

Early assessment of acute coronary syndromes in the emergency department: the potential diagnostic value of circulating microRNAs

Martinus I. F. J. Oerlemans¹, Arend Mosterd^{1,2,3}, Marieke S. Dekker^{2,3,4}, Evelyn A. de Vrey³, Alain van Mil^{1,5}, Gerard Pasterkamp¹, Pieter A. Doevendans^{1,5}, Arno W. Hoes², Joost P. G. Sluijter^{1,5*}

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Previous studies investigating the role of circulating microRNAs in acute coronary syndrome (ACS) were based on small patient numbers, performed no comparison with established markers of cardiac injury and did not have appropriate controls. We determined the potential diagnostic value of circulating microRNAs as novel early biomarkers in 332 suspected ACS patients on presentation to the emergency department (ED) in a prospective single-centre study including cardiac miRNAs (miR-1, -208a and -499), miR-21 and miR-146a. Levels of all miRNAs studied were significantly increased in 106 patients diagnosed with ACS, even in patients with initially negative high-sensitive (hs) troponin or symptom onset <3 h. MiR-1, miR-499 and miR-21 significantly increased the diagnostic value in all suspected ACS patients when added to hs-troponin T (AUC 0.90). These three miRNAs were strong predictors of ACS independent of clinical co-variables including patient history and cardiovascular risk factors. Interestingly, the combination of these three miRNAs resulted in a significantly higher AUC of 0.94 than hs-troponin T (0.89). Circulating microRNAs hold great potential as novel early biomarkers for the management of suspected ACS patients.

INTRODUCTION

Acute coronary syndrome (ACS) remains one of the leading causes of morbidity and mortality in the Western world. Early diagnosis of ACS is essential because of improvement in prognosis following timely interventions. Currently, the diag-

nosis of ACS is based on elevation of (high-sensitive) cardiac troponin I or T (cTnI or cTnT), in the context of clinical and electrocardiographic (electrocardiogram, ECG) findings (Anderson et al, 2011; Thygesen et al, 2007). Unfortunately, these biomarkers are not consistently elevated within the first hours after symptom onset, requiring repetitive measurements and hindering early diagnosis (Dekker et al, 2010). In the setting of typical symptoms and ST segment elevation on the ECG, the diagnosis and subsequent management is straightforward (Kushner et al, 2009). In daily clinical practice, however, this scenario only pertains to a minority of patients presenting to the emergency department (ED) with symptoms suggestive of an ACS, making the early diagnosis of ACS a challenge and new diagnostic markers to improve early recognition of ACS are required.

MicroRNAs (miRNAs) are short, non-coding small RNAs that regulate the expression of proteins by translational repression or

- (1) Department of Cardiology, University Medical Center Utrecht, The Netherlands
- (2) Julius Center for Health Sciences and Primary Care, University Medical Center, Utrecht, The Netherlands
- (3) Department of Cardiology, Meander Medical Center, Amersfoort, The Netherlands
- (4) Department of Cardiology, Isala Clinics, Zwolle, The Netherlands
- (5) Interuniversity Cardiology Institute Netherlands (ICIN), Utrecht, The Netherlands

*Corresponding author: Tel: +31 88 755 7155; Fax: +31 30 252 2693; E-mail: j.sluijter@umcutrecht.nl

degradation of messenger RNAs (mRNAs; Lewis et al, 2003). Over the last few years, it has been established that miRNAs play a crucial role in cardiac development and homeostasis and that miRNA expression is altered in the diseased heart (Sluijter et al, 2010; Thum et al, 2007; van Rooij et al, 2006). Interestingly, miRNAs were also found to be present in human serum and plasma and altered expression profiles were observed in cancer and other diseases like diabetes (Chen et al, 2008; Mitchell et al, 2008; Zampetaki et al, 2010). This led to the hypothesis that miRNAs might be released upon cardiac injury and that the detection of cell-free miRNAs – including cardiac-related miR-1, miR-499 and miR-208a – could be used for the diagnosis of ACS (Adachi et al, 2010; Ai et al, 2010; Cheng et al, 2010; Corsten et al, 2010; D'Alessandra et al, 2010; Gidlof et al, 2011; Ji et al, 2009; Kuwabara et al, 2011; Olivieri et al, 2012; Wang et al, 2010a,b; Widera et al, 2011). Previous studies investigating miRNAs for detection of cardiac injury have been relatively small (mostly including <100 patients) and were not carried out in the clinically relevant patient population (*i.e.* unselected patients suspected of ACS presenting to the ED), but typically included patients already known to have coronary artery disease [*e.g.* those undergoing primary percutaneous coronary intervention (PCI)] and compared these with healthy controls (Corsten et al, 2010; D'Alessandra et al, 2010; De Rosa et al, 2011; Wang et al, 2010a,b). In addition, several studies used samples taken at the time of reperfusion to determine miRNAs, rather than samples taken upon initial presentation to the ED, when diagnostic uncertainty is most evident.

Our aim was to determine the diagnostic value of circulating miRNAs, in particular cardiac-related miR-1, miR-499 and miR-208a, and stress-related miR-21 and miR-146a (Dong et al, 2009; Horie et al, 2010; van Rooij et al, 2008) in the early assessment of suspected ACS patients presenting at the ED. In addition, we determined the diagnostic value of miRNAs in subgroups of suspected ACS patients with initially negative high-sensitive

(hs-) troponin levels and in those presenting within 3 h of symptom onset since in these patients, the diagnostic accuracy of available biomarkers is limited.

RESULTS

Patient characteristics

The median onset of chest pain in the 332 suspected ACS patients was 3.2 h [interquartile range (IQR) 1.8–8.0] prior to presentation (Table 1). ACS was diagnosed in 106 (31.2%) patients, consisting of UA ($n = 24$, 22.6%) and NSTEMI ($n = 82$, 77.4%). Of the ACS-patients, 21.7% (23/106) had a hs-troponin level <0.14 pg/ml at initial presentation with a median hs-troponin of 7.25 (IQR 3.1–11.3) whereas in the hs-troponin >14 pg/ml this was 41.2 (IQR 18.7–108.2).

In 226 (68.1%) patients an ACS could be ruled out; in the non-ACS population, the following diagnoses were established by the expert-panel: stable angina ($n = 39$), rhythm disorders ($n = 9$), heart failure ($n = 3$), pericarditis ($n = 2$), other cardiac diagnosis (unlisted, $n = 12$) and non-cardiac chest pain ($n = 161$).

Circulating miRNA levels in ACS patients

Compared to the non-ACS population, circulating levels of all cardiac-expressed miRNAs studied (miR-1, miR-499 and miR-208a) were higher in patients with ACS (Fig 1A–C). Furthermore, circulating levels of miR-21 (18.9-fold) and miR-146a (11.7-fold) were markedly elevated in ACS patients as well ($p < 0.001$; Fig 1D and E). None of the circulating miRNAs were significantly associated with age, gender or other relevant clinical co-variables (Supporting Information Table S1). Furthermore, heparin treatment or platelet inhibition (aspirin, clopidogrel) at presentation was not significantly correlated with any of the circulating miRNAs as depicted in Supporting Information Table S2.

Table 1. Patient characteristics of 332 patients with chest pain

Characteristic	Non-ACS	ACS	All	p-value
N (%)	226 (68.1)	106 (31.2)	332 (100)	
Age (yrs)	60.2 ± 14.3	68.7 ± 12.6	62.9 ± 14.4	0.00
Male sex (% male)	120 (53.1)	70 (66.0)	190 (57.2)	0.03
Onset of chest pain, median (IQR)	3.2 (1.9–9.0)	3.1 (1.6–8.0)	3.2 (1.8–8.0)	0.64
Risk factors				
Current smoker	62 (27.7)	24 (22.9)	86 (26.1)	0.42
Former smoker	71 (31.8)	32 (30.8)	103 (31.5)	0.90
Hypertension	90 (39.8)	61 (58.7)	151 (45.8)	0.02
Hypercholesterolemia	65 (28.9)	44 (41.9)	109 (33.0)	0.02
Diabetes mellitus	31 (13.8)	21 (20.0)	52 (15.8)	0.19
Body mass index (kg/m ²)	26.4 ± 6.1	26.0 ± 5.1	26.3 ± 5.8	0.34
Parental CVD	89 (39.7)	44 (42.7)	133 (40.7)	0.63
History of CVD	97 (42.9)	62 (58.5)	159 (47.9)	0.01
Cardiac Troponin I (µg/L)	0.02 (0.01–0.03)	0.07 (0.04–0.21)	0.03 (0.02–0.05)	0.00
Cardiac hs-Troponin T (pg/ml)	3.3 (1.1–8.5)	23.7 (10.6–80.8)	6.0 (2.2–18.7)	0.00

Data presented as mean ± SD for age, body mass index and as median (25th to 75th IQR) for onset of chest pain and cardiac troponin levels. All other variables are presented as n (%); ACS, acute coronary syndrome; CVD, cardiovascular disease; Mann–Whitney (continuous variables) or χ^2 test (categorical variables), ACS ($n = 106$) versus non-ACS ($n = 226$).

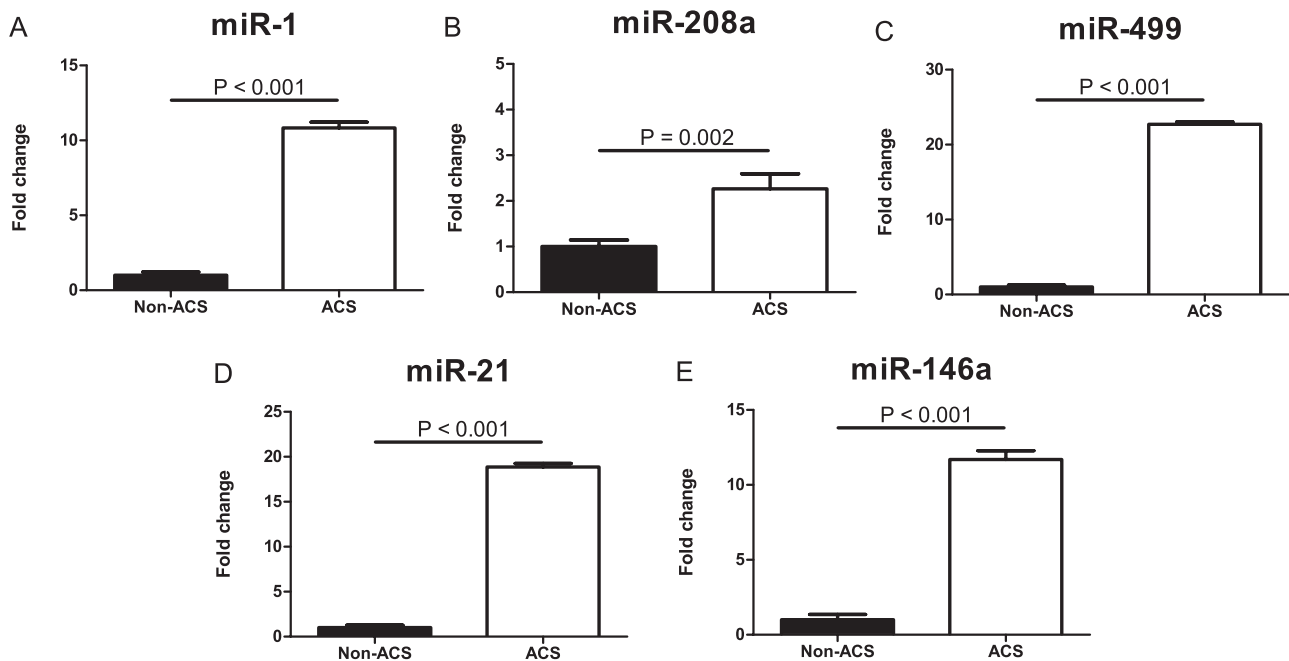


Figure 1. Expression levels of circulating miRNAs in serum of ACS and non-ACS patients. Data are presented as mean \pm SEM, *p*-values versus non-ACS patients, Mann–Whitney test.

A–C. Cardiac-specific miR-1, miR-208a and miR-499 were significantly increased in ACS patients.

D,E. Both levels of miR-21 (D) and miR-146a (E) were markedly elevated in ACS patients compared to non-ACS patients.

Expression pattern of circulating miRNAs in UA and NSTEMI patients

Levels of both miR-1 and miR-499 were elevated in UA and NSTEMI patients when compared to non-ACS patients ($p < 0.001$; Fig 2A and C), while circulating miR-208a levels were significantly increased in NSTEMI patients only (Fig 2B). MiR-21 and miR-146a were significantly increased in both ACS subcategories (Fig 2D and E). For miR-208a and miR-146a, circulating miRNA levels seemed to be the highest in NSTEMI patients, while miR-21 levels were comparable between patients with unstable angina (UA) and NSTEMI patients. Levels of miR-1 and miR-499 were higher in UA patients.

Circulating miRNA levels in suspected ACS patients with a negative hs-troponin at first presentation and in patients presenting within 3 h of symptom onset

In 194 suspected ACS patients (53.6% men, mean age 58.4 ± 12.7 years), initial hs-troponin levels were negative (< 0.14 pg/ml). Of these, 23 (11.9%) were subsequently classified as having an ACS [median hs-troponin level 2.81 (IQR 1.0–6.1) pg/ml], including 18 UA and 5 NSTEMI patients. In this troponin-negative subgroup of suspected ACS patients, cardiac-related miRNAs were considerably higher in patients with ACS (Table 2). Circulating levels of miR-21 and miR-146a were also elevated.

In 152 suspected ACS patients (55.3% men, mean age 62.9 ± 14.5 years), the onset of chest pain was < 3 h prior to presentation. In this subgroup, circulating levels of miR-1, miR-

499 and miR-208a were also higher in ACS patients than non-ACS patients (Table 3). MiR-21 and miR-146a were markedly elevated in ACS patients as well.

Additional diagnostic value of circulating microRNAs in ACS patients

The area under the ROC curve (AUC) of both cardiac troponin I [0.85; 95% confidence interval (CI) 0.80–0.90] and hs-troponin T (0.86; 95% CI 0.82–0.91) was higher than that of any of the miRs (Table 3). Of the miRs, miR-1, miR-499 and miR-21 had the highest AUC. When combining these miRs, the diagnostic value became significantly higher than that of hs-troponin T in the total suspected ACS patients and in the subgroup of suspected ACS patients with initially negative troponin levels (Table 3). Furthermore, combining miR-1, miR-499 or miR-21 with hs-troponin T significantly improved the diagnostic value by increasing the AUC to 0.90 ($p < 0.001$; Table 3).

As expected, the AUC of hs-troponin was low (0.61; 95% CI 0.47–0.75) in the subgroup of suspected ACS patients with initially negative hs-troponin levels (Table 3). Interestingly, the AUC of miR-1 and miR-499 were higher than any of the other miRs or cardiac (hs-)troponin. The AUC of miR-21 was comparable with hs-troponin (0.75; 95% CI 0.65–0.85). Addition of miR-1, miR-499 or miR-21 further increased the diagnostic discriminatory value significantly compared to hs-troponin alone (Table 3). Combining miR-1, miR-499 and miR-21 with hs-troponin T significantly improved the diagnostic value by increasing the AUC to 0.88 (95% CI 0.83–0.94). In patients with

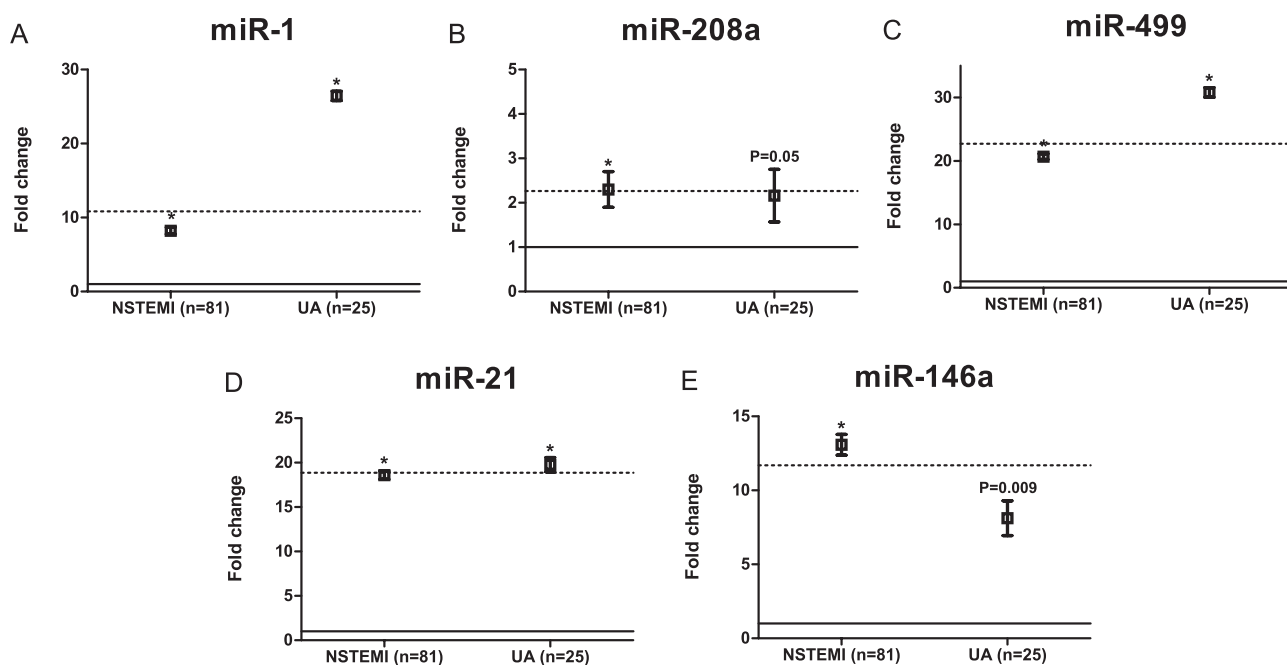


Figure 2. Expression pattern of circulating miRNAs in STEMI and UA patients. Circulating miRNAs displayed different expression levels within the ACS population. Dotted lines represent fold increase in all ACS patients. Data are presented as mean \pm SEM. *p*-values and **p* < 0.001 versus non-ACS patients, Mann-Whitney test.

A–C. While the increase in levels of miR-1 (A) and miR-499 (C) was relatively high in UA patients, miR-208a levels were only increased in NSTEMI patients (B). **D,E.** MiR-21 levels were comparable between NSTEMI and UA patients (D), the increase in levels of miR-146a (E) was most pronounced in NSTEMI patients.

symptoms <3 h, a similar increase in AUC was observed with the addition of miR-1, miR-499 and miR-21 (Table 3).

Circulating miRNAs are independent predictors for ACS

The clinical model with age, sex and cardiovascular risk factors resulted in an AUC of 0.72 (95% CI 0.66–0.78) for all suspected ACS patients (Table 4), which increased to 0.89 after addition of hs-troponin T. Addition of miR-1, miR-499 or miR-21 to the clinical model with hs-troponin T significantly increased the AUC to 0.92 (*p* < 0.001). Importantly, after correction for the clinical model and (hs-)troponin, miR-1, miR-499 and miR-21 were still strong predictors for ACS as illustrated by their odds ratio (Table 4). Interestingly, the combination of the three miRNAs resulted in an even higher AUC of 0.94 (95% CI 0.92–0.97).

In patients with negative hs-troponin at presentation, addition of miR-1, miR-499 and miR-21 also resulted in a

significant increase in AUC to 0.92 (Table 5), whereas miR-208a and miR-146a showed no additional effect. Despite negative hs-troponin levels at presentation, the combination of three miRNAs (miR-1, miR-499 and miR-21) resulted in a very high-AUC value of 0.96 (95% CI 0.93–0.99), again independent of other clinical risk factors (Table 5). In patients with symptom onset <3 h, these three miRNAs were the strongest predictors for ACS, increasing the AUC significantly as well (Supporting Information Table S3).

As a final step, we compared the combination of miR-1, miR-499 and miR-21 with the combination of three established markers of myocardial necrosis (hs-troponin, myoglobin and CK-MB) in the model with clinical risk factors (Supporting Information Table S4). In the total population, hs-troponin negative patients and early presenting patients, the combination of miRNAs performed better than the combination of these three

Table 2. Relative expression of circulating miRNAs in serum of suspected ACS patients with a negative hs-troponin (n = 194) or with onset of symptoms <3 h (n = 152) compared to non-ACS patients (n = 226)

MicroRNA	Non-ACS	ACS patients with negative hs-troponin	<i>p</i> -value	ACS patients with symptom onset <3 h	<i>p</i> -value
miR-1	1.0 \pm 0.2	14.9 \pm 0.8	0.00	6.0 \pm 0.6	0.00
miR-208a	1.0 \pm 0.2	2.8 \pm 0.7	0.03	2.4 \pm 0.5	0.01
miR-499	1.0 \pm 0.3	34.2 \pm 0.7	0.00	24.0 \pm 0.5	0.00
miR-21	1.0 \pm 0.3	15.5 \pm 0.8	0.00	11.0 \pm 0.5	0.00
miR-146a	1.0 \pm 0.4	6.2 \pm 1.3	0.04	11.9 \pm 0.8	0.00

Data are presented as mean \pm SEM. *p*-value versus non-ACS patients.

Table 3. Diagnostic value of cardiac troponin and circulating microRNAs in suspected ACS patients

Marker	All patients (n = 332)		Hs-troponin negative patients (n = 194)		Patients with symptoms <3 h (n = 152)	
	AUC	95% CI	AUC	95% CI	AUC	95% CI
Cardiac troponin I	0.85	0.80–0.90	0.61	0.47–0.75	0.82	0.75–0.90
Cardiac hs-troponin T	0.86	0.82–0.91	0.74	0.62–0.85	0.86	0.80–0.92
miR-1	0.75	0.70–0.81	0.79 ^b	0.69–0.89	0.67	0.28–0.77
miR-208a	0.61	0.54–0.67	0.64	0.52–0.77	0.62	0.53–0.72
miR-499	0.79	0.74–0.84	0.83 ^c	0.76–0.90	0.79	0.71–0.86
miR-21	0.76	0.71–0.82	0.75	0.65–0.85	0.72	0.64–0.80
miR-146a	0.68	0.62–0.74	0.64	0.52–0.75	0.70	0.61–0.78
miR-1 + miR-499 + miR-21	0.89 ^a	0.85–0.94	0.88 ^a	0.83–0.94	0.82	0.76–0.89
Cardiac hs-troponin T with						
miR-1	0.90 ^a	0.86–0.93	0.86 ^a	0.79–0.92	0.88 ^c	0.83–0.94
miR-208a	0.86	0.82–0.91	0.78	0.68–0.89	0.86	0.81–0.92
miR-499	0.90 ^a	0.86–0.93	0.89 ^a	0.83–0.94	0.90 ^a	0.84–0.94
miR-21	0.89 ^a	0.86–0.93	0.84 ^a	0.77–0.91	0.89 ^b	0.83–0.94
miR-146a	0.87	0.83–0.91	0.79	0.70–0.88	0.86	0.80–0.92

AUC, area under the ROC Curve; 95% CI, 95% confidence interval;

^a*p* < 0.001,

^b*p* = 0.03,

^c*p* = 0.003 versus hs-troponin T.

Table 4. AUCs and Odds ratios of miRNAs in suspected ACS patients in a clinical model (n = 332)

Marker	AUC	95% CI	OR ^b	95% CI
Clinical model (CM)	0.72	0.66–0.78	NA	NA
CM + cardiac troponin	0.88	0.85–0.92	NA	NA
CM + cardiac hs-troponin T	0.89	0.85–0.92	NA	NA
CM + cardiac hs-troponin T with				
miR-1	0.92 ^a	0.90–0.95	1.30	1.17–1.42
miR-208a	0.89	0.85–0.93	1.16	1.03–1.30
miR-499	0.92 ^a	0.89–0.95	1.28	1.18–1.40
miR-21	0.92 ^a	0.89–0.95	1.28	1.18–1.39
miR-146a	0.90	0.87–0.94	1.14	1.08–1.21
miR-1 + miR-499 + miR-21	0.94 ^a	0.92–0.97	NA	NA

CM, clinical model (age, sex, hypertension, hypercholesterolemia, family history, current and former smoking, diabetes mellitus, and history of myocardial infarction, PCI or coronary bypass surgery); AUC, area under the ROC curve; 95% CI, 95% confidence interval;

^a*p* < 0.001 versus hs-troponin T. NA, not applicable;

^bAdjusted for clinical model and cardiac hs-troponin T.

necrosis markers. However, only in the hs-troponin negative population, this led to a statistically significant difference in AUC.

DISCUSSION

Circulating miRNAs have been proposed as potentially useful novel biomarkers for detecting cardiac injury. We selected three miRNAs – miR-1, miR-499 and miR-208 – because of their known high expression in cardiac tissue to determine their diagnostic value in suspected ACS patients upon first presentation in the ED. Our results demonstrate that the levels of miR-1 and miR-499 are markedly elevated in patients with ACS compared to non-ACS patients (Fig 1A and C). These data confirm and extend previous reports showing high

levels in patients with myocardial damage (Adachi et al, 2010; Ai et al, 2010; Cheng et al, 2010; Corsten et al, 2010; D’Alessandra et al, 2010; Kuwabara et al, 2011; Wang et al, 2010a,b). The increase in miR-1 levels was less pronounced than that of miR-499. This might explain why a previous study failed to demonstrate a significant increase in miR-1 levels compared to miR-499, which was markedly elevated in patients suffering from acute MI (Corsten et al, 2010). MiR-208a only increased slightly in the ACS group (Fig 1B). This is probably explained by the fact that miR-208a was undetectable in 86% of our samples (286/332), supported by an earlier study (Kuwabara et al, 2011). Also because of known difficulties in detecting miR-208a (and miR-208b) levels (Adachi et al, 2010; D’Alessandra et al, 2010; Widera et al, 2011), miR-208(a/b) appears to be the least suitable of the selected cardiac-expressed candidates.

Table 5. AUCs and Odds ratios of miRNAs in suspected ACS patients with a negative hs-troponin in a clinical model (n = 194)

Marker	AUC	95% CI	OR ^b	95% CI
Clinical model (CM)	0.84	0.76–0.93	NA	NA
CM + cardiac troponin	0.85	0.77–0.94	NA	NA
CM + cardiac hs-troponin T	0.86	0.79–0.93	NA	NA
CM + cardiac hs-troponin T with				
miR-1	0.92 ^a	0.87–0.96	1.44	1.19–1.73
miR-208a	0.87	0.78–0.95	1.12	0.95–1.35
miR-499	0.93 ^a	0.87–0.99	1.38	1.19–1.61
miR-21	0.92 ^a	0.88–0.97	1.34	1.15–1.55
miR-146a	0.86	0.78–0.93	1.06	0.97–1.15
miR-1 + miR-499 + miR-21	0.96 ^a	0.93–0.99	NA	NA

CM, clinical model (age, sex, hypertension, hypercholesterolemia, family history, current and former smoking, diabetes mellitus, and history of myocardial infarction, PCI or coronary bypass surgery); AUC, area under the ROC curve; 95% CI, 95% confidence interval;

^a $p < 0.001$ versus hs-troponin T; NA, not applicable;

^bAdjusted for clinical model and cardiac hs-troponin T.

In addition, we investigated the expression of miR-21 and miR-146a in ACS-suspected patients based on reports showing that these miRNAs were clearly related to cardiac injury or myocyte cell death and in patients suffering from ACS (Dong et al, 2009; Guo et al, 2010). Although further research is necessary to identify why these two miRNAs are upregulated, their involvement in cardiac disease has been established. Inhibition of miR-21 (depending on the anti-miR used) inhibited extensive fibrosis in the failing heart, mainly by decreasing fibroblast survival (Patrick et al, 2010; Thum et al, 2011). Additionally, miR-21 exerted cardioprotective effects as over-expression reduced myocardial cell death (Cheng et al, 2009; Dong et al, 2009) and miR-146a was reported to be upregulated in Doxorubicin-induced cardiotoxicity (Horie et al, 2010). Circulating levels of miR-21 (diastolic dysfunction) and miR-146a (diastolic dysfunction and viral myocarditis) showed no changes compared to control patients (Corsten et al, 2010). In line with our data, two recent studies reported increased miR-21 levels post-MI (Olivieri et al, 2012; Zile et al, 2011).

Analysis of miRNA levels in ACS subcategories revealed an increase of all miRNAs studied in UA and NSTEMI patients, except for miR-208a (only in NSTEMI patients). Interestingly, UA patients had relatively high levels of both miR-1 and miR-499, although this was not statistically different from NSTEMI patients. MiR-146a levels were the highest in NSTEMI patients; miR-21 levels were comparable between UA and NSTEMI patients. It is not unlikely that both miR-21 and miR-146a are subjected to more dynamic changes in the ischemic heart. This is supported by the fact that different profiling studies reported an increase in miR-21 and miR-146a after MI while miR-1 and miR-499 levels (already highly expressed) showed a more stable expression over time (Roy et al, 2009; van Rooij et al, 2008). Furthermore, levels of miR-146a in UA patients were below the mean level of all ACS patients, which might suggest that miR-146a could be helpful in discriminating NSTEMI from UA patients. Larger diagnostic studies will be necessary to validate these observations. These differences raise the question whether miRNAs are released on a regular base (*i.e.* intercellular communication upon stress signals) or simply reflect myocardial damage leading to

uncontrolled leakage from damaged myocardium (Gupta et al, 2010).

It was previously reported that miRNAs in serum can be found in exosomes and microvesicular bodies (Wang et al, 2010a,b; Zampetaki et al, 2012). As their release can be affected by any form of cellular stress, cardiac necrosis is not necessary to evoke this response. This could explain why we observed elevated miRNA levels in patients found to have an ACS whose troponin was negative on initial presentation (*i.e.* patients with UA pectoris – in whom troponin remains negative by definition – and MI patients presenting too early after symptom onset for troponin to be positive). These observations suggest that miRNAs holds potential to improve the prognosis of both NSTEMI and UA patients who are known to benefit from swift initiation of treatment.

A recent study with a final diagnosis of ACS suggested that circulating miRs provide rather poor prognostic and diagnostic information (Widera et al, 2011). However, as this was designed as a prognostic study, a control group consisting of suspected ACS patients, but ultimately not having an ACS, was absent. A very recent study elegantly demonstrated that the transcatheter concentration of several miRs, including miR-499, are increased in patients undergoing catheterization (De Rosa et al, 2011). Importantly, our data further extend these reports, as we included non-ACS patients and analysed blood collected at first presentation. The increase in AUC when adding miRNAs to the current ACS biomarker high-sensitive troponin, the fact that a combination of miRNAs resulted in a higher AUC than high-sensitive troponin, and the AUC of single miRs in the troponin-negative population, underscore the potential diagnostic value of these novel biomarkers in clinical practice. Finally, in a clinical model with important co-variables including age, sex and cardiovascular risk factors, miRNAs were shown to be strong independent predictors of ACS. Importantly, circulating miRs were not significantly correlated with age, hypertension and cholesterol levels.

Especially three of the investigated miRs (miR-1, miR-499 and miR-21) seem very promising, as their combined diagnostic performance is statistically better than hs-troponin. Furthermore, this miR-combination also led to higher AUC values

than the combination of three myocardial necrosis markers (hs-troponin, myoglobin and CK-MB).

The main strength of our study is the evaluation of miRNAs isolated from serum collected immediately upon presentation to the ED in a large group ($n = 332$) of patients, in a clinically relevant setting: *i.e.* patients suspected of an ACS in whom clinical and ECG findings are inconclusive. Apart from comparing miRNA levels in patients with and without an ACS, we also evaluated the added diagnostic value of miRNA compared to high-sensitive troponin and in a clinical model with relevant co-variables. Importantly, a combination of three miRNAs showed better diagnostic performance than hs-troponin T alone.

Several limitations have to be taken into account. From the original 470 patients, 138 were excluded because of poor RNA integrity indicating that the starting material was not optimal for our analysis; but importantly, the patient characteristic of these 138 patients and the 332 included patients were similar, indicating that this is very unlikely to have changed our findings. The material was collected in 2007 when optimal methods for processing and storage of serum for miRNAs isolation was largely unknown. Measurement of circulating miRNAs requires RT-PCR, which is currently the limiting factor in terms of rapid detection. However, as miRNAs have clear diagnostic potential, newer and less expensive techniques to detect serum miRNA levels more rapidly can be expected in the near future. This would also facilitate additional research investigating the incremental value of circulating miRNAs when added to a clinical risk score of ACS like the GRACE Risk Score or HEART score, which might be clinically very relevant (Backus et al, 2011).

Given the inherent limitations of our study, they remain to be confirmed in a larger, prospective, multicentre diagnostic study and in a large independent population of hs-troponin negative patients.

In patients suspected of ACS presenting to the ED within 24 h of symptom onset, circulating microRNA levels (miR-1, miR-208a, miR-499, miR-21 and miR-146a) are higher in those with an ACS and are already increased in suspected ACS patients with initially negative troponin levels and in those presenting within 3 h of symptom onset. Addition of miR-1, miR-499 or miR-21 significantly increased the diagnostic value compared to hs-troponin T and these three miRNAs were independent predictors of ACS. Interestingly, the combination of these three miRNAs resulted in a higher AUC than hs-troponin T, including the hs-troponin negative patients. These findings demonstrate that circulating miRNAs hold great potential as novel early biomarkers of cardiac injury. MiRNAs might be useful for better management of suspected ACS patients, in particular those with UA pectoris and NSTEMI in whom diagnostic uncertainty is high.

MATERIALS AND METHODS

Study population

The present study a single-centre, prospective diagnostic study among patients presenting to the ED within 24 h of onset of chest pain

suggestive of ACS. Patients were enrolled at the ED of the Meander Medical Center (Amersfoort, the Netherlands) between May 2007 and November 2007 ST-elevation myocardial infarction (STEMI) – these patients underwent immediate PCI – or refusal to provide informed consent were the only exclusion criteria, 470 patients were evaluated. All included patients provided written informed consent, the protocol was approved by the local ethics committee and the study was conducted in accordance with the Declaration of Helsinki. RNA extraction and analysis of stored serum samples was performed in all 470 patients. Despite several attempts to isolate good quality RNA, we had to exclude 138 patients because of poor RNA integrity, mostly due to errors in sample processing at time of collection and/or storage. The current analyses thus included 332 patients with chest pain. Importantly, the mean age (61.7 ± 14.9) and median onset of symptoms (3.2 h; IQR 2.0–6.7) in the 138 excluded patients and in the 332 included patients were similar (Supporting Information Table S5).

Routine clinical assessment

All patients underwent routine clinical assessment, including medical history, physical examination, serial 12 lead ECGs, pulse oxymetry and chest radiography. Immediately upon presentation, venous blood was drawn to determine cardiac Troponin I (cTnI) and other routine laboratory parameters. At the same time, additional samples were taken and stored for future analyses, including serum miRNA analysis (Supporting Information Fig S1). cTnI levels were measured by sandwich chemoluminescence immunoassay (Synchron Lxi 725 integrated clinical chemistry, Beckman Coulter). The lower detection limit for cTnI was 0.01 $\mu\text{g/L}$ and the cut-off level for positivity was $\geq 0.04 \mu\text{g/L}$. High-sensitive cardiac Troponin T (hs-cTnT) was determined *post hoc* in serum stored at -80°C . The lower detecting limit for hs-cTnT was 3 pg/ml, measured by the Elecsys troponin T high-sensitive assay fourth generation (Roche Diagnostics) with the 99th-percentage cut-off point of $\geq 14 \text{ pg/ml}$.

Blood processing

Blood samples were collected via a venous puncture at the time of presentation at the ED and 5 ml of blood was collected in a standard serum tube (BD #368968). Blood was centrifuged for 10 min after which serum was aspirated, aliquoted in RNase-free tubes and stored at -80°C until further processing.

RNA isolation

RNA was extracted of fixed volumes of serum (250 μl) using TRIzol LS reagent (Invitrogen 10296-028) as described previously (Chen et al, 2008; Kuwabara et al, 2011). Genomic DNA contamination was eliminated using DNase I kit (Ambion) and RNA concentration quantified with a spectrophotometer (NanoDrop ND-1000, Thermo Scientific, Wilmington, DE). The RNA integrity of small RNAs was determined using small RNA Chip analysis (5067-1548) for an Agilent Bioanalyzer 2100 (Agilent Technologies).

Quantitative RT-PCR

A total of 20 ng DNA-free RNA was used as input in the reverse transcription reaction (RT) using the TaqMan miRNA Reverse Transcription Kit and miRNA-specific primers (Applied Biosystems) as described previously (Kroh et al, 2010). Real-time PCR (RT-PCR)

The paper explained

PROBLEM:

Currently, the diagnosis of ACS is based on elevation of (high-sensitive) cardiac troponins, which are not consistently elevated within the first hours after symptom onset. In daily clinical practice, typical symptoms together with ST segment elevation on the ECG is only present in a minority of patients presenting to the emergency department (ED), making the early diagnosis of ACS a challenge. New diagnostic markers to improve early recognition of ACS are therefore required, for which microRNAs were previously investigated. MicroRNAs are short, non-coding small RNAs that regulate the protein expression by translational repression or degradation of messenger RNAs. Previous studies investigating the role of circulating microRNAs in ACS were based on small patient numbers, performed no comparison with established markers of cardiac injury or did not have appropriate controls.

RESULTS:

We determined the potential diagnostic value of circulating microRNAs as novel early biomarkers in 332 suspected ACS patients on presentation to the ED. Circulating microRNA levels (miR-1, miR-208a, miR-499, miR-21 and miR-146a) were higher in patients diagnosed with an ACS. Moreover, these miRNAs were

also increased in patients diagnosed with ACS when initial troponin was still negative or with symptom onset <3 h. Especially miR-1, miR-499 and miR-21 significantly increased the diagnostic value in all suspected ACS patients when added to high-sensitive troponin T (AUC 0.90). MiR-1, miR-499 and miR-21 were strong predictors of ACS independent of clinical co-variables, including patient history (age, sex, previous MI, PCI or surgery) and cardiovascular risk factors. Interestingly, the combination of these three miRs resulted in a significantly higher AUC of 0.94 than hs-troponin T (0.89).

IMPACT:

Using a real-world population of suspected ACS patients presenting to the ED, our study shows that circulating miRNAs hold great potential as novel early biomarkers of cardiac injury. These findings have important clinical implications as miRNAs might be useful for better management of suspected ACS patients than can be achieved by the current biomarker of choice, high-sensitive troponin T. This is of particular interest in patients with unstable angina pectoris and NSTEMI in whom diagnostic uncertainty is high.

amplification was performed in duplicate using 1:5 diluted RT products for the following miRNAs: miR-1 (#002222), miR-208a (#000511), miR-499 (#002427), miR-21 (#000397) and miR-146a (#000468). Currently, no consensus exists on the use of an internal control for RT-PCR analysis of circulating miRNAs. All RT-PCR data were standardized to U6, which was used by others before (Tijssen et al, 2010; Zile et al, 2011), moreover, U6 levels did not differ between ACS and non-ACS patients. After normalization for RNU6, relative gene expression was calculated by the $\Delta\Delta C_t$ method (Noort et al, 2012; Oerlemans et al, 2010).

Outcome

The primary outcome of this study was ACS, including UA and non-ST-elevation myocardial infarction (NSTEMI). Final diagnosis was made by an expert panel of three cardiologists, based on all available clinical information including serial troponin measurements, serial ECG findings, coronary angiography, echocardiography, cardiac exercise tests and information from hospital discharge letters.

Early invasive coronary angiography was performed in 30 (36.6%) out of 82 NSTEMI-ACS patients. Most important reasons were recurrent or non-resolving symptoms ($n = 10$), dynamic ECG changes ($n = 8$), prior CABG or recent PCI ($n = 11$) and ventricular tachycardia ($n = 1$). From the remaining 52 patients, in 38 (73.1%) patients symptoms resolved upon medicinal treatment. Of the 14 patients with non-resolving symptoms, 4 patients requested conservative therapy, in 5 patients a conservative strategy was chosen considering co-morbidity (aged >85 years, renal failure, CVA) and in 5 patients no coronary

angiography was performed due to known multivessel disease (>2 coronary arteries) or graft or stent failure requiring CABG surgery. The presence of ACS was determined according to the universal definition of myocardial infarction (Thygesen et al, 2007). NSTEMI was diagnosed when there was evidence of myocardial necrosis together with clinical signs and symptoms of myocardial ischemia, according to the current guidelines (Anderson et al, 2011; Hamm et al, 2011). Myocardial necrosis was diagnosed by a rising and/or fall in cardiac troponin with at least one value above the 99th percentile. UA was diagnosed in the presence of clinical signs and symptoms of myocardial ischemia, including cardiac exercise test, without elevation of cardiac biomarkers.

Statistical analysis

Data are presented as mean \pm SEM unless otherwise indicated. Differences between groups were analysed by Mann-Whitney U Test or Kruskal-Wallis Test (>2 groups) when appropriate using Bonferroni correction for multiple comparisons. Spearman rank correlation was used to compare patient characteristics with circulating microRNAs. Receiver-operating-characteristic (ROC) curves were constructed to of each miRNA using the area under the ROC curve (AUC) to assess their ability to diagnose ACS (Reichlin et al, 2009). To compare diagnostic discriminatory ability of miRNAs with cardiac (hs-)troponin, likelihood ratio tests were performed after logistic regression to assess the additional diagnostic value of miRNA to the information provided by (hs-) troponin. Multivariate logistic regression was used to investigate whether miRNAs were independent predictors of ACS after adjustment for relevant co-variables including patient history (age, sex, previous MI,

PCI or surgery) and cardiovascular risk factors (hypertension, hypercholesterolemia, family history, current and former smoking and diabetes mellitus). All tests were two-sided, using a significance level of $p < 0.05$ (SPSS Statistics v17, Chicago, United States).

Author contributions

MIFJO, AM, AvM, GP, PAD, AWH and JPGS designed, analysed and interpreted and were responsible for drafting the article; MIFJO, AM, MSD and EAdV were responsible for data acquisition. All authors critically revised the article and finally approved the manuscript prior to publication.

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Supporting Information is available at EMBO Molecular Medicine online.

The authors declare that they have no conflict of interest.

References

- Adachi T, Nakanishi M, Otsuka Y, Nishimura K, Hirokawa G, Goto Y, Nonogi H, Iwai N (2010) Plasma microRNA 499 as a biomarker of acute myocardial infarction. *Clin Chem* 56: 1183-1185
- Ai J, Zhang R, Li Y, Pu J, Lu Y, Jiao J, Li K, Yu B, Li Z, Wang R, *et al* (2010) Circulating microRNA-1 as a potential novel biomarker for acute myocardial infarction. *Biochem Biophys Res Commun* 391: 73-77
- Anderson JL, Adams CD, Antman EM, Bridges CR, Califf RM, Casey DE, Jr, Chavey WE, Fesmire FM, Hochman JS, Levin TN, *et al* (2011) 2011 ACCF/AHA Focused Update Incorporated Into the ACC/AHA 2007 Guidelines for the Management of Patients With Unstable Angina/Non-ST-Elevation Myocardial Infarction: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. *Circulation* 123: e426-e579
- Backus BE, Six AJ, Kelder JH, Gibler WB, Moll FL, Doevendans PA (2011) Risk scores for patients with chest pain: evaluation in the emergency department. *Curr Cardiol Rev* 7: 2-8
- Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, Guo J, Zhang Y, Chen J, Guo X, *et al* (2008) Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res* 18: 997-1006
- Cheng Y, Liu X, Zhang S, Lin Y, Yang J, Zhang C (2009) MicroRNA-21 protects against the H(2)O(2)-induced injury on cardiac myocytes via its target gene PDCD4. *J Mol Cell Cardiol* 47: 5-14
- Cheng Y, Tan N, Yang J, Liu X, Cao X, He P, Dong X, Qin S, Zhang C (2010) A translational study of circulating cell-free microRNA-1 in acute myocardial infarction. *Clin Sci (Lond)* 119: 87-95
- Corsten MF, Dennert R, Jochems S, Kuznetsova T, Devaux Y, Hofstra L, Wagner DR, Staessen JA, Heymans S, Schroen B (2010) Circulating MicroRNA-208b and MicroRNA-499 reflect myocardial damage in cardiovascular disease. *Circ Cardiovasc Genet* 3: 499-506
- D'Alessandra Y, Devanna P, Limana F, Straino S, Di CA, Brambilla PG, Rubino M, Carena MC, Spazzafumo L, De SM, *et al* (2010) Circulating microRNAs are new and sensitive biomarkers of myocardial infarction. *Eur Heart J* 31: 2765-2773
- De Rosa S, Fichtlscherer S, Lehmann R, Assmus B, Dimmeler S, Zeiher AM (2011) Transcoronary concentration gradients of circulating microRNAs. *Circulation* 124: 1936-1944
- Dekker MS, Mosterd A, van't Hof AW, Hoes AW (2010) Novel biochemical markers in suspected acute coronary syndrome: systematic review and critical appraisal. *Heart* 96: 1001-1010
- Dong S, Cheng Y, Yang J, Li J, Liu X, Wang X, Wang D, Krall TJ, Delphin ES, Zhang C (2009) MicroRNA expression signature and the role of microRNA-21 in the early phase of acute myocardial infarction. *J Biol Chem* 284: 29514-29525
- Gidlof O, Andersson P, van der PJ, Gotberg M, Erlinge D (2011) Cardiospecific microRNA plasma levels correlate with troponin and cardiac function in patients with ST elevation myocardial infarction, are selectively dependent on renal elimination, and can be detected in urine samples. *Cardiology* 118: 217-226
- Guo M, Mao X, Ji Q, Lang M, Li S, Peng Y, Zhou W, Xiong B, Zeng Q (2010) miR-146a in PBMCs modulates Th1 function in patients with acute coronary syndrome. *Immunol Cell Biol* 88: 555-564
- Gupta SK, Bang C, Thum T (2010) Circulating microRNAs as biomarkers and potential paracrine mediators of cardiovascular disease. *Circ Cardiovasc Genet* 3: 484-488
- Hamm CW, Bassand JP, Agewall S, Bax J, Boersma E, Bueno H, Caso P, Dudek D, Gielen S, Huber K, *et al* (2011) ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: the Task Force for the management of acute coronary syndromes (ACS) in patients presenting without persistent ST-segment elevation of the European Society of Cardiology (ESC). *Eur Heart J* 32: 2999-3054
- Horie T, Ono K, Nishi H, Nagao K, Kinoshita M, Watanabe S, Kuwabara Y, Nakashima Y, Takanabe-Mori R, Nishi E, *et al* (2010) Acute doxorubicin cardiotoxicity is associated with miR-146a-induced inhibition of the neuregulin-ErbB pathway. *Cardiovasc Res* 87: 656-664
- Ji X, Takahashi R, Hiura Y, Hirokawa G, Fukushima Y, Iwai N (2009) Plasma miR-208 as a biomarker of myocardial injury. *Clin Chem* 55: 1944-1949
- Kroh EM, Parkin RK, Mitchell PS, Tewari M (2010) Analysis of circulating microRNA biomarkers in plasma and serum using quantitative reverse transcription-PCR (qRT-PCR). *Methods* 50: 298-301
- Kushner FG, Hand M, Smith SC, Jr, King SB III, Anderson JL, Antman EM, Bailey SR, Bates ER, Blankenship JC, Casey DE, Jr, *et al* (2009) Focused updates: ACC/AHA Guidelines for the Management of Patients With ST-Elevation Myocardial Infarction (updating the 2004 Guideline and 2007 Focused Update) and ACC/AHA/SCAI Guidelines on Percutaneous Coronary Intervention (updating the 2005 Guideline and 2007 Focused Update): a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. *Circulation* 120: 2271-2306
- Kuwabara Y, Ono K, Horie T, Nishi H, Nagao K, Kinoshita M, Watanabe S, Baba O, Kojima Y, Shizuta S, *et al* (2011) Increased microRNA-1 and microRNA-133a levels in serum of patients with cardiovascular disease indicate myocardial damage. *Circ Cardiovasc Genet* 4: 446-454
- Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB (2003) Prediction of mammalian microRNA targets. *Cell* 115: 787-798
- Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogossova-Agadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A, *et al* (2008) Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci USA* 105: 10513-10518
- Noort WA, Oerlemans MI, Rozemuller H, Feyen D, Jaksani S, Stecher D, Naaijken B, Martens AC, Buhning HJ, Doevendans PA, *et al* (2012) Human versus porcine mesenchymal stromal cells: phenotype, differentiation potential, immunomodulation and cardiac improvement after transplantation. *J Cell Mol Med* 16: 1827-1839

- Oerlemans MI, Goumans MJ, van Middelaar B, Clevers H, Doevendans PA, Sluijter JP (2010) Active Wnt signaling in response to cardiac injury. *Basic Res Cardiol* 105: 631-641
- Olivieri F, Antonicelli R, Lorenzi M, D'Alessandra Y, Lazzarini R, Santini G, Spazzafumo L, Lisa R, La SL, Galeazzi R, et al (2012) Diagnostic potential of circulating miR-499-5p in elderly patients with acute non ST-elevation myocardial infarction. *Int J Cardiol* DOI: 10.1016/j.ijcard.2012.01.075
- Patrick DM, Montgomery RL, Qi X, Obad S, Kauppinen S, Hill JA, van RE, Olson EN (2010) Stress-dependent cardiac remodeling occurs in the absence of microRNA-21 in mice. *J Clin Invest* 120: 3912-3916
- Reichlin T, Hochholzer W, Bassetti S, Steuer S, Stelzig C, Hartwiger S, Biedert S, Schaub N, Buergle C, Potocki M, et al (2009) Early diagnosis of myocardial infarction with sensitive cardiac troponin assays. *N Engl J Med* 361: 858-867
- Roy S, Khanna S, Hussain SR, Biswas S, Azad A, Rink C, Gnyawali S, Shilo S, Nuovo GJ, Sen CK (2009) MicroRNA expression in response to murine myocardial infarction: miR-21 regulates fibroblast metalloprotease-2 via phosphatase and tensin homologue. *Cardiovasc Res* 82: 21-29
- Sluijter JP, van Mil A, van Vliet P, Metz CH, Liu J, Doevendans PA, Goumans MJ (2010) MicroRNA-1 and -499 regulate differentiation and proliferation in human-derived cardiomyocyte progenitor cells. *Arterioscler Thromb Vasc Biol* 30: 859-868
- Thum T, Chau N, Bhat B, Gupta SK, Linsley PS, Bauersachs J, Engelhardt S (2011) Comparison of different miR-21 inhibitor chemistries in a cardiac disease model. *J Clin Invest* 121: 461-462
- Thum T, Galuppo P, Wolf C, Fiedler J, Kneitz S, van Laake LW, Doevendans PA, Mummery CL, Borlak J, Haverich A, et al (2007) MicroRNAs in the human heart: a clue to fetal gene reprogramming in heart failure. *Circulation* 116: 258-267
- Thygesen K, Alpert JS, White HD, Jaffe AS, Apple FS, Galvani M, Katus HA, Newby LK, Ravkilde J, Chaitman B, et al (2007) Universal definition of myocardial infarction. *Circulation* 116: 2634-2653
- Tijssen AJ, Creemers EE, Moerland PD, de Windt LJ, van der Wal AC, Kok WE, Pinto YM (2010) MiR423-5p as a circulating biomarker for heart failure. *Circ Res* 106: 1035-1039
- van Rooij E, Sutherland LB, Thatcher JE, DiMaio JM, Naseem RH, Marshall WS, Hill JA, Olson EN (2008) Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. *Proc Natl Acad Sci USA* 105: 13027-13032
- van Rooij E, Sutherland LB, Liu N, Williams AH, McAnally J, Gerard RD, Richardson JA, Olson EN (2006) A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. *Proc Natl Acad Sci USA* 103: 18255-18260
- Wang GK, Zhu JQ, Zhang JT, Li Q, Li Y, He J, Qin YW, Jing Q (2010a) Circulating microRNA: a novel potential biomarker for early diagnosis of acute myocardial infarction in humans. *Eur Heart J* 31: 659-666
- Wang K, Zhang S, Weber J, Baxter D, Galas DJ (2010b) Export of microRNAs and microRNA-protective protein by mammalian cells. *Nucleic Acids Res* 38: 7248-7259
- Widera C, Gupta SK, Lorenzen JM, Bang C, Bauersachs J, Bethmann K, Kempf T, Wollert KC, Thum T (2011) Diagnostic and prognostic impact of six circulating microRNAs in acute coronary syndrome. *J Mol Cell Cardiol* 51: 872-875
- Zampetaki A, Kiechl S, Drozdov I, Willeit P, Mayr U, Prokopi M, Mayr A, Weger S, Oberhollenzer F, Bonora E, et al (2010) Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes. *Circ Res* 107: 810-817
- Zampetaki A, Willeit P, Drozdov I, Kiechl S, Mayr M (2012) Profiling of circulating microRNAs: from single biomarkers to re-wired networks. *Cardiovasc Res* 93: 555-562
- Zile MR, Mehurg SM, Arroyo JE, Stroud RE, Desantis SM, Spinale FG (2011) Relationship between the temporal profile of plasma microRNA and left ventricular remodeling in patients following myocardial infarction. *Circ Cardiovasc Genet* 4: 614-619