Use of Circulating MicroRNAs to Diagnose Acute Myocardial Infarction

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BACKGROUND: Rapid and correct diagnosis of acute myocardial infarction (MI) has an important impact on patient treatment and prognosis. We compared the diagnostic performance of high-sensitivity cardiac troponin T (hs-cTnT) and cardiac enriched microRNAs (miRNAs) in patients with MI.

METHODS: Circulating concentrations of cardiac-enriched miR-208b and miR-499 were measured by quantitative PCR in a case-control study of 510 MI patients referred for primary mechanical reperfusion and 87 healthy controls.

RESULTS: miRNA-208b and miR-499 were highly increased in MI patients (>10^5-fold, P < 0.001) and nearly undetectable in healthy controls. Patients with ST-elevation MI (n = 397) had higher miRNA concentrations than patients with non–ST-elevation MI (n = 113) (P < 0.001). Both miRNAs correlated with peak concentrations of creatine kinase and cTnT (P < 10^-8). miRNAs and hs-cTnT were already detectable in the plasma 1 h after onset of chest pain. In patients who presented <3 h after onset of pain, miR-499 was positive in 93% of patients and hs-cTnT in 88% of patients (P = 0.78). Overall, miR-499 and hs-cTnT provided comparable diagnostic value with areas under the ROC curves of 0.97. The reclassification index of miR-499 to a clinical model including several risk factors and hs-cTnT was not significant (P = 0.15).

CONCLUSIONS: Circulating miRNAs are powerful markers of acute MI. Their usefulness in the establishment of a rapid and accurate diagnosis of acute MI remains to be determined in unselected populations of patients with acute chest pain. A rapid diagnosis of acute myocardial infarction (MI) is critical for appropriate management of patients with chest pain. Clinical presentation and echocardiography findings are often nonspecific in patients with chest pain, and the measurement of cardiac markers is required. The diagnostic performance of cardiac markers has recently been improved by the introduction of high-sensitivity assays for troponins (1, 2). The assay for high-sensitivity cardiac troponin T (hs-cTnT) is positive early after ischemia, but serial testing is required because hs-cTnT is also largely positive in patients with chronic stable coronary artery disease and can be positive in apparently healthy controls (3). Therefore, the ideal biomarker for rapid and reliable diagnosis of acute MI is still lacking.

MicroRNAs (miRNAs) are small, single-stranded RNA molecules that regulate gene expression mostly by messenger RNA destabilization (4). Since their discovery in Caenorhabditis elegans in 1993, miRNAs have been described in many species and almost 1000 human miRNAs have been identified so far (5). Each miRNA can regulate hundreds of messenger RNA targets and this underscores the regulatory role of miRNAs in all biological processes, including cancer, metabolism, stem cell regulation, and immune function. Because miRNAs have been shown to regulate genes in numerous oncogenic pathways, the expression of miRNAs in cancerous tissue has been used to characterize patients with various types of malignancies (6). Moreover, it was recently shown that miRNAs circulating in the blood of cancer patients can also be useful for diagnosis and prognosis (7).

A recent report indicates that cardiac-enriched miR-208b and miR-499 are released in the peripheral circulation of rats as early as 1 h after occlusion of the artery (8). The same study showed that cardiac-enriched miRNAs are increased in patients with acute...
MI within 4 h of symptom onset. The findings in humans with acute MI have been confirmed by several other reports, including one from our group (8–17). These studies, although small and preliminary, have indicated that miRNAs hold potential as new cardiac biomarkers (18). Therefore, the aim of the present study was to compare the diagnostic performances of hs-cTnT and miRNAs in a large group of patients with acute MI. We focused our investigations on miR-208b and miR-499 because these 2 miRNAs are very robustly increased in the circulation after acute MI (17).

Materials and Methods

STUDY POPULATION
A total of 510 consecutive patients with an MI referred to the Luxembourg Heart Institute for emergent percutaneous coronary intervention (PCI) with acute and ongoing chest pain for <12 h and clinically significant ST-T changes were included in the study.

Acute ST-segment–elevation MI (STEMI) was defined by the following: (a) clinically significant ST elevation (>1 mm); (b) completely occluded major coronary artery: TIMI (thrombolysis in MI), 0 flow in the left anterior descending, circumflex, or right coronary artery; (c) peak creatine kinase (CK) activity >600 U/L (3 times higher than the upper limit of the reference interval).

Non-STEMI (NSTEMI) was defined by the following: (a) no significant ST-elevation but clinically significant ST depression (>1 mm); (b) clinically significant lesion in a major coronary artery requiring PCI; (c) positive troponin concentrations after 24 h (>0.03 μg/L). Most patients with NSTEMI presented with a severe or subocclusive lesion in the left anterior descending, circumflex, or right coronary artery. Patients with a completely occluded artery were excluded from this group.

Blood samples were obtained at presentation and twice daily thereafter for determination of peak values of cardiac markers. Plasma samples were extracted from citrated tubes and stored at −80 °C. Patients had echocardiography at 4 months. The protocol was approved by the local ethics committee and all study participants signed an informed consent form. Controls were 87 healthy male volunteers recruited from a national observational study of cardiovascular risks. Blood samples used to measure cardiac markers were collected during a medical visit at the hospital. Clinical characteristics are shown in Table 1.

PLASMA miRNA DETERMINATION
We extracted total RNA from plasma samples using the mirVana PARIS kit (Ambion, Applied Biosystem) without enrichment for small RNAs. A mix of 3 supplemented synthetic C. elegans miRNAs (Qiagen), which lacked sequence homology to human miRNAs, was added to plasma samples for correction of extraction efficiency. Potential genomic DNA contamination was eliminated by use of DNase (Qiagen). Reverse transcription of RNA was performed with the miScript reverse transcription kit (Qiagen). The resulting cDNA was diluted 10-fold before quantitative PCR performed by using the miScript SYBR-green PCR kit (Qiagen). miRNA-specific miScript primer sets were obtained from Qiagen. Expression values were normalized by using the mean threshold cycle (Ct) obtained from the spiked-in controls [calculation formula: 2exp(mean Ct spiked-in controls − Ct target miRNA)] and log transformed. The detection limit of the PCR assay was −7.2, which was the log transformation of the minimum expression detected divided by 10. An expression value of −7.2 therefore indicated that the miRNA expression level was below the detection threshold of the assay.

The TaqMan® MicroRNA Assay (Applied Biosystems) was used in a subcohort of patients. This quantitative miRNA stem loop reverse-transcription PCR technology uses gene-specific stem loop reverse-transcription primers and TaqMan® probes to detect mature miRNA transcripts. The TaqMan Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems) was used for PCR. A pool of RNA was used as interrun calibrator, and expression values were calculated with the δ threshold method (Bio–Rad CFX Manager, Bio–Rad).

Information regarding technical considerations about mRNA determination by PCR can be found in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol58/issue3.

BIOCHEMICAL ANALYSES
We measured hs-cTnT by using the Roche highsensitivity assay performed on the Cobas e601 system with a detection limit of 0.003 μg/L, a 99th-percentile cutoff point of 0.014 μg/L, and a CV of <10% at 0.013 μg/L. hs-cTnT concentrations below 0.003 μg/L were given a value of −3.5 (log 0.003/10). The cTnT assay used to establish the diagnosis of MI was a fourth-generation assay from Roche that was also performed on Cobas e601 equipment, with 0.01 μg/L being the lower detection limit and 0.03 μg/L being the troponin T concentration reproducibly measured with a CV below 10%. CK activity was measured with a Roche IFCC-recommended method on a Cobas c501 instrument.

STATISTICAL ANALYSES
Before analysis, all data were subjected to a normality test (Shapiro-Wilk). Mann–Whitney and t-tests were used to compare 2 groups of continuous variables. A χ²
test was used for categorical variables. ROC curves and their corresponding areas under the curve (AUCs) were used for evaluation of diagnostic accuracy. AUCs were compared by the method of DeLong.

Reclassification analyses were performed to test the additive value of miR-499 to a multiparameter clinical model or hs-cTnT. If expression of either marker was higher than its corresponding threshold, the sample was assigned to “test positive” class. If expression was lower than the threshold, the sample was assigned to “test negative” class. For each classification, a confusion table was calculated and used for benchmarking the performances of biomarkers. The net reclassification index was used to validate the classification performance changes for biomarkers with respect to classical hs-cTnT. Statistical significance was evaluated as described elsewhere.

Results

INCREASED CIRCULATING CONCENTRATIONS OF miR-208B AND miR-499 AFTER MI

Concentrations of miR-208b and miR-499 were measured in the plasma of 87 healthy controls and 113 NSTEMI and 397 STEMI patients (Fig. 1). In controls, 5% had detectable concentrations of miR-208b and 14% had detectable concentrations of miR-499. How-

<table>
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<th>Table 1. Clinical characteristics of the study population.</th>
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*All myocardial infarction patients had successful mechanical reperfusion and stenting of the infarct artery within 12 h of chest pain onset. All patients with a large thrombus burden received aspirin, clopidogrel, heparin, and abciximab.
ever, miRNA expression in these controls was weak and very close to the limit of detection of the assay. Concentrations of miR-208b were detectable in 80% of MI patients and concentrations of miR-499 were detectable in 96%. In 3% of the patients concentrations of both miR-208b and miR-499 were undetectable. The mean miR-208b expression was $5 \times 10^3$-fold higher in MI patients than in controls and miR-499 was $3 \times 10^5$-fold higher than in controls (both $P < 0.001$). miR-208b and miR-499 had 8-fold and 2-fold higher expression in STEMI than in NSTEMI patients, respectively ($P < 0.001$). There was a clear discrimination between MI patients and controls (Fig. 1).

From these data, we determined the cutoff values of miRNAs corresponding to the 99th percentiles. Cutoff values of $-5.1$ and $-4.8$ were obtained for miR-208b and miR-499, respectively (Fig. 1).

miRNA concentrations correlate with concentrations of traditional cardiac markers

We next determined whether plasma miRNA concentrations measured at presentation in MI patients correlated with infarct size as determined by peak concentrations of CK and cTnT. We must acknowledge that CK may not be an optimal marker of infarct size after primary PCI. By multiple linear regression miR-208b and miR-499 were associated with peak concentrations of CK and cTnT (approximately 24 h). Correlations between miRNAs and peak concentrations of CK and cTnT were highly significant ($P < 10^{-9}$; Table 2). In addition, we observed significant correlations between miRNAs and initial concentration of hs-cTnT ($r > 0.4$, $P < 10^{-20}$; Table 2). These correlations were independent of the duration of chest pain (see online Supplemental Fig. 1).
MicroRNAs in Myocardial Infarction

miRNAs ARE DETECTABLE AS EARLY AS 1 h AFTER THE ONSET OF CHEST PAIN

Overall, all 3 markers of myocardial injury, hs-cTnT, miR-208b, and miR-499 were detectable as soon as 1 h after the onset of chest pain. However, this was not the case for all patients. After 1 h of chest pain, 91% of patients had detectable miR-499 and a concentration of hs-cTnT above the 0.014 µg/L cutoff (corresponding to the 99th percentile as recommended by the manufacturer of the hs-cTnT assay and by other investigators (2)). There were only 11 patients who presented that early after onset of chest pain.

Percentages of patients positive for the 3 markers (i.e., greater than the cutoff points) were determined according to the delay between the onset of chest pain and blood sampling (see online Supplemental Fig. 2). Overall, fewer patients were positive for miR-208b or cTnT than for miR-499 or hs-cTnT. We therefore focused our analyses on the comparison between miR-499 and hs-cTnT. In patients who presented early [<3 h after onset of pain (n = 187)], miR-499 was positive in 93% of patients and hs-cTnT in 88% of patients (P = 0.78). In patients who presented early and who turned out to have substantial infarcts (CK >1000) (n = 113), 96% of patients were positive for miR-499 and 94% were positive for hs-cTnT (P = 0.99). In patients who presented >3 h after onset of pain (n = 323), the percentage of patients positive for miR-499 and hs-cTnT was also above 90%, independent of the presence of ST-elevation (see online Supplemental Data Fig. 2).

DIAGNOSTIC ACCURACY OF miRNAs AND hs-cTnT

We compared the diagnostic accuracy of miRNAs and hs-cTnT. Overall, miR-208b provided a lower diagnostic accuracy than miR-499 and hs-cTnT (Fig. 2). miR-499 and hs-cTnT were both able to accurately discriminate MI from controls (AUC 0.97) with comparable diagnostic sensitivity and specificity (see online Supplemental Data Table 1). Combined determination of miR-499 and hs-cTnT did not significantly improve the diagnostic value of single markers.

RECLASSIFICATION PERFORMANCE OF miR-499

We evaluated the capacity of miR-499 to reclassify patients misclassified by a model that included several risk factors—age, sex, hypertension, hypercholesterolemia, smoking habit, and hs-cTnT (Table 3). The addition of miR-499 to the clinical model did not significantly improve the diagnosis.

We next evaluated the ability of miR-499 to reclassify patients misclassified by a single measurement of hs-cTnT (see online Supplemental Table 2). Adding miR-499 to hs-cTnT did not result in an improvement of diagnosis.

miRNAs ARE MODERATE PREDICTORS OF LEFT VENTRICULAR DYSFUNCTION

In our cohort of MI patients, the ejection fraction measured by echocardiography at follow-up was available in 362 subjects (278 patients with STEMI and 84 patients without STEMI). miR-208b and miR-499 were inversely correlated to the ejection fraction, with correlation coefficients of −0.18 (P = 0.0008) and −0.17 (P = 0.001), respectively (see online Supplemental Fig. 3). The correlation was not significant in NSTEMI patients. A linear regression model attested that miR-208b was the best predictor of the ejection fraction (P < 0.001). Analysis of ROC curves revealed AUCs of 0.69 for miR-208b and 0.64 for miR-499 (P = 0.02) for prediction of left ventricular dysfunction defined as ejection fraction <40% (see online Supplemental Data Fig. 3). These data show that miR-208b and miR-499 are modest prognostic biomarkers of left ventricular dysfunction after MI.

Discussion

Our results show that circulating miRNAs are increased in patients with acute MI and barely detectable in healthy controls. miRNAs are present in the plasma as early as 1 h after the onset of chest pain and their diagnostic accuracy is robust.

Table 2. Correlation between miRNAs and cardiac markers in MI patients.*

<table>
<thead>
<tr>
<th></th>
<th>CK</th>
<th>cTnT</th>
<th>hs-cTnT</th>
<th>miR-208b</th>
<th>miR-499</th>
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<tr>
<td>CK</td>
<td>1</td>
<td>0.78 (&lt;1 × 10⁻²⁰)</td>
<td>0.25 (1 × 10⁻⁷)</td>
<td>0.37 (1 × 10⁻¹⁷)</td>
<td>0.31 (1 × 10⁻¹²)</td>
</tr>
<tr>
<td>cTnT</td>
<td>1</td>
<td>0.36 (3 × 10⁻¹⁵)</td>
<td>0.36 (7 × 10⁻¹⁶)</td>
<td>0.29 (4 × 10⁻¹⁶)</td>
<td></td>
</tr>
<tr>
<td>hs-cTnT</td>
<td>1</td>
<td>0.49 (&lt;1 × 10⁻²⁶)</td>
<td>0.41 (&lt;1 × 10⁻²⁶)</td>
<td>0.41 (&lt;1 × 10⁻²⁶)</td>
<td></td>
</tr>
<tr>
<td>miR-208b</td>
<td>1</td>
<td>0.88 (&lt;1 × 10⁻²⁶)</td>
<td>0.88 (&lt;1 × 10⁻²⁶)</td>
<td>0.88 (&lt;1 × 10⁻²⁶)</td>
<td></td>
</tr>
<tr>
<td>miR-499</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
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</table>

*Correlation coefficients with P values are indicated. CK: creatine kinase. Peak values of CK and cTnT, and miRNAs and hs-cTnT values at presentation, are considered in these analyses.
In the present study, cardiac-enriched miRNAs, particularly miR-499, provided an accurate diagnosis of MI, comparable to hs-cTnT. These observations may become clinically important. Indeed, in the absence of ST elevation, the diagnosis of MI requires prolonged monitoring because serial testing is usually necessary to establish the diagnosis of myocardial injury with both standard and high-sensitivity assays. This is attributable to the fact that the troponin assays are either not sensitive or not specific enough for the diagnosis of cardiac damage. A single measurement of circulating miRNAs may overcome this limitation and shorten the triage of patients with chest pain.

In patients presenting within 3 h of chest pain onset, there was positive expression of miR-499 in 93% of patients at presentation and positive expression of hs-cTnT in 88% of patients. Of note, all these patients had acute MI documented by typical serial electrocardio-

Fig. 2. ROC curves, which take the time of chest pain onset into account, of miRNAs and hs-cTnT.

miRNAs and hs-cTnT concentrations for 87 controls and 510 MI patients determined at presentation (113 NSTEMI and 397 STEMI). Left panels, ability of markers to discriminate MI from controls. Right panels, ability of markers to discriminate NSTEMI from controls. AUCs and P values are indicated.
gram changes and clinically significant release of cardiac markers, and all underwent mechanical reperfusion. In studies by Reichlin et al. (1) and Keller et al. (2), high-sensitivity troponin assays performed within 3 h of chest pain onset provided maximal diagnostic accuracies, with AUCs between 0.92 and 0.95, and AUCs remained stable for patients presenting later. Our data confirm that the diagnosis of MI with hs-cTnT is very accurate within the 3 h following onset of chest pain and extend this observation to miR-499. In addition, we found that miRNAs are detectable in human plasma as soon as 1 h after chest pain onset, supporting the evidence that miRNAs can be very early biomarkers of MI. However, this observation was limited by the low number of patients who presented that early after chest pain onset.

In our cohort of healthy volunteers, expression of miR-499 was hardly detectable, very close to the detection limit of the assay. In the same cohort, increased concentrations of hs-cTnT (above the 0.014 µg/L cutoff point) were observed in 2%, which is lower than has been reported for patients with stable coronary artery disease (11%) (3). The difference may be explained by the fact that our volunteers had no evidence of left ventricular hypertrophy or reduced renal clearance, which are associated with increases of troponins.

In the present study, miR-499 appeared clearly superior to miR-208b for detecting cardiac damage. Both miRNAs are expressed by myosin heavy chains in cardiac or skeletal muscle (21, 22). The importance of miR-499 in the heart is supported by the observation that miR-499 belongs to a group of 28 miRNAs that are upregulated in the failing heart and normalized after implantation of a left ventricular assist device (23). Although peripheral concentrations of miR-208b are increased in patients with coronary artery disease, this is not the case for miR-499 (22). miR-499 and miR-208b, which share many targets, are associated with cardiac differentiation (24, 25). Therefore, several lines of evidence indicate that miRNAs are not simple bystanders of cardiac damage.

The observation that miRNAs correlate with the ejection fraction at follow-up indicates that miRNAs may also provide information about prognosis. This is consistent with the findings that miRNAs are functionally implicated in the course of cardiac remodeling and heart failure (26). However, in our study, miR-208b and miR-499 provided only a modest prognosis of left ventricular dysfunction, with AUCs below 0.70. These findings do not exclude the possibility that other miRNAs can be good prognostic biomarkers of heart failure, as recently reported for miR-423–5p (27). Interestingly, miRNAs were correlated only with the ejection fraction in STEMI patients.

The current study had some limitations. First, the study cohort may not adequately reflect the population of patients presenting to the emergency department for acute chest pain. All patients had clinically significant electrocardiogram changes and significant myocardial injury, which can explain the increased AUC of hs-cTnT (0.97). However, a similar AUC (0.96) was reported by Reichlin et al. (2). Our study also did not include patients with small MI, unstable angina pectoris, heart failure, arrhythmias, or chronic cardiac disorders. Second, the group of patients with NSTEMI was

### Table 3. Reclassification performance of miR-499 over a multiparameter clinical model.a

<table>
<thead>
<tr>
<th>Class</th>
<th>Clinical model</th>
<th>Clinical model + miR-499</th>
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<tbody>
<tr>
<td></td>
<td>Test negative</td>
<td>Test positive</td>
</tr>
<tr>
<td>Whole cohort</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n = 87)</td>
<td>85b 2c</td>
<td>97.7</td>
</tr>
<tr>
<td>Case (n = 510)</td>
<td>8 502b</td>
<td>98.4</td>
</tr>
<tr>
<td>STEMI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n = 87)</td>
<td>84b 3c</td>
<td>96.6</td>
</tr>
<tr>
<td>Case (n = 397)</td>
<td>5c 392b</td>
<td>98.7</td>
</tr>
<tr>
<td>NSTEMI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n = 87)</td>
<td>85b 2c</td>
<td>97.7</td>
</tr>
<tr>
<td>Case (n = 113)</td>
<td>5c 108b</td>
<td>95.6</td>
</tr>
</tbody>
</table>

a Parameters included in the clinical model were: age, sex, hypertension, hypercholesterolemia, smoking habit, and hs-cTnT. Net reclassification indexes (NRI) (95% CI) and P values are indicated.

b No. of correct classifications.

c No. of false classifications.
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rather small. This was attributable to the fact that emergent PCI (at <12 h of chest pain) is mainly performed in patients with ST-elevation. This may change in the near future if the use of early biomarkers such as hs-cTnT and miRNAs makes earlier diagnosis possible in patients without ST-elevation. Third, the control group consisted of volunteers who were relatively young, healthy males (median age 53 years), whereas 25% of MI patients were females. However, circulating concentrations of miRNAs were not influenced by age and sex in this cohort, nor in our previous study cohort (17). Fourth, kidney function plays a role in the clearance of many biomarkers. However, we have observed that kidney function does not affect circulating miRNAs, at least not for patients who do not require hemodialysis, and we have shown that urine concentrations of miRNAs are very low (17). In our cohort, only 3 patients were on chronic hemodialysis. Whether miRNAs are chronically increased in this patient population to the same extent as cTnT remains to be determined. Fifth, although the concentration of hs-cTnT can be measured in 9 min, determination of circulating miRNAs by PCR requires at least 2 h. Nevertheless, it can be expected that substantial progress will be made in this area, and current research is focusing on rapid detection of miRNAs.

References


Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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Honoraria: None declared.

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