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EHJ BRIEF COMMUNICATION

Acute coronary syndromes

Profiling and validation of circulating microRNAs for cardiovascular events in patients presenting with ST-segment elevation myocardial infarction

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Aims MicroRNAs (miRNA) are important non-coding modulators controlling patterns of gene expression. However, profiling and validation of circulating miRNA levels related to adverse cardiovascular outcome has not been performed in patients with an acute coronary syndrome (ACS).

Methods and results In a multicentre, prospective ACS cohort, 1002 out of 2168 patients presented with ST-segment elevation myocardial infarction (STEMI). Sixty-three STEMI patients experienced an adjudicated major cardiovascular event (MACE, defined as cardiac death or recurrent myocardial infarction) within 1 year of follow-up. From a miRNA profiling in a matched derivation case–control cohort, 14 miRNAs were selected for validation. Comparing 63 cases vs. 126 controls, 3 miRNAs were significantly differentially abundant. In patients with MACE, miR-26b-5p levels ($P = 0.038$) were decreased, whereas miR-320a ($P = 0.047$) and miR-660-5p ($P = 0.01$) levels were increased. MiR-26b-5p has been suggested to prevent adverse cardiomyocyte hypertrophy, whereas miR-320a promotes cardiomyocyte death and apoptosis, and miR-660-5p has been related to active platelet production. This suggests that miR-26b-5p, miR-320a, and miR-660-5p may reflect alterations of different pathophysiological pathways involved in clinical outcome after ACS. Consistently, these three miRNAs reliably discriminated cases from controls [area under the receiver-operating characteristic curve (AUC) in age- and sex-adjusted Cox regression for miR-26b-5p = 0.707, miR-660-5p = 0.683, and miR-320a =0.672]. Combination of the three miRNAs further increased AUC to 0.718. Importantly, addition of the three miRNAs to both, the Global Registry of Acute Coronary Events (GRACE) score and a clinical model increased AUC from 0.679 to 0.720 and 0.722 to 0.732, respectively, with a net reclassification improvement of 0.20 in both cases.

Conclusion This is the first study performing profiling and validation of miRNAs that are associated with adverse cardiovascular outcome in patients with STEMI. MiR-26b-5p, miR-320a, and miR-660-5p discriminated for MACE and increased risk prediction when added to the GRACE score and a clinical model. These findings suggest that the release of

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specific miRNAs into circulation may reflect the activation of molecular pathways that impact on clinical outcome after STEMI.

Introduction

MicroRNAs (miRNAs), a class of non-coding RNAs, are important post-transcriptional modulators.[1] Recently, experimental studies demonstrated that circulating miRNAs are involved in cell-to-cell communication. As miRNAs are bound to transport proteins or encapsulated in microvesicles and exosomes, they are protected from degradation and can be reliably measured in plasma samples.[2,3] Differences in miRNA levels between healthy subjects and patients with acute coronary syndrome (ACS)[4] indicate that miRNAs may serve as biomarkers predicting outcome beyond the currently used scores in patients presenting with ACS and unravel molecular pathways involved in the disease.

We aimed to assess changes in miRNA levels in ST-segment elevation myocardial infarction (STEMI) patients that are associated with cardiovascular outcome. Differential expression levels of miRNAs may lead to improving our understanding of pathophysiological mechanisms that are related to clinical outcomes in this patient population.

Methods

All experimental procedures and computational analyses are described in detail in Supplementary material online.

Results

The study population included STEMI patients with available EDTA-plasma sample ($n = 1002$) derived from a previously described multicentre, prospective ACS-cohort (NCT01000701).[5]

Derivation cohort

To detect whether miRNAs are associated with cardiovascular outcome in patients presenting with STEMI (*Figure 1A*), a miRNA profiling was conducted in plasma samples from patients with a major adverse cardiovascular event (MACE, defined as cardiac death or recurrent myocardial infarction) and without a MACE within 1-year follow-up. Patients with confounding variables known to influence miRNA levels (see Supplementary material online, *Table S1*) were excluded. Cases with MACE and controls without MACE were matched (see Supplementary material online, *Table S2*) according to age, gender, symptom onset to balloon angioplasty time, and dual-antiplatelet therapy. Using a miRNA-profiling approach, 752 miRNAs were assessed with the miRCURY LNA™ Universal RT miRNA PCR Human panel I+II (Exiqon, Vedbaek, Denmark). The 752 miRNAs comprise miRNAs that were—based on array, qPCR, and published literature data—most consistently expressed. We identified 92 miRNAs in all samples, with an average of 290 miRNAs detectable per sample. Differences of miRNA levels between cases ($n = 7$) and controls ($n = 7$) were found for 15 miRNAs ($P < 0.05$; Supplementary material online, *Table S3*). MiRNAs detected in <10 samples (miR-663a, miR-27a-3p, and miR-211-5p) were excluded from further analysis. Due to their known role in cardiomyocyte apoptosis and adverse hypertrophy, miR-320a[6] and miR-150a[7] were included. Finally, 14 miRNAs were carried over to the validation phase. Baseline characteristics and medication of the derivation cohort are shown in Supplementary material online, *Tables S4 and S5*.

Validation cohort

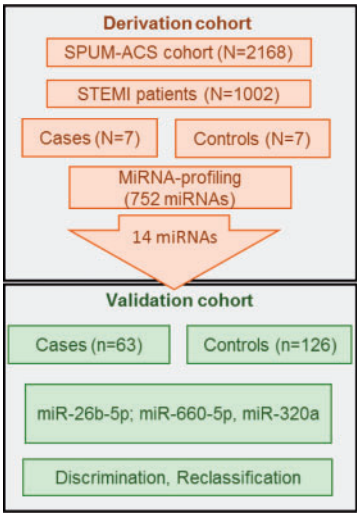
For validation of differentially abundant miRNAs, we conducted a case–control study in 1002 STEMI patients from the multicentre, prospective SPUM-ACS-Cohort. The 14 miRNAs identified in the screening phase were investigated in 63 cases and 126 matched controls. Patient characteristics and medication are shown in Supplementary material online, *Tables S6 and S7*. Cases were older and presented more frequent with renal failure, whereas controls had higher cholesterol levels. The other variables, including medication, showed no significant differences. Of note, the comparison of baseline characteristics and medication did not reveal significant differences between the validation and derivation cohort (Supplementary material online, *Tables S8 and S9*). In addition, no significant differences were found between storage time of case and control samples in the derivation ($P = 0.235$) and validation ($P = 0.815$) cohort.

Out of 14 miRNAs, 9 miRNAs were reliably detected in the majority of samples. Of note, UniSp6 that was used as a spike-in control for cDNA synthesis showed no difference between samples.

Levels of three miRNAs, miR-26b-5p, miR-660-5p, and miR-320a, differed in cases as compared with controls (all $P < 0.05$, Welch's *t*-test, Benjamini–Hochberg corrected). Patients experiencing MACE had lower levels of miR-26b-5p, but higher levels of miR-660-5p and miR-320a (*Figure 1B*).

To test the association of miR-26b-5p, miR-660-5p, and miR-320a with MACE, we calculated the area under the receiver-operating characteristic curves (AUC) (*Figure 1C*) of Cox regression models. MiR-26b-5p (AUC 0.707), miR-660-5p (AUC 0.683), and miR-320a (AUC 0.672) discriminated patients with MACE from patients without MACE. Importantly, combination of these three miRNAs yielded an increased AUC of 0.718. Of note, high-sensitivity troponin T (AUC 0.666) and pro B-type natriuretic peptide (pro-BNP) (AUC 0.674) showed lower discriminatory power as compared with the three miRNAs (*Figure 1C*). We further assessed whether miR-26b-5p, miR-660-5p, and miR-320a may add predictive value to a clinical model (hypertension, diabetes mellitus, smoking, hypercholesterolemia, previous myocardial infarction, and family history of coronary artery disease) or the Global Registry of Acute Coronary Events (GRACE)-

A



B

	Mean Controls	Mean Cases	Fold-Change	P-value
let7b-3p	2.8	2.4	-0.1	0.120
miR-150-5p	-2.0	-1.6	-0.2	0.073
miR-181a-5p	-0.4	-0.1	-0.7	0.234
miR-22-5p	2.4	2.2	-0.1	0.406
miR-26b-5p	0.1	0.5	6.4	0.038
miR-320a	-2.5	-2.9	0.2	0.047
miR-342-3p	-0.3	-0.2	-0.4	0.455
miR-574-3p	0.8	0.7	-0.1	0.682
miR-660-5p	0.9	0.3	-0.6	0.010

C

	AUC	95% CI
MiRNAs		
miR-26b-5p	0.707	(0.68 - 0.74)
miR-660-5p	0.683	(0.66 - 0.70)
miR-320a	0.672	(0.65 - 0.70)
miR-26b-5p + miR-320a + miR-660-5p	0.718	(0.68 - 0.75)
Biomarkers		
hsTnT	0.666	(0.64 - 0.69)
pro-BNP	0.674	(0.64 - 0.71)

D

	AUC	95% CI	NRI	95% CI
GRACE score	0.679	(0.66 - 0.70)		
+ miR-26b-5p	0.714	(0.68 - 0.75)	0.16	(0.14 - 0.18)
+ miR-660-5p	0.685	(0.66 - 0.71)	0.10	(0.08 - 0.11)
+ miR-320a	0.679	(0.65 - 0.71)	-0.01	(-0.03 - 0.00)
+ (miR-26b-5p + miR-660-5p + miR-320a)	0.720	(0.68 - 0.76)	0.20	(0.18 - 0.22)
Clinical Model	0.722	(0.69 - 0.76)		
+ miR-26b-5p	0.724	(0.68 - 0.76)	0.11	(0.09 - 0.13)
+ miR-660-5p	0.715	(0.67 - 0.76)	0.09	(0.07 - 0.11)
+ miR-320a	0.721	(0.69 - 0.75)	0.04	(0.02 - 0.05)
+ (miR-26b-5p + miR-660-5p + miR-320a)	0.732	(0.69 - 0.77)	0.20	(0.19 - 0.22)

Figure 1 (*A*) Study design and workflow. Cases are patients who experienced a major cardiovascular event (defined as cardiac death or recurrent myocardial infarction) within 1 year; controls did not experience a major cardiovascular event within 1 year. (*B*) Fourteen miRNAs identified in the miRNA-profiling from the derivation cohort were analysed in the validation phase. In the validation cohort (cases, $n = 63$; controls, $n = 126$), miR-26b-5p, miR-660-5p, and miR-320a were significantly ($P < 0.05$) differently abundant between cases and controls after Welch's *t*-test and Benjamini–Hochberg correction. Since fold-changes (log2) are calculated for dCt-values, negative fold-changes imply higher miRNA levels in cases as compared with controls. (*C*) Area under the receiver-operating characteristic curve values for miRNAs and biomarkers when classifying cases vs. controls in Cox regression models adjusted for sex and age. MiR-26b-5p (area under the receiver-operating characteristic curve 0.707), miR-660-5p (area under the receiver-operating characteristic curve 0.683), and miR-320a (area under the receiver-operating characteristic curve 0.672) reliably discriminate cases from controls. Combination of the three miRNAs enhances discriminatory power to an area under the receiver-operating characteristic curve of 0.718. (*D*) Addition of miR-26b-5p, miR-660-5p, and miR-320a increases the predictive value of the Global Registry of Acute Coronary Events score and a clinical model. Shown are area under the receiver-operating characteristic curve and net reclassification improvement for the clinical model and the Global Registry of Acute Coronary Events score with and without the three miRNAs. CI, confidence interval; hsTNT, high-sensitivity troponin T; pro-BNP, pro B-type natriuretic peptide.

Score (*Figure 1D*). MiR-26b-5p most profoundly increased AUC, whereas miR-660-5p and miR-320a showed only a moderate or no increase in AUC (*Figure 1D*). Addition of the three miRNAs to a clinical model or the GRACE score, however, further moderately increased AUC from 0.722 to 0.732 and 0.679 to 0.720, respectively. Comparing the GRACE score to the miRNA-extended GRACE score and the clinical model to the miRNA-extended clinical model directly yielded the highest increase for miR-26b-5p (NIR for miR-26b-5p-extended GRACE score- = 0.16; NIR for miR-26b-5p-extended clinical model = 0.11) when miRNAs were assessed individually and a further moderately increased net reclassification improvement (NRI) of 0.2 in both cases when the three miRNAs were combined (*Figure 1D*). After integration of renal function (estimated glomerular filtration rate (eGFR)) in the miRNA-extended clinical model, the NRI remained significantly increased (NRI of clinical model + eGFR + (miR-26b-5p + miR-660-5p + miR-320a) = 0.17 [95% confidence interval (CI) 0.15–0.19)]. In addition, when the analysis was limited to cases only used in the validation cohort, the added prognostic value of the three miRNAs remained significant [NRI for clinical model + (miR-26b-5p + miR-320a + miR-660-5p) = 0.16 (95% CI, 0.14–0.18); NRI for GRACE score + (miR-26b-5p + miR-320a + miR-660-5p) = 0.16 (95% CI, 0.14–0.19)].

Discussion

This is the first study that performed profiling and validation of miRNAs that are associated with adverse cardiovascular outcome using a large multicentre, prospective ACS cohort. We identified three miRNAs (i.e. miR-26b-5p, miR-660-5p, and miR-320a) that discriminate patients experiencing MACE from those without it within the first year after STEMI. As these miRNAs have been reported as modulators of adverse cardiac hypertrophy,[8] cardiomyocyte apoptosis,[6] and active platelet count[9] in experimental studies, our findings shed light onto a miRNA-mediated modulation of molecular pathways that may be related to adverse clinical outcomes.

Previous studies focused on selected and mostly cardiac-derived miRNA panels or examined surrogate outcomes. We conducted an miRNA profiling of 752 miRNAs in a derivation cohort to identify miRNAs that are related to adjudicated MACE (defined as cardiac death or recurrent myocardial infarction). Validation in a matched case–control design involving 63 patients with MACE identified three miRNAs (miR-26b-5p, miR-660-5p, and miR-320a) that are differentially abundant between cases and controls. A decreased level of miR-26b-5p and higher levels of miR-660-5p and miR-320a were detected in patients experiencing a MACE. Interestingly, experimental studies suggest that these three miRNAs are involved in pathophysiological mechanisms relevant for cardiovascular outcome. Overexpression of miR-26b-5p reduced cardiac hypertrophy in an experimental transverse aortic constriction model in vivo.[8] Therefore, it is conceivable that lower miR-26b-5p levels in our study predict MACE. Elevated levels of circulating miR-320a as observed in patients with MACE in our study have previously been reported in cardiac tissues derived from end-stage heart failure as compared with control samples.[10,11] Interestingly, overexpression of miR-320 increased cardiomyocyte apoptosis, potentially through the inhibition of heat-shock protein 20.[6] For miR-660, overexpression of miR-660 increased production of active platelets in vitro,[9] indicating a potential role for thrombotic events, as in recurrent myocardial infarction. These experimental studies suggest that differential expression of miR-26b-5p, miR-660-5p, and miR-320a are related to pathophysiological mechanisms that trigger heart failure, recurrent myocardial infarction, and cardiac death. Consistently, miR-26b-5p, miR-660-5p, and miR-320a were good discriminators of MACE. Combination of these three miRNAs improved discriminatory power. In predictive models using the GRACE score and a clinical model, addition of miR-26b-5p resulted in the highest increase in AUC and NRI, whereas miR-660-5p and miR-320a did not substantially improve prognostication when added alone to these models. However, combination of the three miRNAs moderately increased AUC and NRI as compared with the miR-26b-5p-extended models, potentially reflecting the involvement of different pathophysiological pathways relevant for clinical outcome

Of note, several studies have examined acute miRNA regulation in patients with STEMI (Supplementary material online, *Table S10*) in different cell types[12] or in plasma samples using restricted panels of muscle-enriched miRNAs,[13,14] or miRNA profiling.[12,15,16] However, these miRNAs detected in diagnostic studies of STEMI were not associated with MACE in our prognostic study. Notably, studies that used a profiling and validation approach for the *diagnosis* of ACS have shown promising results to establish miRNAs as early diagnostic biomarkers.[4] The present study, however, for the first time has performed a miRNA profiling and validation approach to assess miRNA related to adverse *prognosis* in a secondary prevention cohort of STEMI patients. Furthermore, we show that miRNAs may improve risk prediction when added to currently used models and scores. In a different clinical setting, i.e. in a primary prevention cohort analysing 19 candidate miRNAs, Zampetaki *et al.*[17] have observed that three endothelial cell or platelet-enriched miRNAs (i.e. miR-126, miR-223, and miR-197) were associated with the incident myocardial infarction. However, no initial miRNA profiling related to myocardial infarction and cardiovascular death was performed, and these three miRNAs were not tested for improvement of net reclassification.

Whether differential abundance of circulating miRNAs is a result of different release mechanisms or modulated intracellular production has to be determined in future studies. Notably, Hergenreider *et al.*[18] and others have shown that circulating miRNAs exert important functions after cellular uptake, indicating that miRNAs continuously modulate pathophysiological pathways depending on their abundances. Therefore, an miRNA-targeted therapy may evolve as a strategy to counteract heart failure or thrombotic events and improve outcome in patients presenting with STEMI. However, to date, no clinical therapies targeting these miRNAs exist.

In conclusion, using a profiling and validation approach, we identified miR-26b-5p, miR-660-5p, and miR-320a to be associated with adverse cardiovascular events in patients presenting with STEMI. Importantly, in experimental studies, these miRNAs are involved in pathways regulating cardiomyocyte apoptosis, adverse cardiac remodelling, and active platelet production. Therefore, these three miRNAs may reflect pathophysiological mechanisms relevant for clinical outcome and may be further examined for improvement of risk stratification in patients with STEMI.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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Conflict of interest: none declared.

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