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Refining Diagnostic MicroRNA Signatures by Whole-miRNome Kinetic Analysis in Acute Myocardial Infarction

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BACKGROUND: Alterations in microRNA (miRNA) expression patterns in whole blood may be useful biomarkers of diverse cardiovascular disorders. We previously reported that miRNAs are significantly dysregulated in acute myocardial infarction (AMI) and applied machine-learning techniques to define miRNA subsets with high diagnostic power for AMI diagnosis. However, the kinetics of the time-dependent sensitivity of these novel miRNA biomarkers remained unknown.

METHODS: To characterize temporal changes in the expressed human miRNAs (miRNome), we performed here the first whole-genome miRNA kinetic study in AMI patients. We measured miRNA expression levels at multiple time points (0, 2, 4, 12, 24 h after initial presentation) in patients with acute ST-elevation myocardial infarction by using microfluidic primer extension arrays and quantitative real-time PCR. As a prerequisite, all patients enrolled had to have cardiac troponin T concentrations <50 ng/L on admission as measured with a high-sensitivity assay.

RESULTS: We found a subset of miRNAs to be significantly dysregulated both at initial presentation and during the course of AMI. Additionally, we identified novel miRNAs that are dysregulated early during myocardial infarction, such as miR-1915 and miR-181c*.

CONCLUSIONS: The present proof-of-concept study provides novel insights into the dynamic changes of the human miRNome during AMI.

The early and sensitive diagnosis of acute myocardial infarction (AMI)[6] is crucial for risk stratification of chest pain patients and guidance of therapy. Of the many biomarkers proposed for AMI diagnosis, cardiac troponin T (cTnT) or cTnI are the preferred diagnostic markers for the detection of myocardial necrosis owing to their cardiospecificity and superior sensitivity *(1, 2)*. Recently, we and others have further increased the analytical sensitivity of the troponin assays, allowing an even earlier and more sensitive AMI diagnosis *(3, 4)*. As a consequence, risk stratification in acute coronary syndrome (ACS) patients has markedly improved by use of the high-sensitivity assays *(5, 6)*. Despite these major advancements, troponin-based AMI diagnosis still requires loss of myocardial cell integrity and leakage of cardiac constituents, which is a rather late event in the pathophysiological cascade of AMI. In the past there have been numerous attempts to detect biomarkers that reflect disease processes in ACS which precede coronary artery occlusion, such as plaque inflammation and rupture, activation of coagulation, or severe myocardial ischemia *(7–11)*. However, none of the biomarkers proposed provided the required preanalytical and analytical characteristics and hence all failed to translate into routine clinical practice.

MicroRNAs (miRNAs) are small noncoding nucleotides that influence the expression of a wide variety of target genes *(12, 13)*. They not only regulate physiological mechanisms, such as differentiation, proliferation, or apoptosis, but also enable the cell to adapt to pathophysiological conditions like ischemia, hypertrophy, or arrhythmias *(14–18)*. Hence, miRNAs might also serve as noninvasive biomarkers for cardiovascu-

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[6] Nonstandard abbreviations: AMI, acute myocardial infarction; cTnT, cardiac troponin T; ACS, acute coronary syndrome; miRNA, microRNA; miRNome, the expressed miRNAs in the genome; STEMI, ST-elevation myocardial infarction; LBBB, left bundle branch blockage; hs-cTnT, high-sensitivity cTnT; PCA, principal component analysis, AUC, area under the curve.

lar disorders *(19–21)*. miRNA signals derived either from whole blood or plasma/serum may reflect different disease pathways in ACS. Although serum miRNA changes in ACS are considered to result predominantly from cardiomyocyte necrosis, miRNA patterns in whole blood may also reflect pathophysiological changes in noncardiac cell types.

In a genomewide approach, we recently reported alterations of all of the expressed miRNAs (miRNome) in unfractionated peripheral blood of AMI patients *(20)*. A total of 121 miRNAs were found to be significantly dysregulated in AMI. However, these were single point measurements and blood samples, obtained at different times after the onset of symptoms. Therefore, we embarked on the current proof-of-concept study to investigate the kinetic changes of the whole blood miRNome in the early stage of suspected myocardial infarction at 5 predefined time points using microfluidic primer extension arrays and to replicate the results by using an independent methodology in an independent cohort of early-stage AMI patients *(22)*. We hypothesized that miRNA patterns may not only indicate myocardial cell necrosis, as reflected by cTnT, but also provide independent, early diagnostic information.

Materials and Methods

STUDY DESIGN

Sixty consecutive patients with suspected ST-elevation myocardial infarction (STEMI) with chest pain and ST-segment elevation of >0.2 mV in at least 2 leads of the 12-lead electrocardiogram or new left bundle branch blockage (LBBB) were recruited during the course of this study. From those 60 patients, 18 patients met our inclusion criterion of cTnT concentration <50 ng/L on admission as measured by a high sensitivity cTnT assay. Although the 99th percentile concentration is the cutoff value of high-sensitivity cTnT (hs-cTnT) assays in serial testing, an hs-cTnT >50 ng/L on admission is the discriminator for myocardial infarction in our institution if a single point determination is used. Of the 60 patients, 42 were excluded because their admission hs-cTnT concentrations exceeded 50 ng/L or a diagnosis of AMI was rejected on clinical grounds and invasive angiography, e.g., due to an underlying myocarditis or LBBB with thoracic pain of other cause. All patients with suspected STEMI underwent immediate heart catheterization and percutaneous coronary intervention of the culprit lesion. The 21 patients of the control group were investigated by routine coronary angiography for suspected coronary artery disease. For each control, a single blood sample was collected before the invasive procedure. Controls had neither significant coronary artery disease (<50% coronary artery stenosis) nor increased hs-cTnT concentrations. Patients and controls had given written informed consent and the study has been approved by the local ethics committee.

BLOOD SAMPLING AND hs-cTnT ASSAY

Venous blood samples were obtained at initial presentation to the hospital. For miRNA isolation, peripheral whole blood samples were collected in PAXgene tubes and RNA was extracted within 3 days as described previously *(23)*. RNA integrity was tested using the Bioanalyzer method. hs-cTnT concentrations were measured using the Elecsys high-sensitivity troponin T assay (Roche Diagnostics) *(4)*. All patients enrolled were required to have hs-cTnT concentrations <50 ng/L at baseline, and 6 patients had hs-cTnT concentrations <14 ng/L, which is the cutoff for fifth-generation troponin assays in serial testing.

miRNA EXPRESSION PROFILING

To quantify the complete miRNome in the "kinetics" cohort, we used microfluidics microarrays in combination with a primer extension assay [MPEA (microfluidics primer extension assay)] *(22)*. Biochips with 7 replicates of all known miRNAs (miRbase 15) *(24)* were hybridized with the unlabeled and nonamplified RNA using the Geniom RT analyzer (febit biotech). On top of each capture probe on the biochip, a polythymidin-tail has been synthesized. Klenow polymerase then binds to the hybridized miRNA/probe complex and extends the miRNA with biotinylated adenins. The signals were measured by a charge-coupled device camera and signals were analyzed with the Geniom Wizard software (febit). All signals were background corrected, and the median expression of the 7 capture probes was computed. Finally, quantile normalization *(25)* was applied, and for all further computations the normalized expression intensities were used.

For the replication experiments, we performed quantitative real-time PCR as described before (including DNase digestion) *(20)* for miRNA-1254, -380*, -455, -566, -636, -7-1*, and -1291 using miRNA primer assays obtained from Qiagen. The small RNA RNU-6b served as a reference, and data were analyzed according to the deltaCT method.

PRINCIPAL COMPONENT ANALYSIS

To visualize the high-dimensional distribution of miRNAs in a 2-dimensional subspace, we applied principal component analysis (PCA). In brief, an eigenvalue decomposition of the miRNA expression covariance matrix was carried out to compute the PCs. The first few PCs, which represent linear combinations of the original patients' profiles, carry the largest portion of the overall information. In our case we computed the first and second PC for each patient separately.

Then, for each time point the mean from all patients and controls and the SD of the PCs was plotted. This type of representation is well suited to indicate proximity of miRNA expression levels at the different time points, including those in control patients.

z SCORES OF BIOMARKER SIGNATURES

To define whether a patient has a positive or a negative miRNA profile for AMI, an easily interpreted statistical measure is essential. Here, classification technologies are a common approach in the basic research field, although they are not commonly used currently in clinical practice. To address this point we decided to use *z* scores. A *z* score indicates how many SDs a patient's biomarker value is above or below a population's mean. A single threshold then can be applied to determine whether patient results are positive or negative. To extend this concept to sets of miRNAs, we considered the median *z* score of a selected miRNA to represent a stable estimate of the overall degree of dysregulation. Hence, to compute a score for a specific patient *a*, we computed a mean *z* score Z_a comprising the median *z* scores of all signature miRNAs. This mean measurement accounts for the fact that we expect positive *z* scores for upregulated miRNAs and negative *z* scores for downregulated miRNAs for AMI patients. Thus, the overall score computes as:

$$Z_a = \frac{1}{p}\left[\sum_{i \in up}[Z_{a,i}] + \sum_{j \in down}[(-1)Z_{a,j}]\right]$$

Here, $Z_{a,i}$ corresponds to the *z* score of patient *a* for each upregulated miRNA*i*, and $Z_{a,j}$ corresponds to the *z* score for each patient *a* and each downregulated miRNA*j*. The overall number of miRNAs in the signature is provided by *p*. To make the estimation more robust against outliers, the single *z* scores have been cut at the 5% quantiles, i.e., all *z* scores were in the interval between −1.645 and 1.645.

Results

STUDY DESIGN AND PATIENT CHARACTERISTICS

In this proof-of-concept study, we analyzed both serial whole-genome miRNA measurements in a kinetics cohort and 1-point candidate miRNA measurements in a replication cohort consisting of patients with STEMI and controls without relevant coronary artery disease or evidence for ACS (Table 1; also see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol59/issue2). In the AMI group, more patients were current smokers (61% and 14%, respectively, $P = 0.002$). Other cardiovascular risk factors were equally distributed between the 2 groups.

Table 1. Patient characteristics.

Characteristics	AMI patients (n = 18)	Controls (n = 21)	*P*
Age, years	63 (17)	60 (13)	0.6
Male/female, n/n	15/3	12/9	0.07
SBP,[a] mmHg	126 (31)	127 (17)	0.8
DBP, mmHg	70 (15)	74 (11)	0.3
Creatinine, mg/dL	0.97 (0.3)	0.93 (0.2)	0.6
Urea, mmol/L	5.95 (2)	5.78 (2)	0.9
Hypertension, n, %	14 (78)	15 (71)	0.7
Current smoking, n, %	11 (61)	3 (14)	0.002
Hyperlipidemia, n, %	9 (50)	14 (67)	0.3
TG, mg/dL	152 (125)	176 (98)	0.6
HDL, mg/dL	37 (8)	45 (26)	0.5
LDL, mg/dL	101 (38)	96 (38)	0.8

[a] SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglycerides.

ALTERED EXPRESSION LEVELS OF SPECIFIC miRNAS ARE DETECTABLE IN THE EARLY COURSE OF AMI

To comprehensively analyze miRNA expression changes in patients and controls, we first applied whole-genome miRNA measurements for all time points (5 time points) in the kinetics cohort of 6 patients. Next, to assess the kinetic changes of expression patterns of miRNAs, we performed a PCA of the previously identified and most strongly dysregulated AMI miRNAs to visualize their distribution at the specific time points in a 2-dimensional space (Fig. 1). Interestingly, miRNA expression levels in AMI patients were most distant from the controls (denoted as "ctrl" in Fig. 1) on admission (0 h). During the evolution of myocardial infarction expression differences diminish, and 24 h after presentation miRNA levels in AMI patients are almost comparable to those in the control group.

A SIGNATURE CONSISTING OF 7 miRNAS AS AN EARLY MARKER OF AMI

We next evaluated if the most significantly dysregulated miRNAs may be indicative for early myocardial infarction. To this end, we first applied a filtering approach to the 40 most significantly dysregulated (according to *P* values) previously described miRNAs and excluded miRNAs that have low abundance. Thereby, we removed 16 of the 40 miRNAs (40%) with a maximal expression below the limit of 50 fluorescence intensity units. Of the remaining 24 markers, we compared the median fold changes between AMI patients and controls, irrespectively of the time point. We found that 17 of the 24 miRNAs (71%) differed in patients with and without AMI, whereas the remaining 7

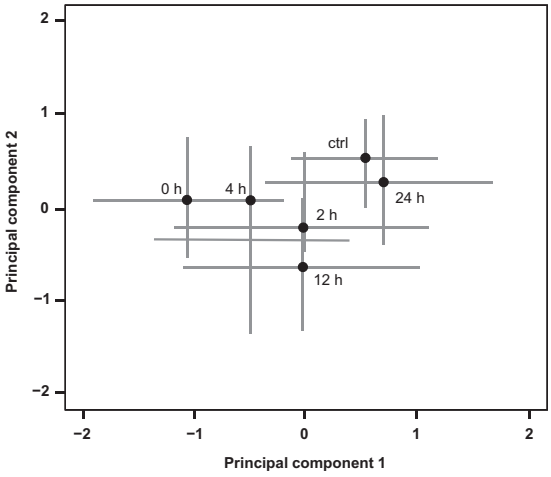

Fig. 1. Changes in miRNA expression in AMI patients are most evident in the early stage of myocardial infarction.

PCA visualizing miRNA expression at all measured time points and in comparison to a control group (ctrl).

miRNAs (29%) showed no significant concordance. These 17 miRNA markers, of which 2 were upegulated and 15 were downregulated in AMI patients, showed a high consistency between the different time points. In detail, 5 of the miRNA markers (29%) were significantly different in 2 of 5 time points, and a further 5 showed a discrepancy in 1 of 5 time points. Most remarkably, the remaining 7 markers were dysregulated in the same direction at all time points. These miRNAs include human miR-636, miR-7-1*, miR-380*, miR-1254, miR-455-3p, miR-566, and miR-1291 (Table 2 and Fig. 2A).

Although each of these 7 miRNAs may individually represent a promising biomarker for the early detection of AMI, the analysis of the entire signature may further increase the diagnostic power. To visualize the diagnostic power of such a signature, we computed *z* scores as described above for all patients at all time points. As shown in Fig. 2B, the mean *z* scores of AMI patients showed highest values at time points 1 and 2, corresponding to 0 h and 2 h after presentation to the hospital. For the last time point, the mean *z* scores of the 7 miRNAs returned to almost normal values, as also found by the PCA for all miRNAs (Fig. 1). Thus for AMI patients values at all time points except time point 5 were significantly different from those of controls. The respective 2-tailed *P* values were 0.004, 0.002, 0.013, 0.007, and 0.082, respectively.

Interestingly, the 2 earliest time points after admission were the most distinct. Although the sample size of the kinetics cohort was rather small and hence conclusions on the discriminatory power have to be made carefully, we computed ROC curves for the early AMI signature and found values for the area under the curve (AUC) for the first and second time points of 0.89 (CI, 0.74–1.00) and 0.92 (CI, 0.79–1.00), respectively (see online Supplemental Fig. 1).

IDENTIFICATION OF NOVEL miRNAS ASSOCIATED WITH EARLY-STAGE AMI

In addition to the validation and temporal exploration of previously identified miRNAs in AMI patients, the assessment of the whole miRNome of patients and controls represents a comprehensive source of information for the screening of novel diagnostic miRNAs. After ranking all miRNAs and time points according to *P* values, we additionally found miR-1915 (Fig. 3A) and miR-181c* (see online Supplemental Fig. 2) to be associated with early AMI. Although miR-1915 showed downregulation at all 5 time points, miR-181c* was significantly upregulated during AMI with the exception of the 4-h blood samples. Other miRNAs such as miR-339-3p (Fig. 3B) showed a slower response, with downregulation beginning at the 2-h time point.

Table 2. MiRNAs comprising an early biomarker signature for AMI.

miRNA	Nucleotide sequence	Replication *P* value, hs-cTnT <50 ng/L	Replication *P* value, hs-cTnT <14 ng/L
miR-636	UGUGCUUGCUCGUCCCGCCCGCA	0.0005	0.0143
miR-7-1*	CAACAAAUCACAGUCUGCCAUA	0.0108	0.0200
miR-380*	UGGUUGACCAUAGAACAUGCGC	0.0036	0.0052
miR-1254	AGCCUGGAAGCUGGAGCCUGCAGU	0.0023	0.0108
miR-455-3p	GCAGUCCAUGGGCAUAUACAC	0.0075	0.0539
miR-566	GGGCGCCUGUGAUCCCAAC	0.0006	0.0004
miR-1291	UGGCCCUGACUGAAGACCAGCAGU	0.3933	0.6961

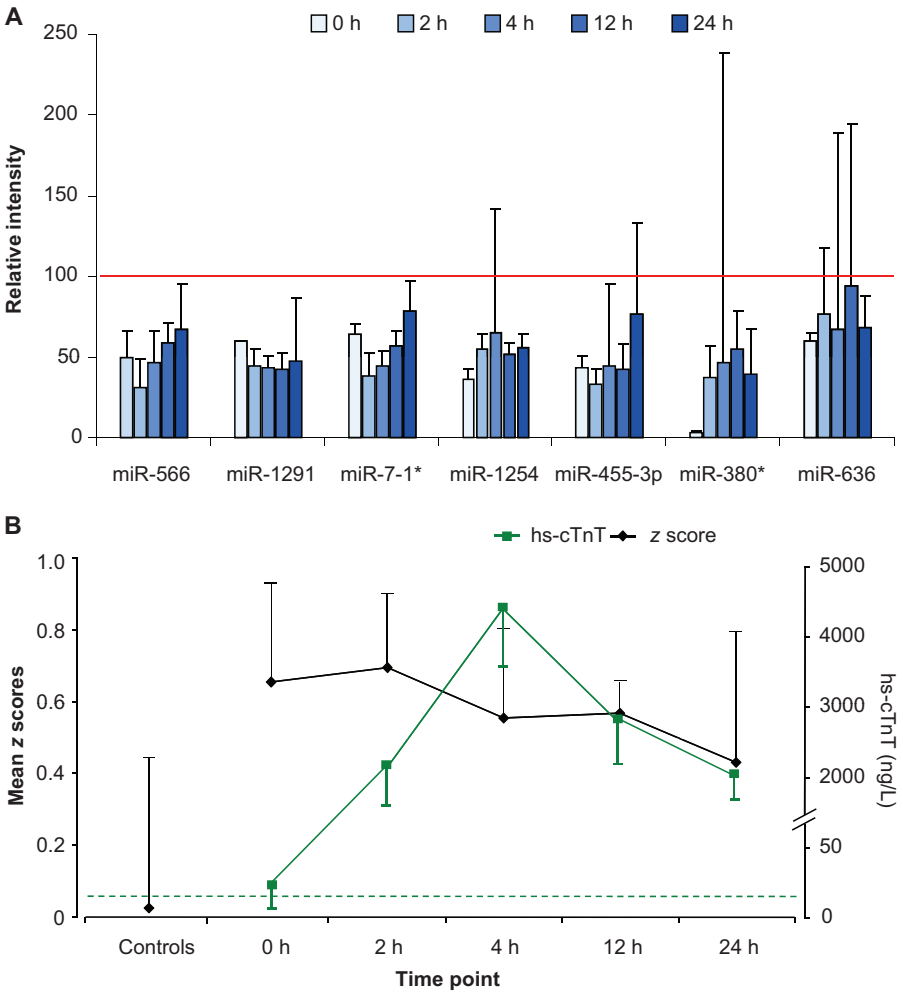

Fig. 2. Signature of 7 consistently dysregulated miRNAs is an early marker of AMI.

(A), Bar graphs showing miRNAs that are dysregulated at each time point studied. Bars show relative intensity values of miR-566, -1291, -7-1*, -1254, -455-3p, -380*, and -636 at different time points normalized to controls (red line). (B), The black line represents the mean *z* scores of the 7-miRNA signature for AMI patients at different time points, as well as for controls. The green line denotes the corresponding hs-cTnT concentrations in AMI patients. Dashed green line, 99th percentile of the hs-cTnT assay used.

INDEPENDENT REPLICATION OF miRNAS IN EARLY-STAGE AMI

In the next step, we applied quantitative real-time PCR measurements of the 7 AMI miRNAs in an independent cohort of 12 AMI patients and 10 controls. This biological and technical replication showed concordant dysregulation of miRNA-1254, -380*, -455, -566, -636, and -7-1*, all reaching statistical significance (Fig. 4A and Table 2). Only miR-1291 did not reach significance. Very interestingly, when we looked at the patient subgroup with hs-cTnT concentrations <14 ng/L (n = 4) we still found significant downregulation for miR-1254, -380*, -566, -636, and -7-1* ($P < 0.05$), and a tendency toward downregulation for miR-455, underlining the early nature of these miRNAs.

We next computed combined *z* scores of the 6 miRNA signature and found significantly higher *z* scores for AMI patients compared to the controls (Fig. 4B). Importantly, the miRNA signature reached higher AUC values in ROC analysis than each of the single replicated miRNAs alone (Fig. 4C; see also online Supplemental Fig. 3) consistent to the serial whole-genome measurements.

Discussion

We recently identified miRNAs as potential novel biomarkers for AMI *(20)* and demonstrated their power in discriminating AMI patients from control patients. However, these single point measurements did not

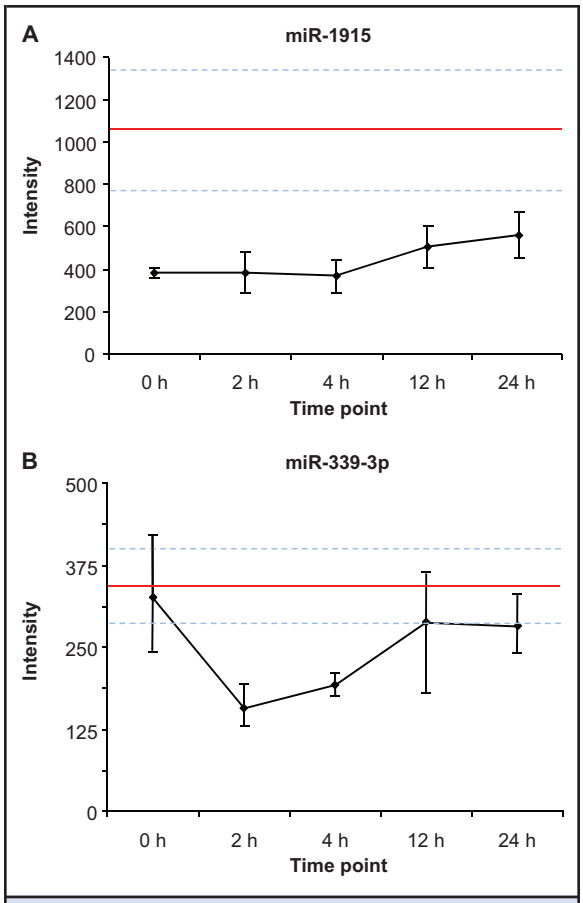

Fig. 3. MiR-1915 and miR-339–3p are novel miRNAs associated with myocardial infarction.

Mean intensity values in AMI patients are given for miR-1915 (A) and miR-339-3p (B) at the different time points studied. The red line indicates the mean intensity values in the control group; dashed lines indicate the corresponding SE.

provide data regarding the kinetic changes of the novel biomarkers or their value in early AMI diagnosis. Furthermore, until the current study, these findings had not been replicated in an independent cohort or by an independent methodology. To investigate the kinetics of miRNA dysregulation, we performed here the first serial whole-miRNome expression study of which we are aware in AMI patients. We were able to validate AMI miRNAs in an independent patient cohort and confirmed their discriminatory power in the early phase of myocardial infarction. Finally, a signature of 6 miRNAs was confirmed by a replicate analysis to be associated with AMI.

miRNAs have been shown to be associated with cancer and its outcome, neurological disorders, and cardiovascular diseases *(19)*. However, many studies on miRNA expression patterns in human disorders were restricted to measurements made at a single time point, neglecting the dynamic nature of most diseases and the kinetics of the respective biomarkers. Hence, the time-dependent changes of the human miRNome in many diseases are largely unknown. Since the precise description of the temporal changes of miRNA networks has marked implications when these tests are applied in routine clinical practice, in our current approach we investigated the kinetics of the entire miRNome in patients with AMI. We observed very distinct patterns of miRNAs in the very early phase of AMI that resolved within the first 2 days in patients who were treated by successful reperfusion therapy and reestablished coronary blood flow. Surprisingly, the most significant differences of 7 miRNAs were seen at the 2 earliest time points of the kinetic analysis. Notably, at time point 1, hs-cTnT concentrations were still <50 ng/L (cutoff for 4th-generation cTnT assays) in all patients and <14 ng/L (cutoff for the hs-cTnT assay) in 2 patients of the kinetics cohort. In the replication cohort, the hs-cTnT mean (SD) value was as low as 17.7 (6.8) ng/L, and in 4 patients (30%) the value was even below 14 ng/L. Hence, these miRNAs appear to be early indicators of AMI, and their expression may even change before macromolecules like troponins egress from injured cardiomyocytes; the latter possibility has to be confirmed in larger studies that also include patients with other conditions, e.g., unstable angina or non–ST-segment elevation myocardial infarction. To fully understand the complete behavior of the miRNAs described herein, it would be important to investigate their long-term levels following AMI to determine whether their observed dysregulation completely resolves. In addition to our confirmation of miRNAs observed to be dysregulated in previous AMI studies, in the current study we have identified reduced expression of miR-1915 and upregulation of miR-181c* to be present in the very early phase of myocardial infarction.

The biological functions of the miRNAs considered in the current study are still unknown. It is likely that the miRNAs comprising the early myocardial infarction signature are not heart muscle specific, but may be derived from various cell types, including inflammatory cells, activated thrombocytes, or damaged endothelium. To our knowledge, none of the peripheral whole blood miRNAs studied here have been previously implicated for a specific disease. Some have been described to be misexpressed in cancerous tissue *(26)*. For instance, miR-455-3p is associated with temozolomide resistance in glioblastoma multiforme *(27)*, and miR-1915 modulates Bcl-2–mediated drug resistance in human colorectal carcinoma cells *(28)*. Interestingly, miR-181c*, which we identified as a novel marker in this study, was very recently shown to

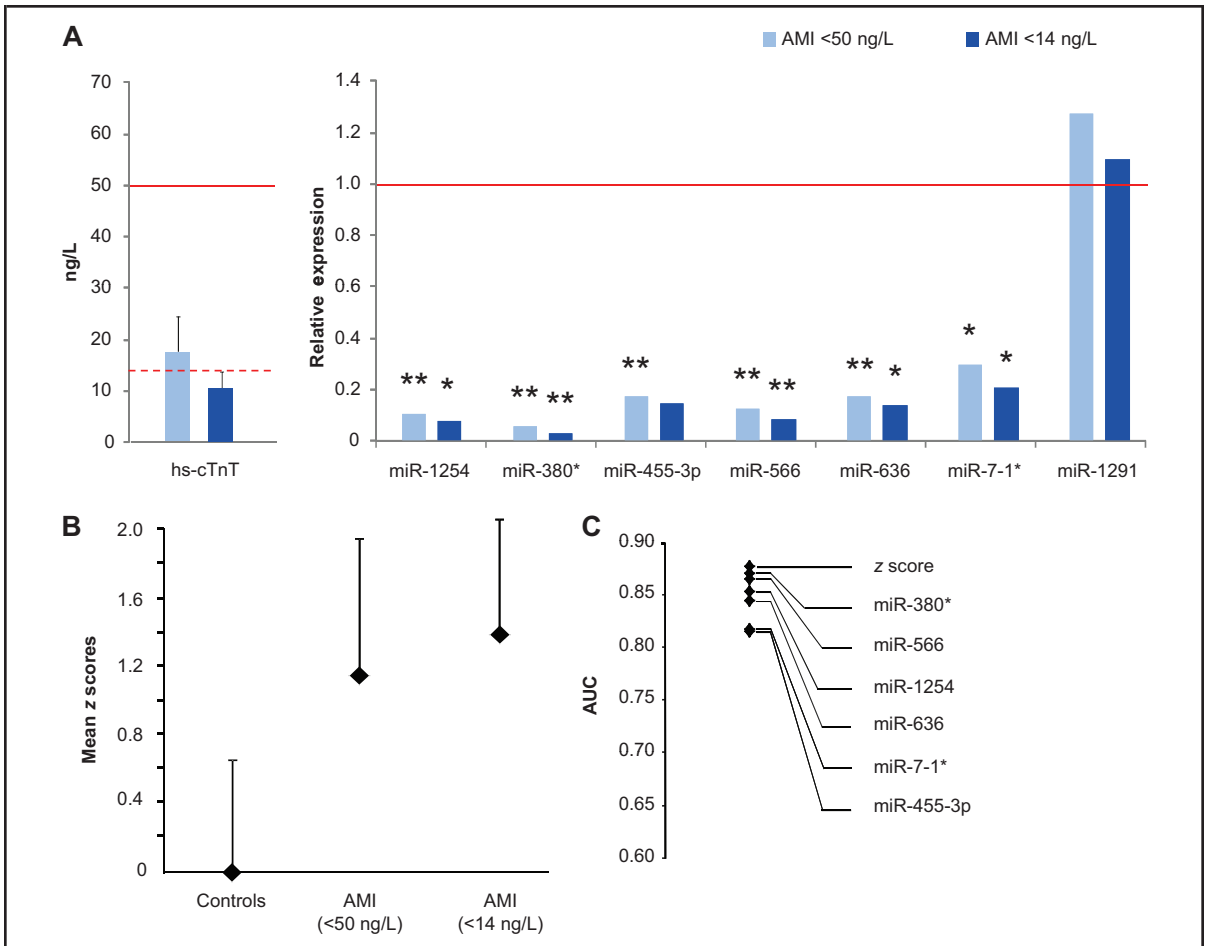

Fig. 4. Replication of early AMI miRNAs.

Independent biological and technical replication using real-time PCR for the 7 AMI miRNAs. (A), The left panel shows the hs-cTnT concentrations of all AMI patients (n = 12) of the replication cohort and the concentrations in the subgroup of n = 4 patients with hs-cTnT <14 ng/L (red line, inclusion criterion hs-cTnT <50 ng/L; dashed red line, 99th percentile of the used hs-cTnT assay). The right panel shows bar graphs depicting the relative expression values (normalized to the control group indicated by a red line) of the 7 miRNAs identified in the kinetics cohort (*$P < 0.05$; **$P < 0.01$). (B), Mean *z* scores and corresponding SDs of the signature comprising the 6 replicated miRNAs that discriminate AMI patients from controls. (C), AUC values for each of the successfully replicated miRNAs and for the combined *z* score.

be dysregulated in response to cerebral ischemia in mice *(29)*. These authors found an increase of miR-181 in the ischemic core area of the brain and decreased levels in the penumbra. In the future, detailed studies in cellular and animal model systems, including experimental AMI, will be needed to definitively determine their origin and functional role. However, alterations in the expression patterns of whole-blood miRNAs seem to be rather specific for myocardial infarction. Accordingly, in a large multicenter, multidisciplinary study we have recently shown that miRNA expression in patients with AMI can be easily discriminated from miRNA expression associated with various other diseases *(19)*.

So far, most miRNA studies have measured specific candidate miRNAs released from damaged myocardium into the serum of AMI patients. In a very recently published study, for example, Devaux et al. found miR-208b and miR-499 to be highly increased in serum samples of patients with AMI as determined by quantitative PCR *(30)*. In another study, Wang and coworkers showed that 4 muscle-enriched or cardiac-specific miRNAs (miR-1, miR-133a, miR-499, and miR208a) were upregulated in the plasma of AMI pa-

tients *(31)* and decreased within 2 months after myocardial infarction. However, the authors focused on a single measurement within the first 12 h after the onset of symptoms (4.8 ± 3.5 h), when patients already showed highly increased troponin concentrations. D'Alessandra et al. found similar kinetics of cardiac miRNA vs cardiac troponin release into circulation *(32)*. The authors assessed plasma levels of circulating miRNAs in a total of 41 AMI patients. In a subgroup of 8 patients, these investigators performed serial measurements of both circulating serum miRNAs and troponins from 156 min up to 69 h after the onset symptoms, showing similar kinetics for both. They found miR-1, miR-133a, and miR-133b to be upregulated in AMI patients, with a peak level shortly before the peak of troponin I, whereas miR-499-5p showed a slower time course.

Because the release of molecules from damaged myocardium into the blood may be similar for miRNAs and proteins, miRNA determination from serum or plasma may not provide novel information over that given by existing biomarkers of cardiomyocyte necrosis. However, we hypothesized that a whole-blood approach including circulating blood cells might provide added information *(33)* because it would reflect the disease processes involved in the pathogenesis of ACS, such as plaque rupture, inflammation, coagulation, or vascular injury *(34, 35)*, rather than solely detecting myocardial necrosis. Hence, it is conceivable that expression profiles of miRNAs may provide independent information and reflect specific disease mechanisms of ACSs in individual patients. If this hypothesis is confirmed in larger trials, preventive treatment options could eventually be selected that specifically address plaque instability or prothrombotic activation.

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References

1. Thygesen K, Alpert JS, Jaffe AS, Simoons ML, Chaitman BR, White HD. Third universal definition of myocardial infarction. J Am Coll Cardiol 2012;60:1581–98.
2. Jaffe AS, Ravkilde J, Roberts R, Naslund U, Apple FS, Galvani M, Katus H. It's time for a change to a troponin standard. Circulation 2000;102:1216–20.
3. Giannitsis E, Becker M, Kurz K, Hess G, Zdunek D, Katus HA. High-sensitivity cardiac troponin T for early prediction of evolving non-ST-segment elevation myocardial infarction in patients with suspected acute coronary syndrome and negative troponin results on admission. Clin Chem 2010; 56:642–50.
4. Giannitsis E, Kurz K, Hallermayer K, Jarausch J, Jaffe AS, Katus HA. Analytical validation of a high-sensitivity cardiac troponin T assay. Clin Chem 2010;56:254–61.
5. Keller T, Zeller T, Peetz D, Tzikas S, Roth A, Czyz E, et al. Sensitive troponin I assay in early diagnosis of acute myocardial infarction. N Engl J Med 2009;361:868–77.
6. Reichlin T, Hochholzer W, Bassetti S, Steuer S, Stelzig C, Hartwiger S, et al. Early diagnosis of myocardial infarction with sensitive cardiac troponin assays. N Engl J Med 2009;361:858–67.
7. Lin S, Yokoyama H, Rac VE, Brooks SC. Novel biomarkers in diagnosing cardiac ischemia in the emergency department: a systematic review. Resuscitation 2012;83:684–91.
8. Kehl DW, Iqbal N, Fard A, Kipper BA, De La Parra Landa A, Maisel AS. Biomarkers in acute myocardial injury. Transl Res 2012;159:252–64.
9. Kim JS, Hwang HJ, Ko YG, Choi D, Ha JW, Hong MK, Jang Y. Ischemia-modified albumin: is it a reliable diagnostic and prognostic marker for myocardial ischemia in real clinical practice? Cardiology 2010;116:123–9.
10. Liyan C, Jie Z, Yonghua W, Xiaozhou H. Assay of ischemia-modified albumin and C-reactive protein for early diagnosis of acute coronary syndromes. J Clin Lab Anal 2008;22:45–9.
11. Hjortshoj S, Kristensen SR, Ravkilde J. Diagnostic value of ischemia-modified albumin in patients with suspected acute coronary syndrome. Am J Emerg Med 2010;28:170–6.
12. Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 1993;75:843–54.
13. Lee CT, Risom T, Strauss WM. Evolutionary conservation of microRNA regulatory circuits: an examination of microRNA gene complexity and conserved microRNA-target interactions through metazoan phylogeny. DNA Cell Biol 2007;26: 209–18.
14. Cai B, Pan Z, Lu Y. The roles of microRNAs in heart diseases: a novel important regulator. Curr Med Chem 2010;17:407–11.
15. Dong S, Cheng Y, Yang J, Li J, Liu X, Wang X, et al. MicroRNA expression signature and the role of microRNA-21 in the early phase of acute myocardial infarction. J Biol Chem 2009;284:29514–25.
16. Yang B, Lin H, Xiao J, Lu Y, Luo X, Li B, et al. The muscle-specific microRNA miR-1 regulates cardiac arrhythmogenic potential by targeting GJA1 and KCNJ2. Nat Med 2007;13:486–91.
17. Terentyev D, Belevych AE, Terentyeva R, Martin

MM, Malana GE, Kuhn DE, et al. miR-1 overexpression enhances Ca(2+) release and promotes cardiac arrhythmogenesis by targeting PP2A regulatory subunit B56alpha and causing CaMKII-dependent hyperphosphorylation of RyR2. Circ Res 2009;104:514–21.
18. Meder B, Katus HA, Rottbauer W. Right into the heart of microRNA-133a. Genes Dev 2008;22:3227–31.
19. Keller A, Leidinger P, Bauer A, Elsharawy A, Haas J, Backes C, et al. Toward the blood-borne miRNome of human diseases. Nat Methods 2011;8:841–3.
20. Meder B, Keller A, Vogel B, Haas J, Sedaghat-Hamedani F, Kayvanpour E, et al. MicroRNA signatures in total peripheral blood as novel biomarkers for acute myocardial infarction. Basic Res Cardiol 2011;106:13–23.
21. Tijsen AJ, Creemers EE, Moerland PD, de Windt LJ, van der Wal AC, Kok WE, Pinto YM. MiR423-5p as a circulating biomarker for heart failure. Circ Res 2010;106:1035–9.
22. Vorwerk S, Ganter K, Cheng Y, Hoheisel J, Stahler PF, Beier M. Microfluidic-based enzymatic on-chip labeling of miRNAs. N Biotechnol 2008;25:142–9.
23. Keller A, Leidinger P, Borries A, Wendschlag A, Wucherpfennig F, Scheffler M, et al. miRNAs in lung cancer - studying complex fingerprints in patient's blood cells by microarray experiments. BMC Cancer 2009;9:353.
24. Griffiths-Jones S, Saini HK, van Dongen S, Enright AJ. Mirbase: tools for microRNA genomics. Nucleic Acids Res 2008;36:D154–8.
25. Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 2003;19:185–93.
26. Erdogan B, Facey C, Qualtieri J, Tedesco J, Rinker E, Isett RB, et al. Diagnostic microRNAs in myelodysplastic syndrome. Exp Hematol 2011;39:915–926.e2.
27. Ujifuku K, Mitsutake N, Takakura S, Matsuse M, Saenko V, Suzuki K, et al. miR-195, miR-455-3p and miR-10a(*) are implicated in acquired temozolomide resistance in glioblastoma multiforme cells. Cancer Lett 2010;296:241–8.
28. Xu K, Liang X, Cui D, Wu Y, Shi W, Liu J. miR-1915 inhibits Bcl-2 to modulate multidrug resistance by increasing drug-sensitivity in human colorectal carcinoma cells. Mol Carcinog 2013;52:70–8.
29. Ouyang YB, Lu Y, Yue S, Xu LJ, Xiong XX, White RE, et al. miR-181 regulates GRP78 and influences outcome from cerebral ischemia in vitro and in vivo. Neurobiol Dis 2012;45:555–63.
30. Devaux Y, Vausort M, Goretti E, Nazarov PV, Azuaje F, Gilson G, et al. Use of circulating microRNAs to diagnose acute myocardial infarction. Clin Chem 2012;58:559–67.
31. Wang GK, Zhu JQ, Zhang JT, Li Q, Li Y, He J, et al. Circulating microRNA: a novel potential biomarker for early diagnosis of acute myocardial infarction in humans. Eur Heart J 2010;31:659–66.
32. D'Alessandra Y, Devanna P, Limana F, Straino S, Di Carlo A, Brambilla PG, et al. Circulating microRNAs are new and sensitive biomarkers of myocardial infarction. Eur Heart J 2010;31:2765–73.
33. Hausler SF, Keller A, Chandran PA, Ziegler K, Zipp K, Heuer S, et al. Whole blood-derived miRNA profiles as potential new tools for ovarian cancer screening. Br J Cancer 2010;103:693–700.
34. Hoekstra M, van der Lans CA, Halvorsen B, Gullestad L, Kuiper J, Aukrust P, et al. The peripheral blood mononuclear cell microRNA signature of coronary artery disease. Biochem Biophys Res Commun 2010;394:792–7.
35. Voellenkle C, van Rooij J, Cappuzzello C, Greco S, Arcelli D, Di Vito L, et al. MicroRNA signatures in peripheral blood mononuclear cells of chronic heart failure patients. Physiol Genomics 2010;42:420–6.