarction. Furthermore, we identify four differentially expressed miRNAs validated from two cohorts studied. Accordingly, the combination of miR-let-7f-5p, miR-146a-5p, miR-151a-3p and miR-223-5p is a promising predictor of acute SCAD. Our data also reveal that the functional profiling of target genes of the four differentially expressed miRNAs regulate genes known to be associated with vascular biology,

TGF-beta signaling and SCAD. Our data also revealed that relevant targets for the vascular pathology such as CXCL-8, TGFB1, TGFBR1 and EDN1 are increased in plasma from acute SCAD patients. These findings support further validation of miRNAs as diagnostic biomarkers as well as their pathophysiological role in larger studies.

Although less common than AT-AMI, SCAD is not rare accounting for up to 35% of events in women under 50 years of age and 43% of pregnancy-associated AMI. As understanding of the condition has moved forward following the publication of the first American and European consensus documents, key differences in management between SCAD and AT-AMI have become increasingly evident. These include different approaches to revascularization and potentially secondary prevention medications. In this context, accurate diagnosis is critical. Currently, invasive coronary angiography is the gold standard diagnostic test, but many SCAD patients present with angiographic patterns that mimic or are actually indistinguishable from atherosclerotic coronary artery disease. Use of intracoronary imaging is required in these cases necessitating mechanical instrumentation of the coronary vessel with inherent risks in patients with SCAD, in which the vessel wall is fragile and vulnerable to iatrogenic complications. [6] This has led to a search for acute SCAD-specific biomarkers which can be used either to support angiographic diagnosis or even to develop a new non-invasive diagnostic paradigm.

In this study we have focused on miRNAs due to their potential as diagnostic, prognostic, and monitoring biomarkers in many different contexts including in AT-AMI. [9−11] As a proof of concept, we first assessed the ability of our approach to reproduce miRNAs differentially expressed in AT-AMI compared to controls subjects. The comparison with other AT-AMI studies is a challenge mainly due to differences in normalization methods; nevertheless, our data show differences in the expression of miR-22-3p, miR-22-5p, miR-26b-5p, miR-30c-5p, miR-125b-5p, miR-106a-5p, and miR-191-5p, as previously reported. [17−22]

Our approach allowed us to identify 4 miRNAs whose presence in plasma is higher in SCAD patients compared to AT-AMI (p value <0.05). One of these miRNAs is miR-146a-5p, the increased presence in plasma of which has also been reported in patients with aortic dissection. [23] In particular, expression of miR-146a-5p, but also the combination of the 4 miRNAs described here, was able to distinguish SCAD from AT-AMI patients. The AUC, specificity, sensitivity, positive predictive value and negative predictive value compare favourably to a plasma protein and lipid-based approach recently published for fibromuscular dysplasia, a condition related-to and overlapping with SCAD. [24] The combination of these miRNAs is therefore a potentially suitable candidate to be studied in larger prospective cohorts as a biomarker for acute SCAD. During the last years, circulating miRNAs have been proposed as potential biomarkers in numerous studies. However, the inherent difficulties of their study and normalization strategies result in limitations in their use as diagnostic tools. [25]

These findings may also provide novel insights into the molecular pathophysiology of acute SCAD. miRNAs participate in different molecular pathways associated with coronary diseases, such as endothelial dysfunction, smooth muscle cell and platelet activation, inflammation or atheromatous plaque formation. [26,27] Interestingly, we identified a statistically significant enrichment of target genes of the miRNAs signature in processes associated to the morphogenesis and development of the circulatory system, for example *LRP2, NOTCH1, TGFB1, TGFBR3, TGFBR1, SMAD2, CXCL8, EDN1, MMP2*, among others. Increased plasma levels of IL-8, EDN1, TGFB1, TGFBR1 and MMP2 in SCAD patients highlight the possible involvement of these molecules in the pathophysiology of SCAD. The high levels of miR-let-7f-5p, miR-146a-5p, miR-151a-3p and miR-223-5p in SCAD patients could be a mechanism to counter-regulate the expression of those molecules. Several functions described for these gene products make them interesting candidates to be explored in further detail in SCAD patients. To our knowledge, no prior studies have compared the blood levels of these molecules between SCAD and AT-AMI patients. In the case of EDN1, an association between lower levels of EDN1 in plasma and the presence of the PHACTR1/EDN1 risk allele rs9349379 does exist. [28] Further investigation is needed to determine whether the plasma levels of EDN1 in SCAD patients are associated to additional factors, and to establish the levels of this molecule in a healthy population.

Mutations in components of TGFB pathway result in a series of diseases collectively known as TGFB vasculopathies. [29,30] Indeed, causal variants in *SMAD2, TGFB1* and *TGFBR2* have been recently reported in SCAD. [31] In this regard, our data identified an enrichment of functional miRNA-targets in the pathway of TGFB response. [32,33] miR-146a-5p regulates *TGFB1* expression, a crucial player in the development and homeostasis of the vascular system mainly through TGFBR1 and TGFBR3. Strikingly, both receptors are regulated by miR-let-7f-5p, which is also a differential marker identified in this study. Dysregulation of the TGFB1 signalling cascade has been associated with syndromic aortic aneurysm and dissection conditions, including heritable connective tissue disorders such as Marfan syndrome (MFS), Loeys-Dietz syndrome and vascular Ehlers-Danlos syndrome (vEDS). [34] TGFB is also related to the onset of fibrosis in different pathologies, which strongly suggests an important function in the mechanical response of the tissue to shear stress. [35] This is an important issue as collagen vascular disorders like MFS or vEDS are predisposing conditions related to SCAD. [36] miR-let-7f-5p also seems regulate the expression of endothelin 1 (EDN1); [37] this is highly expressed in vascular endothelial and smooth muscle cells, macrophages and podocytes among other type of cells, and it has been also recently associated with SCAD. [28,38,39]

The finding of a group of interrelated target genes (WASF2, RHOA, ROCK1, MYLK and MYH9) suggests the involvement of a mechano-active mechanism that controls cell shape and cytoskeleton dynamics in SCAD. In addition, mutations in MYLK cause familial aortic dissection underscoring the role of mechano-sensing and mechanical responses in the maintenance of aortic homeostasis. [40] Very recent data highlight the presence of causal variants in MYLK in SCAD. [41] Whether these biological processes are a cause, or a consequence of SCAD pathophysiology is just prospective. Neither the cells from which these plasma miRNAs are secreted, nor their recipient cells can be elucidated from our results, although both topics are very interesting aspects to address in future investigations.

In summary, our work provides a distinct miRNA signature that, with further prospective validation, could be a useful biomarker to distinguish acute SCAD from AT-AMI. It also provides novel insight into the molecular mechanisms underlying this condition.

4.1. Study limitations

Our data is limited by the sample size, due to the relatively low incidence of acute SCAD and as such it has not been possible to investigate any potential co-variates with the potential to influence the findings.

4.2. Contributors

MLP: Literature search, data collection, writing; DA: Literature search, writing, data interpretation, patients recruitment; MGG: Conceived the presented idea, data interpretation; ASG: Statistical analysis; PVT: samples management for the revised version; data collection FR: Patients recruitment, data interpretation; JC: Patients recruitment; TB: Patients recruitment; AB: samples management, data base; AV samples management; EMG: Figures and network analysis; MVM: Figures and data interpretation; PM: data interpretation; NS: Patients recruitment, data interpretation; FSM: Supervised the findings of this work, data interpretation; FA: Supervised the findings of this work, overall planning; HF: Data collection, analysis, writing

and interpretation. All authors have read and approved the final version of the manuscript. MLP, HF and FSM have verified the underlying data.

5. Data Sharing Statement

The data that support the findings of this study are available from the corresponding author upon request.

Declaration of Competing Interest

DA has received in kind support for SCAD genetics research from Astra Zeneca Inc. and research grants for unrelated research from the same Company. He has received funding from Abbott Vascular Inc. to support a clinical research Fellow and has undertaken unrelated consultancy for GE inc. No other authors have potential conflicts of interest to declare.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2021.103338.

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