Transcoronary Concentration Gradients of Circulating MicroRNAs

Salvatore De Rosa, MD, PhD*; Stephan Fichtlscherer, MD*; Ralf Lehmann, MD; Birgit Assmus, MD; Stefanie Dimmeler, PhD†; Andreas M. Zeiher, MD†

Background—Circulating levels of microRNA (miR) have been proposed as biomarkers for cardiovascular disease. To identify the heart as a potential source for miRs released into the circulation, we measured concentration gradients across the coronary circulation for muscle-enriched (miR-133a, miR-499, miR-208a), vascular (miR-126, miR-92a), leukocyte-related (miR-155), and platelet-enriched (miR-223) miRs.

Methods and Results—Circulating miRs were measured by TaqMan polymerase chain reaction in EDTA-plasma simultaneously obtained from the aorta and the coronary venous sinus in patients without coronary artery disease (n/H11005 7), with stable coronary artery disease (n/H11005 31), and with troponin-positive acute coronary syndromes (n/H11005 19). Circulating levels of the muscle-enriched miR-499 (>20-fold; P<0.01), miR-133a (11-fold; P<0.01), and miR-208a (5-fold; P<0.01) were significantly elevated in the aorta of troponin-positive acute coronary syndrome patients compared with patients with coronary artery disease. Importantly, there was a significant increase in circulating levels of miR-499 and miR-133a across the coronary circulation in troponin-positive acute coronary syndrome patients, suggestive of a release into the coronary circulation during myocardial injury. Indeed, miR-499 concentration gradients were significantly correlated with the extent of myocardial damage as measured by high-sensitivity troponin T (r=0.70, P<0.01). In contrast, circulating levels of miR-126 (P=0.16) decreased during transcoronary passage in patients with evidence of myocardial injury, suggesting consumption during transcoronary passage.

Conclusions—Muscle-enriched miR-499 and miR-133a are released from the heart into the coronary circulation on myocardial injury, whereas the vascular miR-126 is consumed during transcoronary passage. The differential regulation of circulating miRs during the transcoronary passage might provide important insights to exploit their role as cardiac biomarkers.

Clinical Trial Registration—URL: http://www.germanctr.de. Unique identifier: DRKS00000207; in German Clinical Trials Registry. (Circulation. 2011;124:00-00.)

Key Words: acute coronary syndrome ■ coronary circulation ■ coronary artery disease ■ microRNA

Micro RNAs (miRs) are short, noncoding RNAs that mediate posttranscriptional gene regulation by binding to and repressing specific mRNA targets. MiRs are generally considered to act as intracellular mediators involved in many pathophysiological processes, including cardiovascular diseases.1,2 Recent studies have demonstrated that miRs are present in the human circulation in a cell-free form, can be detected in circulating blood, and thus may serve as a new class of blood-based biomarkers.3

Editorial see p ●●●

Clinical Perspective on p ●●●

Numerous studies reported altered plasma and serum levels of various miRs in patients with cardiovascular diseases, including acute myocardial infarction,4,5 myocarditis,6 acute and chronic heart failure,6,8 stable coronary artery disease (CAD),6,8 and type 2 diabetes mellitus.6,9 Importantly, because specific miRs are differentially enriched in the various cell types of the heart, it was suggested that altered circulating levels of selected miRs might reflect different cardiovascular pathologies, and thus could be exploited as biomarkers for cardiovascular disease.11 However, except for miR-208a, which is expressed exclusively in cardiac myocytes, the other muscle-enriched miRs that were proposed as potential biomarkers for acute myocardial infarction, eg, miR-1, miR-133a/b, and miR-499, are not expressed exclusively in cardiac myocytes, but also are detected in skeletal muscle cells.1 Likewise, alterations in levels of the highly endothelial cell

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enriched miR-126 might not reflect alterations in the coronary circulation but rather relate to systemic disturbances in endothelial cell function in patients with CAD and acute coronary syndromes (ACS).12,13 Therefore, we simultaneously obtained blood from the aorta and the coronary sinus for measurement of various circulating miRs to distinguish cardiac-derived and coronary circulation–associated miRs from peripheral systemic alterations in patients with CAD with or without myocardial injury.

Methods

Study Sample
A total of 57 patients undergoing coronary angiography in the catheterization laboratory of the Goethe University of Frankfurt were

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* Indicates coronary artery disease; ACS, acute coronary syndrome; SBP, systolic blood pressure; DBP, diastolic blood pressure; LVEF, left ventricular ejection fraction; hsTNT, high-sensitivity troponin T; CVS, coronary venous sinus; CRP, C-reactive protein; NT-proBNP, N-terminal pro-B-type natriuretic peptide; LDL, low-density lipoprotein; HDL, high-density lipoprotein; PAOD, peripheral arterial occlusive disease; TIMI, Thrombolysis in Myocardial Infarction; AMI, acute myocardial infarction; ASA, acetylsalicylic acid; AT-RB, angiotensin receptor blocker; ACE, angiotensin-converting enzyme; and OAC, oral anticoagulants. All P values represent comparisons between CAD and ACS. Comparisons between groups were performed with ANOVA followed by Bonferroni correction for continuous variables and with the Fisher exact test or χ² test for categorical variables.

*No Bonferroni correction because only 2 groups (CAD and ACS) were available.

†P<0.001 as significance level after Bonferroni correction for multiple testing.
enrolled between October 2009 and September 2010. Patients were classified into groups according to evidence of CAD at angiography and high-sensitivity troponin T (hsTNT) levels as follows: (1) patients with no evidence of CAD at angiography and absence of hsTNT elevation (>100 pg/mL); (2) patients with angiographically documented CAD and absence of hsTNT elevation; and (3) patients with ACS and elevated hsTNT (>100 pg/mL) in the coronary venous blood. hsTNT was measured by means of an electrochemiluminescence immunoassay (sTnT-hs ECLIA; Roche, Germany) in aliquots of the samples that were collected for the RNA isolation and miRNA measurements.

Exclusion criteria were a known history of leukopenia, thrombocytopenia, or severe hepatic or renal dysfunction, as well as ongoing inflammatory or malignant disease. In addition, diagnosis of myocarditis and the presence of cardiogenic shock were also exclusion criteria.

The ethics review board of the Goethe University Frankfurt (Frankfurt, Germany) approved the protocols, and the study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each individual. The study was registered within the German Clinical Trials Registry (http://www.germanctr.de; unique identifier: DRKS00000207).

**Plasma Collection and Storage**

Blood samples were simultaneously obtained from the coronary venous sinus and the aortic bulb through 5F catheters during the cardiac catheterization procedure, before heparin or any contrast agent was administered and before any interventional procedure was started. After centrifugation, samples were transferred to RNase/DNase-free tubes and stored at −80°C.

**RNA Preparation**

Total RNA in plasma was isolated by the use of TRI Reagent BD following the manufacturer’s instructions with modifications. To date, no housekeeping miRNA has been established and validated to normalize for the miRNA content. Therefore, we supplemented the samples (after addition of TRIzol) with 5 nmol/L *Caenorhabditis elegans* miR-39 (cel-miR-39), as described previously.3

**Quantitative Reverse Transcriptase–Polymerase Chain Reaction Analysis**

RNA was obtained as outlined above and diluted 1:10 (for all miRNAs except miR-208a, which was added without dilution). Diluted RNA (5 μL) was reverse transcribed with the TaqMan
miRNA reverse transcription kit (ABI) according to the manufacturer’s instructions. Subsequently, 3 μL of the product was used for detecting miRNA expression by quantitative polymerase chain reaction with TaqMan miRNA assay kits (ABI) for the corresponding miRNA. cel-miR-39 was used for normalizing the data, expressed as 2^{-ΔCt[cel-miR-39]} and normalized for mean value of the plate. MicroRNA levels are expressed as 2^{-ΔCt[miRNA]/normalized cel-miR-39} values.

Statistical Analysis
Quantitative data were analyzed by means of the Mann-Whitney U test or ANOVA followed by Bonferroni correction, as specified in the figure legends and in the footnote of the Table. To account for unequal variance, groups were compared by means of the Welch test if the Levene test confirmed unequal variance. For categorical variables, the Fischer exact test or the χ² test was used. Pearson correlation was used to compare levels of miRNAs with hsTNT levels after logarithmic transformation.

A value of P<0.05 was considered statistically significant (2-sided tests). All statistical calculations were performed with SPSS 16.0 (SPSS, Inc, Chicago, IL).

Results

Study Sample
A total of 57 patients were studied. Seven patients had angiographic absence of CAD (no CAD) and normal hsTNT levels (<100 pg/mL), 31 patients had angiographically documented CAD but normal hsTNT levels (<100 pg/mL) in the coronary venous blood (CAD), and 19 patients presented with ACS and hsTNT levels >100 pg/mL in the coronary venous blood (ACS group). The clinical characteristics of the 3 study groups are summarized in the Table. The frequency of antiplatelet therapy with acetylsalicylic acid or clopidogrel was significantly lower in the no-CAD group. Patients with CAD without hsTNT elevation differed from patients with troponin-positive ACS with respect to N-terminal pro-B-type natriuretic peptide serum levels, number of diseased vessels, and use of statins and β-blockers (the Table). Of note, the group of patients without CAD does not represent a healthy control group, which would not be available for invasive blood sampling for ethical reasons, but represents patients who underwent coronary angiography because of clinical suspicion of CAD, which was ruled out at angiography. Because we previously demonstrated that patients with stable CAD have significantly lower levels of systemically circulating miR levels compared with healthy control subjects, we included the no-CAD group in the present analysis to ascertain that differences in circulating miRs between CAD and ACS patients are not driven predominantly by a reduction of miR levels in the CAD group.

MiRs Levels in Aortic and Coronary Sinus Blood
As illustrated in Figure 1A, circulating plasma levels of the muscle-enriched miRs miR-133a, miR-208a, and miR-499 were profoundly elevated in both the aorta and the coronary sinus in patients with troponin-positive ACS compared with patients with CAD but normal TNT levels (Figure 1A). In fact, systemic plasma concentrations in the aorta were elevated ≈11-fold for miR-133a, ≈5-fold for miR-208a, and >20-fold for miR-499 in patients with troponin-positive ACS compared with patients with CAD but no significant troponin elevation.
Plasma levels of the vascular miR-126 (5-fold; \( P = 0.03 \)) and miR-92a (2-fold; \( P = 0.04 \)) were higher in the aorta of patients with ACS compared with patients with stable CAD, whereas no significant differences were detected in the coronary sinus blood (Figure 1B). Circulating levels of miR-155 and miR-223 were modestly but nonsignificantly increased in the aorta of patients with ACS compared with CAD patients without hsTNT elevation (Figure 1C), whereas no differences were observed in the coronary sinus (Figure 1C).

Although some studies demonstrated that vasculoprotective therapies with statins and angiotensin receptor blockers alter inflammation-associated miRNAs in peripheral blood cells,14–16 little is known about potential effects of medication on circulating plasma levels of miRNAs. There was no statistically significant association between various vasculoprotective drug therapies and systemic circulating plasma levels of the measured miRNAs, except for miR-133a, which was significantly lower in patients treated with a statin, and miR-92a, which showed a strong trend (\( P = 0.06 \)) to be lower in patients on statin therapy. However, it should be noted that the number of patients without statin treatment was very small in the CAD group and that doses and duration of statin treatment varied considerably, thus precluding a meaningful multivariate analysis. Thus, patients with troponin-positive ACS exhibit profoundly elevated systemic plasma levels of prototypical muscle-enriched and modestly increased concentrations of endothelial cell–enriched miRs.

Transcoronary Concentration Gradients
To distinguish heart-specific from systemic alterations in circulating miRs levels, we calculated transcoronary concentration gradients by subtracting plasma miRs levels in the aorta from plasma miRs levels in the coronary sinus.

As illustrated in Figure 2, there was a positive transcoronary concentration gradient for both miR-133a and miR-499 in patients with troponin-positive ACS, suggesting that these muscle-derived miRs are indeed released into the coronary circulation during myocardial injury (Figure 2A). The lack of miR-208a plasma levels to demonstrate a significant transcoronary concentration gradient was most likely due to the extremely low concentration of miR-208a in plasma, thereby limiting precise quantitative assessment.

In contrast to the muscle-enriched miRs, the endothelial cell–enriched miR-126 demonstrated an inverse transcoronary concentration gradient (Figure 2B), suggesting either uptake or degradation of miR-126 during passage through the coronary circulation in patients with troponin-positive ACS. A similar pattern was observed for the endothelial cell–enriched miR-92a, although the inverse transcoronary concentration gradient failed to achieve statistical significance (Figure 2B). Although miR-155 and miR-223 concentration tended to be reduced during transcoronary passage, no significant differences were detectable between the groups (Figure 2C).

Correlation Between Circulating miRs Concentration and Extent of Myocardial Injury
To further document an association between circulating concentrations of miRs and myocardial injury, we correlated hsTNT levels in the coronary venous blood with individual miRs levels. As illustrated in Figure 3A, plasma concentra-
tions of miR-499 were positively correlated with hsTNT levels in the coronary venous blood. More important, transcoronary concentration gradients of miR-499 were closely related to the release of troponin into the coronary circulation (Figure 3B). However, the correlation between miR-499 and hsTNT demonstrates a hockey-stick relation, with very little increase apparent in miR-499 levels across the very low troponin values (ln hsTNT $< 5.5$, corresponding to hsTNT values $< 250$ pg/mL). Thus, very minute elevations of hsTNT are not reflected by increases in circulating miR-499 levels. Similar correlations with hsTNT were observed for miR-133a concentrations in the coronary venous blood ($r = 0.75$, $P < 0.01$) and for the miR-133a transcoronary concentration gradient ($r = 0.66$, $P < 0.01$). Moreover, miR-133a and miR-499 transcoronary concentration gradients significantly correlated with the transcoronary TNT concentration gradients (miR-133a: $r = 0.71$, $P < 0.01$; miR-499: $r = 0.74$, $P < 0.01$). Thus, the release of the muscle-enriched miR-499 and miR-133a indeed reflects the extent of myocardial injury as measured by troponin release into the coronary circulation.

In contrast, although the coronary venous plasma concentration of miR-126 was also significantly associated with hsTNT levels in the coronary sinus ($r = 0.31$, $P = 0.02$), the transcoronary concentration gradients of miR-126 were inversely related to the release of TNT into the coronary circulation ($r = -0.54$, $P < 0.01$). Thus, the transcoronary reduction in circulating concentrations of the endothelial cell–enriched miR-126 is directly associated with the extent of myocardial injury.

Plasma concentrations of both the leukocyte-associated miR-155 and the platelet-enriched miR-223 did not show any significant association with circulating troponin levels. Likewise, plasma concentrations of miR-208a and miR-92a were not associated with circulating troponin levels.

Discussion

The results of the present study document that the muscle cell–enriched miR-499 and miR-133a are released from the heart into the coronary circulation on myocardial injury in patients with CAD. In contrast, circulating plasma levels of the endothelial cell–enriched miR-126 are reduced during the transcoronary passage in patients with ACS and evidence for myocardial injury. Thus, the differential regulation of circulating miRs during the transcoronary passage indeed reflects cell type–specific miRs expression and/or release mecha-
nisms associated with myocardial injury in patients with ACS.

Plasma concentrations of circulating miRs may be affected by multiple parameters comprising tissue expression and processing, the release by specific cells into the circulation, and the stability in the plasma. The close correlation between coronary venous blood concentrations of miR-499 and, to a lesser extent, miR-133a with hsTNT levels in the coronary circulation documents that myocardial injury is associated with a significant release of myocyte-specific miRs into the circulation. These data confirm and extend previous reports showing significantly increased systemic plasma concentrations of miR-499 and miR-133a in patients with acute myocardial infarction.4–7 Importantly, even minute elevations in cardiac troponin, as measured by the high-sensitivity assay, were associated with increased miR-499 plasma concentrations, suggesting that miR-499 may be a useful biomarker to detect even minor myocardial injury. Although it was conceivable that myocardial injury induces the release of myocyte-specific miR-499 into the coronary circulation, the negative transcoronary concentration gradient for the endothelial cell–enriched miR-126 was a surprise finding in patients with troponin-positive ACS. However, it should be noted that patients with ACS had profoundly elevated systemic concentrations of miR-126 compared with patients with stable CAD despite the transcoronary concentration reduction. It is well established that patients with ACS exhibit a systemic endothelial activation, as evidenced by impaired endothelium-mediated vasodilator function in the forearm12 and increased systemic circulating levels of endothelial microparticles and remnants of apoptotic endothelial cells.18,19 Because circulating miRs were shown to be vesicle associated,20,21 it is likely that the increased concentration in the systemic circulation of miR-126 in patients with ACS is due to active secretion in microvesicles from the systemically activated endothelium. In contrast, the transcoronary reduction in plasma concentration of miR-126 in patients with troponin-positive ACS indicates a consumption of circulating miR-126 during transcoronary passage through the culprit vessel causing the ACS.

It is difficult to explain the phenomenon that vascular miRNAs are consumed across the coronary circulation in ACS because one may have assumed that even these markers should be released from the coronary circulation during injury. One reason for the observed difference may relate to a different packaging of individual circulating miRNAs. miRNAs are detected in microvesicles that differ in size and composition, including apoptotic bodies (>1 μm) or exosomes (<100 nm).22 Depending on the cell that releases the microvesicles and the vesicle type, the molecular components of the lipid membranes (eg, surface receptors) and the content vary.22,23 Moreover, biochemical fractionation of plasma samples demonstrated that individual miRNAs are detectable in different complexes in the circulation21; whereas some miRNAs are preferentially transported and are protected from RNases by microvesicles, others (such as miR-92a) were shown to be released in protein complexes containing Argonaute-2 or are bound to lipid proteins, suggesting sequence-specific release and transport mechanisms.21,24 These complexes are essential to protect circulating miRNAs from being degraded. However, the different mechanisms of protection may influence the degradation rate; eg, the degradation of protein-conjugated miRNAs is sensitive to proteinases25–27 and RNases28,29 might lead to the degradation of the proteins protecting the vascular miRNAs during passage through the ischemic myocardium, resulting in a reduction in the coronary circulation. Moreover, the uptake or clearance of the circulating miRNAs may be influenced by the nature of the miRNA-protein or miRNA-lipid complexes. Thus, recent experimental studies in mice demonstrated that miR-126 can be delivered by apoptotic

![Figure 3. Correlation between circulating microRNA (miR)-499 and high-sensitivity (hs) troponin T in coronary venous blood.](http://circ.ahajournals.org/)
bodies to atherosclerotic lesions.\textsuperscript{30} Because little is known regarding the biochemical composition of the circulating vascular and muscle-enriched miRNAs and the influence of CAD or ACS on these complexes, future studies are mandatory to elucidate the mechanisms underlying the surprising finding.

It should be noted that the rather limited number of patients studied precluded a comprehensive assessment of potential drug effects on transcoronary concentration gradients of the various miRs analyzed. Likewise, the small number of patients without angiographically detectable CAD studied limits our ability to extend our observations to patients without CAD.

Conclusions

Circulating concentrations of the muscle-enriched miR-499 and miR-133a closely correlate with the extent of myocardial injury, as measured by hsTNT levels in the coronary circulation. In contrast, the endothelial cell–enriched miR-126 is consumed during transcoronary passage in patients with evidence of myocardial injury. Future studies will have to address whether circulating miRs may also function in intercellular communication and whether transfer of miRs between cells occurs, in addition to their potential utility as biomarkers of myocardial injury.

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Disclosures

None.

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RNA improves survival after myocardial infarction reducing vascular permeability, edema formation and myocardial tissue damage [abstract]. Circulation. 2010;122:A20299.


**CLINICAL PERSPECTIVE**

MicroRNAs (miRNAs) are short, noncoding RNAs that control gene expression on a posttranslational level. Recent studies show that miRNA can be detected in circulating blood, and selected miRNAs have been shown to be elevated in the blood after acute myocardial infarction. Here, we demonstrate that muscle-enriched miR-499 and miR-133a are released from the heart into the coronary circulation on myocardial injury and that this release closely correlates with the extent of myocardial injury. In contrast, the endothelial cell–enriched miR-126 is consumed during transcoronary passage in patients with evidence of myocardial injury. These data suggest a differential regulation of muscle versus vascular miRNAs across the coronary circulation. Because muscle-related miRNAs closely correlate with the extent of myocardial injury, future studies should evaluate their usefulness as biomarkers for cardiac injury.
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