Assessment of microRNAs in patients with unstable angina pectoris

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Aims
While cardiac troponin measurements have significantly improved the early diagnosis of myocardial infarction, the timely biomarker-based diagnosis of unstable angina pectoris (UAP) remains a major unmet clinical challenge. The aim of this study was to assess levels of circulating microRNAs (miRNAs) as possible novel biomarkers in patients with UAP.

Methods and results
A three-phase approach was conducted, comprising (i) profiling of miRNAs in patients with UAP and controls groups; (ii) replication of significant miRNAs in an independent patient cohort, (iii) validation of a multi-miRNAs panel in a third cohort. Out of 25 miRNAs selected for replication, 8 miRNAs remained significantly associated with UAP. In a validation phase, a miRNA panel including miR-132, miR-150, and miR-186 showed the highest discriminatory power [area under the receiver-operating-characteristic curve (AUC): 0.91; CI: 0.84–0.98].

Conclusion
Using a profiling-replication-validation model, we identified eight miRNAs, which may facilitate the diagnosis of UAP.

Keywords
Circulating microRNA • Myocardial infarction • Unstable angina pectoris • Acute myocardial infarction • Diagnosis

Introduction
Acute coronary syndrome comprises two entities: acute myocardial infarction (AMI) and unstable angina pectoris (UAP). Their rapid and accurate diagnosis is critical for the initiation of effective evidence-based medical management and treatment but is still an unmet clinical need. Delayed ‘rule-in’ increases morbidity and mortality. Delayed ‘rule-out’ prolongs the time spent in the emergency department, delays the recognition and treatment of the actual cause of chest pain, increases patients’ uncertainty and anxiety, and causes costs for the health care system.

MicroRNAs (miRNAs) are endogenous, small non-coding RNAs involved in the regulation of gene expression. They control expression on a posttranscriptional level as intracellular RNAs and are discussed as therapeutic targets. MicroRNAs are involved in the pathogenesis of various cardiovascular conditions such as angiogenesis, hypertrophy, heart failure, and fibrosis. Recent studies revealed that miRNAs are reliably detected as cell-free RNAs in circulating blood and other body fluids. Because of their stability, miRNAs may serve as novel disease biomarkers.
Assessment of microRNAs in patients with UAP

Methods

Overall study concept and study flow

Selection of samples

The overall study concept consisted of an initial profiling phase of miRNAs in patients with UAP compared with two different control groups, (i) a replication phase and (ii) a subsequent validation phase (Figure 1). For the profiling phase, UAP patients were selected from the stenoCardia study; controls were selected from the stenoCardia study (control group 1) and the population-based Gutenberg Health Study (GHS) (control group 2), respectively. The selection was performed as a ‘case–control’ approach with unstable angina patients considered as cases and non-coronary chest pain patients (NCCP) as control group 1.

Patients were randomly selected from an overall pool of \( n = 240 \) UAP patients and \( n = 1165 \) NCCP patients of the stenoCardia study.\(^\text{18}\) Randomization was done using standard spreadsheet software functions blinded to any patient characteristics or laboratory results without further stratification after patients selection based on the stringent criteria for UAP and NCCP, outlined below. Supplementary material online, Table S1 places the study characteristics of the selected UAP and NCCP patients into the context of the overall stenoCardia cohort’s characteristics. For NCCP, patients without angiographically proven coronary artery disease (CAD) had been preselected. The comparison between the 10 NCCP profiling patients (see below) and the preselected NCCP cohort is provided in Supplementary material online, Table S1.

A second case–control approach was applied selecting control group 2 from the population-based Gutenberg Health Study (GHS). Here, healthy controls were age- and sex-matched to the UAP patients. The R statistical software was used to perform the matching.

Definition of unstable angina, non-coronary chest pain, and acute myocardial infarction

Unstable angina patients had been adjudicated by two independent cardiologists as having unstable angina if they (i) presented with typical clinical symptoms of chest pain, (ii) the electrocardiogram did not show ST-segment elevations or new left bundle block, and (iii) the coronary angiography revealed a culprit lesion with the need for intervention in at least one major coronary artery. (iv) Cardiac troponin I (TnI) assayed by a contemporary-sensitivity test (Tnl-Ultra, Siemens Health- care Diagnostics, Germany) never exceeded the 99th percentile (0.04 ng/mL) at baseline, 3- or 6-h blood draw (troponin-negative). Patients with a history of aortocoronary bypass surgery were excluded.

Criteria for NCCP individuals had been presentation with chest pain, never exceeding the 99th percentile of the contemporary sensitive tropinin I ultra (0.04 ng/mL) during hospital stay and—most importantly—having an angiographically proven exclusion of any coronary stenosis (profiling phase). Additional criteria for this group were normal renal function (creatinine < 1.3 mg/dL) and no alternative cardiac diagnosis explaining the symptoms. Often, a clear alternative cause of chest pain such as pleuritis, pneumonia, or musculoskeletal pain was identified in these patients.

Acute myocardial infarction was defined by electrocardiographic changes indicative of new ischaemia (new ST-T segment changes or new left bundle branch block) and elevated Tnl-Ultra with a clear rising or falling pattern according to the Universal Definition of Myocardial Infarction.\(^\text{19,20}\)

Study phases

Profiling phase

The miRNA profiling phase served as hypothesis generating phase. This phase consisted of 40 individuals: 10 diagnosed with UAP, 10 with NCCP, and 20 healthy, population-based controls. The phenotype ‘unstable angina’ had been selected carefully according to the definitions described above. In the profiling phase, 667 human miRNAs were tested and out of those, 25 were selected to enter phase 2, the replication phase.

Replication phase

Patients for the replication phase had been taken from an independent study cohort, the APACE study (description see below). We selected 49 patients classified as UAP and 48 as NCCP. The selection process had been identical to that described for the profiling phase. Most importantly, the same troponin I test (Tnl-Ultra, 99th percentile at 0.04 ng/mL) had been applied and all patients classified as UAP or NCCP had been an angiographically proven diagnosis of either culprit lesion (UAP) or exclusion of CAD (NCCP). Out of the 25 miRNAs replicated in this phase, 8 entered the validation phase.

Validation phase

The validation phase tested the diagnostic potential of the selected eight miRNAs for the diagnosis UAP, their kinetics over time, and their
behaviour in individuals presenting with AMI. From the stenoCardia study, 46 patients had been selected with the diagnosis UAP, 63 patients with NCCP, both as defined above.

To directly test the kinetics of the eight miRNAs over time in response to myocyte necrosis in 29 AMI patients, we selected the baseline and 6 h blood draw.

Underlying study populations used for sample selection

\textit{Study for evaluation of newly onset chest pain and rapid diagnosis of myocardial necrosis (stenoCardia)}

Patients with acute chest pain presenting consecutively at the chest pain unit of the Johannes Gutenberg-University Medical Centre Mainz between January 2007 and December 2008 were enrolled in this all-comers prospective biomarker assessment registry as described earlier.\textsuperscript{15,16,21} Blood samples were obtained on admission and after 3 and 6 h. Routine laboratory parameters including C-reactive protein were measured immediately after blood withdrawal by standardized methods. Additionally, EDTA plasma and serum samples were collected at each time point, centrifuged, aliquoted, and stored at \(-80^\circ\text{C}\). The study was approved by the local ethics committees. Participation was voluntary; each patient gave written, informed consent.

\textit{Advantageous predictors of Acute Coronary Syndromes Evaluation study (APACE)}

Consecutive patients who presented to the emergency department with symptoms suggestive of AMI between April 2006 and June 2009 were enrolled in this prospective, international, multi-centre study as described previously.\textsuperscript{17} Blood samples were collected in serum or EDTA plasma tubes at the time of presentation and additional samples were obtained 1, 2, 3, and 6 h after presentation. Samples were centrifuged and aliquots were stored at \(-80^\circ\text{C}\). The study was approved by the local ethics committee and each patient gave written informed consent.

\textit{Gutenberg Health Study (GHS)}

Apparently healthy individuals of the Rhine-Main area in Germany were enrolled in this ongoing community-based, prospective, observational single-centre cohort study as described earlier.\textsuperscript{22,23} Participants were selected from the local registry offices. Individuals between 35 and 74 years of age were eligible to participate in the study. The study protocol and sample drawing have been approved by the local ethics committee and each participant gave written informed consent.

Measurement of protein-based biomarkers

In both the stenoCardia and the APACE study, cardiac troponin I using a commercially available contemporary-sensitivity assay (Tnl-Ultra, ADVIA Centaur XP system, Siemens Healthcare Diagnostics, Germany) was used to establish the diagnosis ‘unstable angina’. The assay detection limit was 0.006 ng/mL, the assay range was 0.006–50 ng/mL, and 10% coefficient of variation (CV) was at 0.03 ng/mL. This cardiac TnI-Ultra test detects troponin in \(-20\%\) of the general population.

We determined troponin I in the study samples using a high-sensitivity cardiac troponin I (hsTnl) assay (STAT high sensitive Troponin, Abbott Diagnostics, Abbott Park, USA, ARCHITECT 2000SR). The established limit of detection (LoD) for the assay is 1.9 pg/mL with an assay range between 0 and 50,000 pg/mL. This assay detects troponin I concentrations in \(-90\%\) of the general population and was used as a troponin test to be compared against the diagnostic capability of the 3-miRNA panel.

B-type natriuretic peptide (BNP) was measured in EDTA plasma using the ARCHITECT i2000SR BNP assay (Abbott Diagnostics, Abbott Park, USA). The assay measuring range was 10–5000 pg/mL. Inter-assay CV was 5.96%, and intra-assay CV was 4.67%. Cystatin C was measured by the ARCHITECT c8000 Cystatin C assay (Abbott Diagnostics, Abbott Park, USA) with a measuring range of 0.005–10 mg/dL. The assay measuring range was 10–5000 pg/mL. Inter-assay CV was 3.96%, and intra-assay CV was 2.25%.

Lipid values and C-reactive protein had been determined using routine methods.

Isolation of circulating RNA from serum

Circulating cell-free RNA was isolated from frozen serum samples. The maximum storage time of the samples at \(-80^\circ\text{C}\) was 5 years. Briefly, 3 volumes of TRIzol (Invitrogen) were mixed with 1 volume of serum and incubated for 5 min at room temperature to ensure complete dissociation of nucleoprotein complexes. Chloroform was added, and the mixture was shaken vigorously. After 5 min at room temperature, the RNA was centrifuged at 14 000g and 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 the miRNeasy kit (Qiagen) according to the manufactures’ recommendations. RNA was eluted in 30 μL RNase-free H₂O. For normalization, serum samples were supplemented with 10 nM C. elegans miR-39 (cel-miR-39) after addition of TRizol as previously described.\textsuperscript{24}

Detection of circulating microRNA

For miRNA profiling, total RNA from serum was analysed using the low-density TaqMan Array Human MicroRNA A + B Cards Set v2.0 (Applied Biosystems) according to the manufacturer’s protocol. This array card set contains a total of 384 TaqMan miRNA assays per card and enables assaying of 667 specific human microRNA. Briefly, RNA was reversely transcribed to cDNA with MegaPlex RT primers (MegaPlex RT Primer Pool, Applied Biosystems) followed by a pre-amplification step using MegaPlex PreAmp Primer Pool Set v2.0 (Applied Biosystems). Subsequently, real-time PCR amplification of miRNAs using low-density TaqMan Arrays was performed on an Applied Biosystem 7900 HT system using SDS software v2.3. For validation of miRNAs and miR-39 spike-in, single miRNA assays (Applied Biosystems) were used for miRNA detection.

Statistical analysis

Cycle threshold (Ct) values were normalized to cel-miR-39 by the formula \(2^{-(\text{Ct}[\text{miRNA}] - \text{Ct}[\text{cel-miR-39}])}\) for Ct < 40 and \(2^{-40}\) in the case Ct > 40 (considered as undetermined). Difference in miRNA levels groups was tested by the Mann–Whitney test, the Wilcoxon signed-rank test, or the Skillings–Mack test as appropriate. As a measure of effect strength and direction when using the Mann–Whitney test, the area under the receiver operating characteristic (ROC) curve was computed.\textsuperscript{25}

Selected miRNAs (n = 8) from the profiling and replication phases, together with the biomarkers hsTnl, BNP, C-reactive protein, and Cystatin C were examined in the validation phase with respect to the diagnosis of UAP. For these analyses, the average of the normalized miRNA values measured on admission and at 6 h was used. Combinations of miRNAs and/or biomarker were obtained via logistic regression. In the biomarker combination principal component analysis was used to reduce the number of covariates used in the model. The miRNA combination was produced using logistic regression with variable selection via the elastic net.\textsuperscript{26} Receiver operating characteristic curves and areas under the curve (AUCs) were computed. All biomarkers levels were used after a logarithmic transformation.

A pooled analysis using a random effects model was performed with the selected eight miRNAs. As before, the average of the normalized miRNA values measured on admission and at 6 h was used. The studies combined were APACE (replication phase) and stenoCardia (validation phase).
phase). All statistical testing was two-tailed, and P-values < 0.05 were considered statistically significant. All statistical analyses were performed using R 2.14.2 (http://www.r-project.org).

Results

Profiling of circulating microRNAs in unstable angina pectoris patients

Table 1 provides baseline characteristics of the study cohorts. To determine miRNAs in acute myocardial ischaemia without evident necrosis (= UAP), we compared levels of 667 circulating miRNAs in serum samples of patients adjudicated to have UAP to NCCP patients and healthy controls. The level of various circulating miRNAs differed profoundly and several miRNAs showed significant changes in UAP patients (Supplementary material online, Table S2). Primarily, most miRNA levels were lower in patients with UAP, 19.5% of all miRNAs showed lower levels in UAP patients compared with NCCP (26.5% compared with healthy controls). Circulating levels of miRNAs with known cardiac relevance (miR-1, miR-208a, and miR-208b) did not differ in UAP patients at the time of admission. Of note, levels of miR-208b were mainly undetectable in UAP patients and controls at the time of admission (Ct ≥ 40), whereas miR-208a and miR-1 were detectable but showed no significant difference between patients and controls.

Replication of circulating microRNA in patients adjudicated to have unstable angina pectoris

For replication, 25 miRNAs were selected based on their association with UAP in the initial profiling phase (P ≤ 0.05 for association). In addition, we included miRNAs miR-1, miR-208a, and miR-208b based on their potential role as biomarkers for AMI, although these miRNAs showed no significant difference between UAP patients and controls in our initial profiling phase. The 28 miRNAs were forwarded into the replication phase and circulating miRNA levels were determined in an independent study cohort (APACE). The results of the profiling and replication phases of these 28 miRNAs are presented in Table 2. Eight miRNAs showed significantly differential levels in UAP patients in both the profiling and replication phase and were selected for the validation phase. Those miRNAs included miR-19a, miR-19b, miR-132, miR-140-3p, miR-142-5p, miR-150, miR-186, and miR-210.

Validation of circulating microRNAs

To further explore the applicability of circulating miRNAs as potential diagnostic biomarkers of UAP in troponin-negative patients, subsequent ROC analyses were performed in samples of the validation phase (n = 46 UAP, n = 63 NCCP). First, AUCs were calculated for the eight selected miRNAs individually. Of all miRNAs, miR-186 showed the highest AUC (0.78; CI: 0.67–0.88) (Table 3). In a next step, we selected a ‘miRNA panel’ of the most discriminatory miRNAs out of the eight selected miRNAs. A panel of three miRNAs including miR-132, miR-150, and miR-186 showed the highest AUC (0.91; CI: 0.84–0.98), thus improving the AUC compared with that of miR-186 alone. If the high-sensitivity assayed troponin I (hsTnI) was applied in this validation phase detecting concentrations above the LoD of 1.9 pg/mL in nearly 100% of the study participants, an AUC of 0.57 (CI:0.44–0.70) was achieved for the diagnosis of unstable angina. Even combing the four markers hsTnI, BNP, C-reactive protein, and Cystatin C did not improve the diagnostic sensitivity substantially [AUC:0.63 (CI:0.5–0.76) (Figure 2)]. Thus, the highest diagnostic accuracy for unstable angina was achieved by applying the 3-miRNA combination of miR-132, miR-150, and miR-186.

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**Table 1** Characteristics of the study populations

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>stenoCardia/GHS (profiling)</th>
<th>APACHE (replication)</th>
<th>stenoCardia (validation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UAP</td>
<td>NCCP</td>
<td>HC</td>
</tr>
<tr>
<td>No. of patients</td>
<td>10</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Age (years)</td>
<td>65.3 ± 12</td>
<td>60.6 ± 14.2</td>
<td>60.5 ± 10.3</td>
</tr>
<tr>
<td>Male gender (%)</td>
<td>6 (60)</td>
<td>5 (50)</td>
<td>11 (55)</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>9 (90)</td>
<td>8 (80)</td>
<td>8 (40)</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>26.8 ± 3.7</td>
<td>26.5 ± 3.9</td>
<td>25.9 ± 4.2</td>
</tr>
<tr>
<td>Diabetes mellitus (%)</td>
<td>1 (10)</td>
<td>2 (20)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current smoker (%)</td>
<td>1 (10)</td>
<td>3 (30)</td>
<td>3 (15)</td>
</tr>
<tr>
<td>Former smoker (%)</td>
<td>7 (70)</td>
<td>5 (50)</td>
<td>11 (55)</td>
</tr>
<tr>
<td>Known CAD (%)</td>
<td>4 (44)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>CPO (chest pain onset)</td>
<td>2.59</td>
<td>3.87</td>
<td>–</td>
</tr>
<tr>
<td>time (h)</td>
<td>(1.9/13.6)</td>
<td>(2.6/6.8)</td>
<td>–</td>
</tr>
</tbody>
</table>

Data are summarized by either mean ± standard deviation or 50th (25th/75th) percentiles for continuous variables and n (%) for binary variables. UAP, unstable angina pectoris; HC, healthy controls; NCCP, non-cardiac chest pain; stenoCardia, study for evaluation of newly onset chest pain and rapid diagnosis of myocardial necrosis; APACHE: Advantageous Predictors of Acute Coronary Syndromes Evaluation study, GHS: Gutenberg Health Study.
Kinetic profiling and Influence of ST-elevation myocardial infarction on validated microRNAs identified in unstable angina pectoris

We further addressed the kinetic profiling and the impact of transmural myocardial necrosis on the eight selected miRNAs. For kinetic profiling, we determined circulating levels of the eight validated miRNAs in UAP patients of the validation study sample collected at the time of admission and after 6 h. We found profoundly decreased levels of miR-140-3p and miR-186 in serum of UAP patients 6 h after admission, whereas levels of the remaining six miRNAs did not change considerably (Figure 3). Additionally, miRNAs with known cardiac relevance (miR-1, miR-208a, miR-208b) showed no significant change in abundance between time of admission and after 6 h in UAP patients.

As it is known that miRNAs are released from the cells under conditions of myocardial necrosis, we also addressed the impact of transmural myocardial necrosis on levels of the eight miRNAs in 29 STEMI patients and 63 NCCP controls and compared miRNA levels on admission, after 3 h and after 6 h. Levels of miR-19b, miR-132, miR-186, and miR-210 showed a profound increase within 6 h, whereas circulating miR-140-3p slightly decreased (Supplementary material online, Figure S1). As expected, levels of miR-1, miR-208a, and miR-208b showed significant increase in their abundance in STEMI patients within 6 h after admission.

Discussion

Recent studies revealed that miRNAs are implicated in the pathogenesis of various cardiovascular conditions.1–4 Accumulating evidence
These miRNAs had lower levels in UAP than in control patients. With UAP in both the profiling and the replication cohort. Most of these miRNAs selected from the profiling and replication phases were further validated in 46 UAP patients and n = NCCP controls of the stenoCardia study. CI, confidence interval. AUC, area under the receiver operating characteristic curve. AUC < 0.5 indicates miRNA levels in patients with UAP lower than in controls, AUC > 0.5 indicates miRNA levels in patients with UAP higher than in controls.

### Table 3 Validation of selected circulating microRNAs

<table>
<thead>
<tr>
<th>miRNA</th>
<th>AUC (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-19a</td>
<td>0.44 (0.32–0.56)</td>
</tr>
<tr>
<td>miR-19b</td>
<td>0.42 (0.30–0.54)</td>
</tr>
<tr>
<td>miR-132</td>
<td>0.43 (0.30–0.55)</td>
</tr>
<tr>
<td>miR-140-3p</td>
<td>0.48 (0.35–0.60)</td>
</tr>
<tr>
<td>miR-142-5p</td>
<td>0.46 (0.33–0.58)</td>
</tr>
<tr>
<td>miR-150</td>
<td>0.41 (0.29–0.54)</td>
</tr>
<tr>
<td>miR-186</td>
<td>0.78 (0.67–0.88)</td>
</tr>
<tr>
<td>miR-210</td>
<td>0.43 (0.31–0.55)</td>
</tr>
</tbody>
</table>

Figure 2 Discriminatory power of the 3-miRNA panel. Receiver operator characteristic (ROC) curves and area under the ROC curve (AUC) are given for the 3-miRNA panel compared with troponin I measured by a high-sensitivity assay (hsTnI) and a model including hsTnI, B-type natriuretic peptide (BNP), C-reactive protein, and Cystatin C (4-marker combination). The 3-miRNA panel includes the miRNAs miR-132, miR-150, and miR-186.

Discriminatory power and had moderate accuracy in the early diagnosis of UAP. Third, the discriminatory power of a panel of three miRNAs (miR-132, -150, and -186) was highest, with an AUC of 0.91 (CI: 0.84–0.98), suggesting that a multi-miRNA approach provides more clinically useful information compared with single miRNAs. Of particular importance, in our study the diagnosis UAP had been confirmed by coronary angiography with at least one culprit coronary lesion, accompanied by typical clinical symptoms of chest pain, electrocardiogram without ST-elevations or new left bundle block, and negative troponin I measured by a contemporary-sensitivity assay in serial measurements up to 6 h after admission.

Recently published studies suggest a role in cardiovascular conditions for most of the miRNAs related to UAP in our study: dysregulation of miR-150 under conditions of hypoxia and an upregulation of miR-186, miR-210, and miR-150 in myocardial infarction had been reported. Likewise, a crucial cardiac role has also been implicated for miR-210 in the endothelial cell response to hypoxia. 29, 30 miR-19a and -19b are members of the miR-19-92 cluster; a recent study showed the relation of decreased miR-19a and miR-19b expression with age-related remodelling in the heart 31 supporting a role of these miRNAs during cardiac aging and heart failure. However, conflicting results regarding the regulation (up/downregulation) of miRNA levels are currently available in the literature and need to be investigated further.

In our study, levels of known cardiac-enriched miRNAs miR-1, miR-208a, and miR-208b after serial measurements were not different in UAP patients, but showed increased levels in STEMI patients, indicating different pathophysiological mechanisms of miRNA release during ischaemia and necrosis events. These observations are in line with results of a recently published study, showing higher miR-1 and miR-208b levels in patients with STEMI and NSTEMI than in UAP patients.

From a clinical perspective, a biomarker-based diagnosis of UAP is highly relevant to improve the differential diagnosis of chest pain. Aortic dissection, peri-/myocarditis and pulmonary embolism are the main differential diagnostic aspects in patients presenting with severe chest pain. To date, no specific biomarker diagnosis is available to facilitate differential diagnosis in these clinical conditions. Therefore, a miRNA panel which leads to the assumption that chest pain derives from coronary causes even in the absence of troponin elevation is of immediate clinical interest.

Several limitations of our study merit consideration. Because of the relatively small profiling sample size some miRNAs relevant for UAP diagnosis might not have been detected, although an overestimation of identified miRNAs should be minimized by our multiple phases approach. Further to this, the observed miRNA differences between UAP cases and controls might simply represent the prevalence of CAD. However, as NCCP controls of the replication phase included CAD cases, and the UAP case selection was strictly related to the prevalence of a culprit lesion (in half of the cases even without underlying severe CAD), the observed miRNA differences should mainly be driven by the phenotype UAP. Thus, an appropriate sampling design to ensure representation of the population of interest needs to be implemented in future studies; our results are a first step in that process. Second, we also acknowledge that we did not provide other diagnostic performance characteristics than AUC values such as sensitivity/specificity and predictive values for our
Due to the small sample size, we were not able to compute stable cut-offs for these test performance measures. Thus, a replication of our findings in a larger samples size and the evaluation of the concept of clinical application and usefulness of the 3-miRNA panel in a prospective ‘real-world clinical setting’ needs to be further evaluated.

From a laboratory view, C. elegans miR-39 had been used for normalization of miRNA data in our study. Using other ‘housekeeping’ miRNAs or small RNAs as recently discussed\textsuperscript{24,33,34} might result in differently normalized data and thus might have an influence on the results. Finally, the methodology of RT-PCR used in this study does not allow an easy and rapid application in a clinical setting. However, further development of semi-automated protocols might overcome this limitation in the future.

In conclusion, using a profiling-replication-validation model, we were able to identify miRNAs, which when used in a multi-miRNA panel seem to provide clinically useful information for the early diagnosis of UAP.

**Supplementary material**

Supplementary material is available at European Heart Journal online.
Acknowledgements
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Conflict of interest: Abbott Diagnostics provided test reagents for high-sensitive troponin I determinations within the frame of the stenoCardia Study. S.B. has received honoraria from Abbott Diagnostics, Siemens, Brahms/Thermo Fisher, and Roche Diagnostics and is a consultant for Thermo Fisher. C.M. received research grants from Abbott, Brahms, Nanoshore, Roche and Siemens, consulting fees from Abbott, and lecture fees from Abbott, Biosite, Brahms, Roche and Siemens. All other co-authors report no conflict of interest.

References

Assessment of microRNAs in patients with UAP
A 71-year-old female with scoliosis and multiple laminectomies underwent C3-5 laminectomy with root decompression for bilateral lower extremity pain and weakness. Four days after discharge, while recovering at a skilled nursing facility, she developed vague chest pain and presented to her orthopaedic surgeon’s office who referred her immediately to the emergency department (ED) for suspected pulmonary embolism. Upon presentation to the ED, she transiently required respiratory support with face mask ventilation. Her work-up identified biatrial atrial thrombus connecting through a patent foramen ovale (PFO), saddle pulmonary embolus, multiple bilateral segmental and subsegmental PEs, and residual bilateral lower extremity DVTs. Transoesophageal echocardiography confirmed these findings (Panels A and B; Supplementary material online, Videos 1 and 2). She was transferred to a tertiary-care centre and went urgently to the operating room. In the operating room, the intracardiac thrombus was exposed via the right atrium and the septum secundum was opened to ensure complete removal of the thrombus. The PFO was closed and the pulmonary emboli were removed with separate incisions in each pulmonary artery during a brief period of hypothermic circulatory arrest (Panels C and D). She was discharged 10 days after her operation after an uncomplicated post-operative course. At 1-month follow-up, she was asymptomatic with 97% oxygen saturation on room air and is currently on a 6-month course of Coumadin with a plan for subsequent hypercoagulable work-up. The patient described is extremely fortunate to have not had a stroke. According to the current literature, the PFO closure is still under debate, but this case illustrates the potential catastrophic consequences of a PFO.

(Supplementary material is available at European Heart Journal online.)