

Circulating microRNAs as diagnostic biomarkers for cardiovascular diseases

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Tijssen AJ, Pinto YM, Creemers EE. Circulating microRNAs as diagnostic biomarkers for cardiovascular diseases. *Am J Physiol Heart Circ Physiol* 303: H1085–H1095, 2012. First published August 31, 2012; doi:10.1152/ajpheart.00191.2012.— One of the major challenges in cardiovascular disease is the identification of reliable clinical biomarkers that can be routinely measured in plasma. MicroRNAs (miRNAs) were recently discovered to circulate in the bloodstream in a remarkably stable form. Because of their stability and often tissue- and disease-specific expression and the possibility to measure them with high sensitivity and specificity, miRNAs are emerging as new diagnostic biomarkers. In this review we will provide an overview of the potential of circulating miRNAs as biomarkers for a wide range of cardiovascular diseases such as coronary artery disease, myocardial infarction, hypertension, heart failure, viral myocarditis, and type-2 diabetes mellitus. Furthermore, we will discuss the challenges with regard to further validation in large patient cohorts, and we will discuss how the measurement of multiple miRNAs simultaneously might improve the accuracy of the diagnostic test.

circulating miRNAs; biomarkers; cardiovascular disease

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Introduction

Cardiovascular diseases represent the predominant cause of human morbidity and mortality in developed countries, underscoring the need for therapeutic, diagnostic, and prognostic strategies for this class of diseases (3, 22). One of the major challenges in cardiovascular disease is the identification of reliable biomarkers. MicroRNAs (miRNAs) are short, noncoding RNA sequences that regulate gene expression at the post-transcriptional level by targeting the 3'-untranslated region of mRNA sequences (5). Gene expression studies revealed that miRNAs are differentially expressed in heart disease (46), and loss-of-function studies in mice firmly established that miRNAs control a variety of cellular processes essential to the heart (43).

Although the presence of intact extracellular RNA in plasma was already described in 1947 (24, 32), it was discovered in 2008 that miRNAs are also present in the circulation in all compartments of the blood, including plasma, platelets, erythrocytes, and nucleated blood cells (7, 35). These circulating miRNAs are found to be remarkably stable in plasma even under harsh conditions as boiling, low or high pH, long-term storage at room temperature, and in multiple freeze-thaw cycles (7, 35). Interestingly, circulating miRNAs are protected from endogenous RNase activity (35), and evidence is now accumulating that this protection is achieved by the packaging

of plasma miRNAs into microparticles [e.g., exosomes, microvesicles, or apoptotic bodies (45, 57)], by binding to RNA-binding proteins [e.g., Argonaute 2 and nucleophosmin 1 (4, 50)] or by linkage to high-density lipoprotein (47). Readers interested in the cellular release mechanism of miRNAs, the nature of their stability in the circulation, and their possible biological role in cell-cell signaling are referred to two recent reviews by Fichtlscherer et al. (17) and Creemers et al. (11).

Because of their stability in the circulation, miRNAs are currently explored for their potential as biomarkers in a wide range of cardiovascular diseases. The ideal biomarker fulfills a number of criteria: 1) accessible using noninvasive methods; 2) a high degree of sensitivity and specificity to the disease; 3) allow early detection; 4) sensitivity to relevant changes in the disease; 5) a long half-life within the sample; and 6) the capability of rapid and accurate detection (52). Circulating miRNAs fulfill a number of these criteria. They are stable in the circulation, they are often regulated in a tissue- and pathology-specific manner, and they can be detected with high sensitivity and specificity using sequence-specific amplification. These qualities suggest that the discovery-validation pipeline for miRNA biomarkers will be more efficient than for protein-based biomarkers, where bottlenecks at the point of specific antibody generation are often encountered because of the complexity of protein composition, posttranslational modifications, and the low abundance of many proteins in serum and plasma (35, 52).

In cardiovascular disease, distinctive patterns of circulating miRNAs have thus far been found for coronary artery disease (CAD), myocardial infarction (MI), hypertension, heart failure (HF), viral myocarditis (VM), and type-2 diabetes mellitus (DM) (10, 16, 28, 42, 49, 56). In this review we will summarize and discuss the current knowledge regarding circulating miRNAs as putative biomarkers in these specific diseases.

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Coronary Artery Disease

CAD is caused by atherosclerosis of the coronary arteries. Atherosclerosis is an inflammatory disease of the arteries characterized by the formation of atherosclerotic plaques (30). The growth of these plaques is responsible for narrowing of the lumen of the artery, whereas acute rupture of an unstable plaque can cause thrombus formation and complete obstruction of the lumen (30). All cellular components involved in plaque and thrombus formation (e.g., endothelial cells, macrophages, smooth muscle cells) may potentially release miRNAs in the circulation that may serve as potential biomarker for atherosclerosis. Furthermore, these cellular components may also theoretically take up miRNAs from the circulation or simply secrete less miRNAs, which will both result in decreased miRNA levels in the circulation. Loss of these miRNAs might thereby also serve as potential biomarker for atherosclerosis. In the setting of coronary atherosclerosis, miRNA signatures have been investigated in serum, plasma, whole blood, peripheral blood mononuclear cells (PBMCs), and platelets. These studies are summarized in Table 1.

Serum/plasma miRNAs in CAD. Fichtlscherer et al. (16) were the first to investigate miRNA signatures in plasma and serum of patients with CAD. They included patients with stable angiographically documented CAD and excluded patients with impaired ejection fraction, HF, unstable CAD, and acute MI to reduce the influence of cell death and plaque instability on miRNA levels. Eight miRNAs selected from a microarray were determined in plasma of a cohort of 36 patients with CAD and 17 controls. In this cohort the endothelial enriched miRNAs miR-126, miR-17, and miR-92a; the smooth-muscle cell enriched miR-145; and the inflammatory cell enriched miR-155 were significantly reduced, whereas the cardiomyocyte enriched miRNAs miR-133 and miR-208a were elevated in patients with CAD (16). The aberrant levels of these miRNAs, except for miR-133 and miR-208a, were confirmed in serum of a second cohort consisting of 31 patients with CAD and 14 controls.

Diehl et al. (15) isolated microparticles from plasma of five patients with CAD and five patients with acute coronary syndrome and identified miR-19, miR-21, miR-146, miR-155, and miR-223 as elevated in patients with acute coronary syndrome compared with patients with CAD. This suggests that the reduction in plasma miR-155 in patients with CAD found by Fichtlscherer et al. (16) is due to a reduction in miR-155 in plasma microparticles.

Li et al. (29) measured serum miRNA levels in 104 patients with peripheral artery disease (arteriosclerosis obliterans) and 105 age-matched controls. They identified miR-130a, miR-21, miR-27b, and miR-210 as possible biomarkers with increased levels in peripheral artery disease compared with controls. MiR-130a and miR-27b were correlated with severity of disease indicated by Fontaine stage (29). The lack of overlap between increased miRNAs in coronary and peripheral atherosclerosis shows the specificity of these miRNAs for the site of origin of the disease.

miRNAs in whole blood in CAD. So far, two studies have investigated the potential of circulating miRNAs as biomarkers for CAD in whole blood. Taurino et al. (40) performed microarray analysis in 12 patients with CAD and 12 healthy controls and found miR-140-3p and miR-182 to be enriched in

patients with CAD. They also performed microarrays on 10 patients before and after completion of an exercise-based rehabilitation program after surgical coronary revascularization and revealed an increase in miR-92 levels after the program (40). This is an interesting observation regarding the fact that Fichtlscherer et al. (16) identified miR-92a to be reduced in plasma of patients with CAD.

In the second study, Weber et al. (53) determined the levels of 16 candidate miRNAs in whole blood of 10 patients with CAD and 15 healthy controls by real-time PCR (qRT-PCR) and found the levels of 11 miRNAs (miR-19a, miR-484, miR-155, miR-222, miR-145, miR-29a, miR-378, miR-342, miR-181d, miR-150, and miR-30e-5p) to be reduced in patients with CAD. Further analysis revealed that medication with angiotensin-converting enzyme (ACE) inhibitors within the patient group also resulted in a significant reduction of 7 miRNAs (miR-19a, miR-155, miR-145, miR-222, miR-342, miR-30e-5p, and miR-378) (53). Loss of miR-155 and miR-145 in whole blood of patients with CAD is consistent with the reduced levels found in plasma (16), but since 83% of the patients and none of the controls in the study by Fichtlscherer et al. (16) were using ACE inhibitors, this reduction in plasma might be explained by the use of ACE inhibitors.

miRNA profiling in PBMCs in CAD. Hoekstra et al. (21) determined the miRNA signature in PBMCs of patients with CAD by qRT-PCR-based microarrays. They included 20 healthy subjects, 25 patients with stable angina pectoris (AP), and 25 patients with unstable AP and revealed that miR-135 and miR-147 were downregulated both in patients with stable and unstable AP. Further analysis in this same cohort revealed three miRNAs (miR-134, miR-370, and miR-198) to be significantly upregulated in the mononuclear cells of patients with unstable compared with stable AP, which suggests that the expression of these miRNAs in mononuclear cells might be used as biomarker to identify patients at risk for acute coronary syndromes. An important limitation of this study is the pooling of RNA from eight to nine patients, which results in sample sizes of four per group and no independent validation cohort is included, implicating the need for studies with a larger patient cohort to validate these findings (21).

Takahashi et al. (39) measured the levels of the inflammatory-related miRNAs miR-146a and miR-146b in PBMCs of 66 stable patients with CAD and 33 controls with no CAD. They found both miRNAs to be significantly upregulated in patients with CAD. Within 12 months of follow-up, 13 of the 66 patients with CAD experienced a cardiac event, and miR-146a levels turned out to be an independent predictor of these events (39). This suggests that miR-146a in PBMCs might serve as a prognostic biomarker to identify patients with CAD at risk of cardiac events.

Furthermore, in circulating endothelial progenitor cells, miR-221 and miR-222 (miRNAs highly expressed in endothelial cells) were found to be upregulated in 44 patients with CAD compared with 22 patients with no CAD (34).

Platelet miRNAs in CAD. A miRNA signature in platelets of patients with CAD was recently investigated by Sondermeijer et al. (38). Using microarrays and qRT-PCR, they found two miRNAs, miR-340* and miR-624*, enriched in platelets in two different cohorts consisting of 40 premature CAD and 40 age-matched controls, and 27 atherosclerotic patients and 40 of their family members, respectively. A combination of four

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Table 1. Circulating miRNAs as biomarkers for CAD

Groups	miRNAs	Source	RNA Isolation	miRNA Detection	Normalization	Age/Sex Differences	Multivariate Analysis	Reference
31 controls; 67 CAD	miR-126, miR-17, miR-92a, miR-145, miR-155	EDTA plasma and serum	TRIzol and miRNeasy kit	Taqman probes	cel-miR-39 spike in	Controls > 30 yr younger, more females	No	(16)
12 controls; 12 CAD	miR-140-3p, miR182	Whole blood	miRNeasy kit	Microfluidic array LC sciences	No	No	No	(40)
15 healthy control; 10 CAD	miR-19a, miR-584, miR-155, miR-222, miR-145, miR-29a, miR-378, miR-342, miR-181d, miR-150, miR-30e-5p	Whole blood	PAXgene miRNA kit	Taqman probes	RNU44	Age and sex matched	No	(53)
40 premature CAD; 40 healthy controls and 27 atherosclerosis; 40 family members	miR-340*, miR-624*	Platelets	mirVana PARIS	Sybr-green based, taqman probes	Platelet count, miR-223	Age matched and males only; Controls > 20 yr younger, sex unknown	No	(38)
20 healthy controls; 25 unstable AP; 25 stable AP; 33 non-CAD; 66 stable CAD	miR-135, miR-147	PBMC	Guanidium-thiocyanate	Taqman array	let-7a, miR-16	Age, sex, ethnically, smoking matched	No	(21)
22 non-CAD; 44 stable CAD	miR146a/b	PBMC	mirVana PARIS	Taqman probes	RNU6	No	Yes*	(39)
5 CAD; 5 acute coronary syndrome	miR-221, miR-222	Endothelial progenitors	mirVana PARIS	Taqman probes	RNU6	No	No	(34)
104 arteriosclerosis obliterans; 105 controls	miR-19, miR-21, miR-146, miR-155, miR-223	Microparticles from plasma	miRNeasy kit	Sybr-green based	No	No	No	(15)
	miR-130a, miR-27b, miR-210, miR-21	Serum	QIAamp circulating nucleic acid kit	Sybr-green based	No	Age matched	No	(29)

miRNA, microRNA; CAD, coronary artery disease; AP, angina pectoris; PBMC, peripheral blood mononuclear cells. *Age, sex, culprit lesion, smoking, blood pressure, fasting glucose, HbA_{1c}, LDL cholesterol, high-sensitive C-reactive protein, and history of hypertension, diabetes mellitus (DM), and CAD corrected.

miRNAs (miR-340*, miR-624*, miR-451, and miR-454) was able to distinguish the 40 premature patients with CAD and 40 controls with an area under the receiver-operating-characteristic (ROC) curve (AUC) of 0.71. This study shows that miRNA signatures in platelets differ between controls and patients with CAD. Whether these miRNAs are able to identify patients at risk of cardiovascular events remains to be elucidated.

In conclusion, several studies identified aberrant miRNA signatures in patients with CAD in several components of the blood. The lack of overlap of identified miRNAs in the different studies suggests that these aberrant miRNA signatures are specific for the investigated blood components as a source of the miRNAs (Table 1). Together, numerous miRNAs were identified that may become helpful in diagnosis and risk stratification of patients with CAD, but studies with larger patient cohorts are needed to validate the most promising miRNA or combination of miRNAs and to determine the potential of these miRNAs as biomarker for CAD.

Myocardial Infarction

Several groups hypothesized that necrosis of cardiac cells after MI results in leakage of miRNAs into the circulation and that miRNAs highly, and preferably, specifically expressed in the heart might be used to diagnose acute coronary events. We have summarized these studies in Table 2.

Several groups have investigated the time course of miRNA release after MI. For the cardiomyocyte-specific miR-208, Ji et al. (23) revealed that the levels in plasma were highly comparable with cardiac troponin I (cTnI) levels in their rat model of isoproterenol-induced myocardial injury. They found miR-208 to be undetectable at baseline, increased after 3 h of isoproterenol treatment, and significantly elevated up to 12 h. MiR-208 was also found to be rapidly induced in rodent models of MI where it was undetectable in sham-operated animals, increased at 30 min, peaked at 3 h, and disappeared from plasma again at 24 h (12, 49). The levels of the muscle-enriched miRNAs miR-1, miR-133a, and miR-499 showed a rapid increase within 1 h after MI in rats to peak between 3 and 12 h, decrease at 24 h, and return to basal levels at 3 days after MI (9, 49). Comparable time courses were also found by Gidlof et al. (19) after ischemia-reperfusion injury in pigs. Also in humans, similar time courses of miRNA release are detected; miR-1 and miR-133a and -b were found to peak at 2.5 h after the onset of symptoms in patients with MI, whereas cTnI and miR-499 showed slower time courses and peaked at 6 and 12 h, respectively. Elevated miR-499 levels could still be detected at 48 h after MI, and after 3 days all miRNAs had returned to their normal levels (1, 12).

Diagnostic abilities of circulating miRNAs for MI. The cardiac-specific miR-208 and the muscle-enriched miRNAs miR-1, miR-133 and miR-499-5p have also been investigated for their diagnostic ability in plasma of patients with MI. D'Alessandra et al. (12) found in a cohort of 25 MI and 17 healthy controls that miR-1, miR-133a, miR-133b, and miR-499-5p were elevated in patients with MI, whereas miR-122 and miR-375 were reduced. They also measured miR-208 in a subgroup of this cohort and found it only detectable in three of the nine examined patients with MI, which may be due to the relatively late time point of sample collection, which was in the whole cohort on average 9 h after the occurrence of symptoms.

Wang et al. (49) confirmed that miR-208 was not detectable in plasma of healthy controls or in patients with stable CAD, whereas miR-208 could be detected in 91% of the patients with MI. Strikingly, in a subgroup of 20 patients with MI of which blood samples were collected within 4 h after the onset of symptoms, miR-208 was detected in all patients, whereas cTnI was only detected in 85% of the patients, confirming the superior sensitivity of miR-208 at early time points. In the complete cohort, miR-208 showed a superior ROC curve compared with miR-1, miR-133a, and miR-499 in separating 33 patients with MI from 33 patients with other cardiovascular diseases (49).

Also miR-208b, a family member of miR-208 expressed in heart and skeletal muscle, showed a superior performance in separating patients with MI from controls with atypical chest pain and no cardiac disease compared with miR-499, miR-1, and miR-133 (10, 19). Interestingly, levels of miR-499 and miR-208b could be related to disease severity, as was shown by the correlation with troponin T (TnT) and creatine phosphokinase levels (10, 19). These results were further confirmed by Devaux et al. (14), who found elevated levels of miR-208b and miR-499 and a correlation with TnT and creatine phosphokinase in a cohort of 510 patients with MI and 87 healthy controls. In this cohort miR-499 showed a diagnostic accuracy comparable with TnT and superior to miR-208b. Kuwabara et al. (27) were not able to detect miR-208 in the majority of their 29 patients with MI, but in this study sample collection was performed at a wide range of time points, and it seems likely that of the 5% of patients in which miR-208 was detected, blood was collected at the earliest time points.

The muscle enriched miRNAs miR-1, miR-133, and miR-499 were also found to be elevated in whole blood or plasma of patients with MI (2, 9, 51). In this regard, miR-499 was found to be specifically enriched in plasma of nine patients within 48 h after MI, whereas this miRNA was undetectable in the same patients at discharge from the hospital, in patients with unstable AP, in patients with congestive HF, and in subjects without cardiovascular disease (1).

Olivieri et al. (37) investigated plasma miRNA levels in a cohort of geriatric patients, in which the diagnosis of non-ST-elevation MI is challenging because of atypical symptoms and the presence of a modest TnT elevation in many elderly patients, possibly because of other underlying cardiac pathologies. Olivieri et al. (37) studied the miRNA levels in a cohort of 92 patients with non-ST-elevation MI, 81 patients with acute HF, and 99 healthy controls and found miR-133a, miR-1, miR-499-5p, miR-21, and miR-423-5p elevated in the patients with MI compared with the controls and, except miR-1, in HF patients compared with controls. MiR-21 and miR-499-5p were elevated in patients with MI compared with those with HF, and miR-423-5p was higher elevated in patients with HF than those with MI. As miR-423-5p has been proposed as a biomarker for HF and 74% of patients with MI in this population experience HF, we suggest that the elevation of miR-423-5p in the MI patients is due to the presence of HF in this population.

Meder et al. (33) performed a microarray on whole blood of 20 MI patients compared with 20 control patients. Strikingly, miR-208a/b, miR-133a/b, miR-1, and miR-499 were not among the 20 most highly enriched miRNAs detected in this study. This cannot be explained by the time of sample collection as blood

Table 2. Circulating miRNAs as biomarkers for MI

Groups	miRNAs	Source	RNA Isolation	miRNA Detection	Normalization	Age/Sex Differences	Multivariate Analysis	Reference
7 no CAD; 31 stable CAD; 19 ACS	miR-499, miR-133, miR-208a, miR-126	EDTA plasma	Tri reagent	Taqman probes	cel-miR-39 spike-in	No	No	(13)
36 atypical chest pain; 32 MI	miR-1, miR-133, miR-208b, miR-499	Citrate plasma	mirVana PARIS	Sybr-green based	3 <i>Caenorhabditis elegans</i> spike-ins	No	No	(10)
117 unstable AP; 327 MI	miR-1, miR-133, miR-208b	Plasma	MasterPure kit	Taqman probes	cel-miR-54 spike-in	No	Yes*	(54)
10 controls; 9 MI; 5 unstable AP; 15 HF	miR-499	EDTA plasma	mirVana PARIS	Taqman probes	Synthetic miRNA	Controls > 30 yr younger	No	(1)
66 healthy control; 93 MI	miR-1	Citrate plasma	mirVana PARIS	Sybr-green based	RNU6	No	No	(2)
20 controls; 31 MI	miR-1	Serum	miRNA isolation kit	Sybr-green based	No	Age, sex matched	No	(9)
17 healthy control; 33 MI	miR-1, miR-133a, miR-133b, miR-499-5p, miR-208, miR-122, miR-375	EDTA plasma	mirVana PARIS	Taqman probes	miR-17-5p	Controls > 10 yr younger	Age	(12)
30 healthy control; 33 MI, 16 CAD, 17 other CVD	miR-1, miR-133a, miR-499-5p, miR-208	Plasma	Tri reagent	Taqman probes	cel-miR-39	No	No	(49)
29 MI; 42 nonMI	miR-1, miR-133a	Serum	TRIzol LS	Taqman probes	No	No	No	(27)
11 healthy control; 9 MI	miR-208b, miR-1, miR-133, miR-499-5p	Plasma	TRIzol LS, miRNeasy kit	Sybr-green based	No	No	No	(19)
28 control; 51 MI	miR-133, miR-328	EDTA plasma, whole blood	TRIzol LS	Sybr-green based	RNU6	No	No	(51)
20 control patients; 20 MI	miR-1291, miR-663b, miR-145, miR-30c	Whole blood	miRNeasy kit	Microarray	No	No	No	(33)
92 elderly MI; 81 HF; 99 healthy control	miR-1, miR-133a, miR-423-5p, miR-21, miR-499-5p	EDTA plasma	mirVana PARIS	Taqman probes	miR-17, cel-miR-39	Age matched	Yes†	(37)
510 MI; 87 control	miR-208b, miR-499	Citrate plasma	mirVana PARIS	Sybr-green based	3 <i>C. elegans</i> spike-ins	No	Yes‡	(14)

MI, myocardial infarction; ACS, acute coronary syndrome; HF, heart failure; CVD, cardiovascular disease. *Age, sex, and high-sensitivity troponin T (hsTnT) corrected. †Age and sex corrected. ‡Age, sex, hypertension, hypercholesterolemia, smoking and hsTnT corrected.

was drawn within 3 h after onset of symptoms (33). This indicates that miRNAs in plasma are only a small fraction of the miRNAs in whole blood, which may lead to biomarkers specific for the different components of blood. However, Meder et al. (33) did find 121 miRNAs significantly changed, of which miR-1291 and miR-663b showed the highest AUC of 0.91 and 0.94, respectively, and miR-145 and miR-30c showed the highest correlation (positive) with TnT as a measure of infarct size. A combination of 20 miRNAs even showed an AUC of 0.99, indicating that a miRNA signature might be superior as a diagnostic biomarker for MI.

The different groups investigating circulating miRNAs after MI do not all agree on the suitability of miR-208a as diagnostic biomarker after MI. While its cardiac-specific expression results in high specificity, it might also result in relatively low levels of this miRNA in the blood compared with miRNAs released by other sources, and therefore it might be that miR-208a was below the detection limit in some studies resulting in a low sensitivity. Another explanation might be that blood collection in some studies was too long after the onset of symptoms, since miR-208a is shown to have an early peak and fast reduction to normal levels in animal models of MI (12, 49).

Source of released miRNAs. The first indirect indication that the elevated plasma levels of miR-1, miR-133a, miR-208, and miR-499 in patients with MI are released by the injured myocardium is that the levels of these miRNAs are reduced within the infarcted myocardium of mice compared with myocardium from sham-operated control mice (27). More direct evidence was obtained by De Rosa et al. (13), who simultaneously measured miRNA levels in plasma obtained from the aorta and coronary venous sinus of patients with acute coronary syndromes. MiR-133a and miR-499 showed an increased level in the coronary venous sinus compared with the aorta samples in 19 patients with acute coronary syndromes, which correlated with the increased levels of TnT and suggests their release to reflect the extent of myocardial injury. No differences in coronary venous sinus and aorta plasma levels were demonstrated for miR-208, which was most likely due to the low concentrations in plasma limiting precise quantitative assessment. Interestingly, not all myocardial miRNAs leak into the circulation with the same kinetics. The slower release of miR-499 into the bloodstream compared with several other myocardial miRNAs may suggest that the different miRNAs are bound to different proteins within the cell. It is also striking that two other highly expressed cardiac miRNAs, miR-30c and miR-24, which are expressed at even higher levels than miR-208 in cardiomyocytes, failed to increase in plasma after MI (12). The observation that miR-30c was increased in whole blood after MI and correlated with TnT levels (33), suggests that this elevation is due to upregulation in blood cells instead of leakage by cardiomyocytes.

Prognostic abilities of circulating miRNAs after MI. The studies described above are based on low patient numbers and therefore not able to assess the relation of miRNAs to clinical characteristics and their potential prognostic value. Widera et al. (54) determined levels of six candidate miRNAs in plasma of 444 patients with acute coronary syndromes and found increased levels of miR-1, miR-133a, and miR-208b in plasma of patients with MI ($n = 327$) compared with unstable AP ($n = 117$) and no differences in miR-133b, miR-208a, and miR-499.

During 6 months of follow-up, 34 patients died and miR-133a and miR-208b were significantly related to all-cause mortality. Both miRNAs were not able to enhance the discriminatory ability of high-sensitive (hs)TnT between survivors and non-survivors (54).

In conclusion, several studies fuel the notion that circulating miRNAs might be useful as diagnostic and prognostic biomarkers for MI. In a clinical setting the differences in time courses of release between specific miRNAs and cTnI might be valuable. Especially in the consideration of the fact that the cTnI levels begin to rise only 4 to 8 h after MI (49), diagnosis via biomarkers with a faster cardiac release, such as miR-208, miR-1, and miR-133, might be beneficial. The slow time course of miR-499 might lead to increased diagnostic performance at late time points after MI when cTnI has already returned back to normal levels. Therefore, it may be expected that in the future, a panel of miRNAs, probably in combination with cTnI, has a better potential to offer sensitive and specific diagnostic tests for acute coronary syndromes.

Essential Hypertension

Essential hypertension is a predisposing risk factor for stroke, MI, HF, arterial aneurysm, and chronic renal failure. Li et al. (28) have investigated whether a specific miRNA signature could be identified in patients with essential hypertension (detailed methods are included in Table 4). After initial miRNA array analysis in plasma of hypertensive patients and controls, they were able to confirm the different levels of three miRNAs (hcmv-miR-UL112, let-7e, and miR-296-5p) in a cohort of 127 patients with hypertension and 67 control subjects. Interestingly, one of the successfully validated miRNAs appeared to be a human cytomegalovirus (HCMV)-encoded miRNA, which suggests a novel link between HCMV infection and essential hypertension. Li et al. (28) subsequently measured HCMV titers in their hypertensive patients and controls and showed an elevation of HCMV titers in the patients with hypertension and a correlation between the HCMV titers and the levels of hcmv-miR-UL112 in plasma.

In conclusion, miRNA profiling in plasma of patients with hypertension reveals a possible involvement of HCMV in the pathogenesis of essential hypertension. A possible causal link between HCMV and blood pressure is recently found in mice, where infection with mouse HCMV resulted in higher blood pressure (8). However, as indicated by Li et al. (28), a high degree of interpatient variation was detected in miRNA levels in plasma, which will make it difficult to use these miRNAs as biomarkers for hypertension.

Heart Failure

HF is defined as a complex clinical syndrome that can result from any structural or functional disorder that impairs the ability of the ventricles to fill with or eject blood. Table 3 contains an overview of the published studies that report on circulating miRNAs in HF.

Our laboratory investigated whether circulating miRNAs show aberrant profiles in HF and can be used as a biomarker for this disease (42). In a cohort of 39 healthy controls and 50 dyspnea patients, 30 of whom were diagnosed to have dyspnea because of HF and 20 because of other causes, we determined the levels of 16 miRNAs selected from a microarray. Seven

Table 3. Circulating miRNAs as biomarker for HF

Groups	miRNAs	Source	RNA Isolation	miRNA Detection	Normalization	Age/Sex Differences	Multivariate Analysis	Reference
39 healthy controls; 20 HF cases; 20 non-HF cases with dyspnea	miR-423-5p, miR-18b*, miR-129-5p, HS_202.1, miR-622, miR-654-3p, miR-1254	Citrate plasma	mirVana PARIS	Sybr-green based	miR-1249	Controls > 10 yr younger	Yes*	(42)
30 chronic HF; 30 healthy controls	miR-423-5p, miR-320a, miR-22, miR-92b	Serum	Phenol: chloroform purification	Taqman probes	Based on mean C _t of all miRNAs	Age, sex, ethnically matched	No	(20)
41 right ventricular HF; 10 healthy controls	miR-423-5p	Plasma	MasterPure kit	Taqman probes	cel-miR-54	Age and sex matched	No	(44)
10 HF; 17 asymptomatic control	miR-126	EDTA plasma	mirVana PARIS	Taqman probes	Synthetic spike-in	Controls > 25 yr younger	No	(18)
33 acute HF; 34 healthy controls	miR-499, miR-122	EDTA plasma	mirVana PARIS	Sybr-green based	3 C. elegans spike-ins	Controls > 40 yr younger	No	(10)
19 healthy controls; 19 NIDCM; 15 ICM	miR-107, miR-139, miR-142-5p, miR-142-3p, miR-19b, miR-125b, miR-497	PBMC	TRIzol	Taqman probes	miR-16	Unknown	No	(48)

NIDCM, nonischemic dilated cardiomyopathy; ICM, ischemic cardiomyopathy. *Age and sex corrected.

miRNAs were validated to be enriched in plasma of HF patients in this cohort (miR-423-5p, miR-18b*, miR-129-5p, HS_202.1, miR-622, miR-654-3p, and miR-1254), among which miR-423-5p was most strongly related to the clinical diagnosis of HF. MiR-423-5p distinguished patients with HF from healthy controls with an AUC of 0.91 and from patients with dyspnea but without HF with an AUC of 0.83 (42). The circulating levels of miR-423-5p were related to disease severity as shown by an inverse correlation with ejection fraction and higher levels of miR-423-5p in patients with a higher New York Heart Association (NYHA) classification. MiR-423-5p was also correlated to the levels of the current clinically used biomarker N-terminal pro-brain natriuretic peptide (NT-proBNP). The elevation of circulating miR-423-5p levels in HF was confirmed by Goren et al. (20), who determined the levels of 186 miRNAs in serum of 30 patients with chronic HF compared with 30 age-, sex-, and ethnically matched healthy controls. They found 26 miRNAs to show significantly different levels in patients with HF, of which miR-423-5p showed the strongest increase. In this study miR-423-5p was able to distinguish patients with HF from healthy controls with an AUC of 0.88. A direct correlation between circulating miR-423-5p levels and BNP was also detected in this population. However, no relation to disease severity was found, shown by a lack of correlation between miR-423-5p and ejection fraction or NYHA class (20).

In contrast to these findings in left ventricular HF, Tutarel et al. (44) do not find elevations of miR-423-5p in right ventricular HF. They studied 41 patients with congenital transposition of the aorta and pulmonary artery where the systemic circulation is supported by the right ventricle. When compared with 10 age- and sex-matched controls, they did not find any differences in circulating miR-423-5p between patients and controls. Two possible explanations for these differences could be postulated: 1) the difference in pathophysiology of systemic right ventricular HF compared with left ventricular HF (44) and 2) the difference in severity of affected patients, as the patients in the studies of Tijssen et al. (42) and Goren et al. (20) were more severely affected compared with those in the study by Tutarel et al. These findings indicate the specificity of miR-423-5p elevation for left ventricular HF. Unfortunately, no other miRNAs were measured in patients with right ventricular HF, as this could have resulted in promising biomarkers for this specific disease.

Several important questions about miR-423-5p remain, since it is unanswered how this miRNA is released into the circulation and by which cell type. It has been shown that in the circulation of healthy subjects, miR-423-5p is specifically bound to Argonaute 2 complexes and not associated with microvesicles (4). On the other hand, Goren et al. (20) did find enrichment of miR-423-5p in the exosomal fraction of patients with HF, indicating that the mechanism of miR-423-5p release may be different in patients with HF compared with healthy controls. In the pig, miR-423-5p was shown to be ubiquitously expressed, with high levels in heart, liver, and brain (55). Together with the detected upregulation of miR-423-5p in human failing myocardium (41, 42), this suggests that circulating miR-423-5p in HF is derived from the myocardium. Interestingly, miR-423-5p levels were also found to be increased in serum of patients with specific forms of cancer such as non-small cell lung cancer (7) and gastric cancer (31). The

fact that levels of miR-423-5p were not elevated in patients with dyspnea but without HF argues against the possibility that (damage to) the lung is a source of miR-423-5p release during HF (42).

Three other circulating miRNAs are linked to the diagnosis of HF. The endothelium-specific miR-126 was found to be negatively correlated with age, BNP, and NYHA class in 10 patients with and 17 asymptomatic controls (18). Corsten et al. (10) found both miR-499 and miR-122 to be enriched in the plasma of 33 patients with acute HF compared with 34 healthy controls, of which miR-499 is probably myocardium derived and miR-122 might possibly reflect hepatic venous congestion, as miR-122 is enriched in the liver.

Voellenkle et al. (48) studied the miRNA signature of patients with HF in PBMCs. In a cohort of 19 control subjects, 19 subjects with nonischemic dilated cardiomyopathy and 15 subjects with ischemic cardiomyopathy miR-107, miR-142-5p, and miR-139 were downregulated in both classes of HF, miR-125b and miR-497 in ischemic cardiomyopathy only and miR-142 and miR-29b upregulated in nonischemic dilated cardiomyopathy only (48). That none of the plasma/serum miRNAs were significantly different within these mononuclear cells suggests that these miRNAs are not released by these cells and that the results of the other studies are not influenced by lysis of these cells during sample preparation.

One of the characteristics of an ideal biomarker is changing levels as disease severity changes in response to therapy. For two of the identified circulating miRNAs in HF, miR-499-5p and miR-423-5p, this response to therapy was shown by Montgomery et al. (36) in a rat model of HF. In Dahl salt-sensitive rats HF was hypertension induced by a high salt diet and treatment with anti-miR-208a resulted in improved cardiac function and survival. This anti-miR treatment also blunted the increase of circulating miR-499-5p and miR-423-5p levels (36). Although this response to therapy makes miR-499-5p and miR-423-5p promising biomarkers for HF, the diagnostic performance of these miRNAs is only tested in relatively small patient cohorts; therefore, larger studies are needed to confirm their diagnostic capability.

Viral Myocarditis

Myocarditis is an acute or chronic inflammatory disease of the myocardium, which can be caused by viral infections, postinfectious immune reactions, or organ-specific autoimmune reactions. Corsten et al. (10) determined circulating miRNA levels in plasma of 14 patients with acute VM, 20 patients in the post-VM phase, and 20 healthy control subjects. They identified two cardiac-enriched miRNAs, miR-208b and

miR-499-5p, specifically elevated in patients with acute VM compared with both control groups. A subdivision of patients in the acute VM group into mild, moderate, and severe VM based on TnT levels and ejection fraction revealed that the levels of miR-208b and miR-499-5p are associated with disease severity (10). Detailed methods of this study can be found in Table 4. Both miRNAs, miR-208b and miR-499-5p, are found to be elevated in other cardiovascular diseases as well; miR-208b in MI and miR-499 in MI and HF (10, 49). This may suggest that the release of these miRNAs into the circulation is an indication of cardiac damage, and they are therefore not very specific as a biomarker for the diagnosis of VM.

Diabetes Mellitus Type 2

Type-2 DM is a disease characterized by chronic elevation of blood glucose levels and is one of the major risk factors for cardiovascular disease (56). We have summarized the studies investigating circulating miRNAs in DM in Table 5.

Based on network analysis of microarray results, Zampetaki et al. (56) selected 13 miRNAs for validation in a cohort of 80 patients with DM of the Bruneck study and 80 age- and sex-matched controls. In this cohort 12 miRNAs were significantly associated with DM in a multivariate analysis (miR-24, miR-21, miR-20b, miR-15a, miR-126, miR-191, miR-197, miR-223, miR-320, miR-486, miR-150, and miR-28-3p). In a second validation step, miR-126 emerged as a significant predictor of DM in the entire Bruneck cohort ($n = 822$) and showed a gradual decrease from normal glucose tolerance via impaired glucose tolerance to DM. Using the expression profiles of the five most significantly changed miRNAs (miR-15a, miR-126, miR-320, miR-223, and miR-28-3p), they were able to distinguish patients with DM from healthy controls with a sensitivity of 70% and a specificity of 92% (56). Interestingly, levels of miR-126, miR-15a, miR-29b, miR-223, and miR-28-3p were already altered before manifestation of the disease.

In a study population of 19 patients susceptible for DM (high body mass index and/or family history of DM), 19 prediabetic patients (impaired glucose tolerance and/or impaired fasting glucose), and 18 patients with type-2 DM, Kong et al. (26) measured the serum levels of seven miRNAs previously reported to be involved in insulin regulation (miR-9, miR-29a, miR-30d, miR-34, miR-124, miR-146a, and miR-375, but unfortunately not miR-126). Strikingly, all seven miRNAs were found to be elevated in patients with type-2 DM and not in patients with prediabetes compared with the susceptible controls. Karolina et al. (25) used microarrays to investigate the miRNA signature in whole blood in a cohort of 14 patients with impaired fasting glucose, 21 patients with DM type 2, and

Table 4. Circulating miRNAs as biomarker for hypertension and VM

Disease	Groups	miRNAs	Source	RNA Isolation	miRNA Detection	Normalization	Age/Sex Differences	Multivariate Analysis	Reference
Essential hypertension	89 controls; 151 hypertension	hcmv-miR-UL112, let-7e, miR-296-5p	EDTA plasma	Tri reagent and RNeasy mini kit	Taqman probes	No	No	Yes*	(28)
VM	14 acute VM; 20 post-VM; 20 healthy controls	miR-208b, miR-499	EDTA plasma	mirVana PARIS	Sybr-green based	3 <i>C. elegans</i> spike-ins	Age matched	No	(10)

VM, viral myocarditis. *Age, sex, body mass index, DM, hyperlipidemia, and history of CAD corrected.

Table 5. Circulating miRNAs as biomarker for DM

Groups	miRNAs	Source	RNA Isolation	miRNA Detection	Normalization	Age/Sex Differences	Multivariate Analysis	Reference
80 DM; 80 controls	miR-15a, miR-126, miR-29b, miR-223, miR-28-3p	Plasma	miRNeasy kit	Taqman probes	Unadjusted, miR-454, RNU6b	Age and sex matched	Yes*	(56)
580 controls; 162 impaired glucose; 80 DM	miR-126	Plasma	miRNeasy kit	Taqman probes	Unadjusted, miR-454, RNU6b	Unknown	Yes*	(56)
19 susceptible; 19 prediabetic; 18 DM	miR-29, miR-34a, miR-146a, miR-9, miR-30d, miR-124	Serum	mirVana miRNA isolation kit	Taqman probes	RNU6b	No	No	(26)
15 healthy controls; 14 impaired glucose tolerance; 21 DM	miR-146a, miR-182, miR-30d, miR-144, miR-150, miR-192, miR-29a, miR-320	Whole blood	RiboPure blood kit	Taqman probes	RNU6	Age matched; only males	No	(25)
11 controls; 11 DM with critical ischemia	miR-503	Plasma	TRIzol	Taqman probes	RNU6	No	No	(6)

*Social status, DM family history, body mass index, waist-to-hip ratio, smoking, alcohol, C-reactive protein, physical activity, age, and sex corrected.

15 healthy subjects. They identified circulating miR-144, miR-192, and miR-29a as increased in patients with impaired fasting glucose and further increased in patients with type-2 DM; miR-146 as decreased in both disease states with a higherfold change in type-2 DM; and miR-150 and miR-320 as decreased in patients with impaired fasting glucose and increased in type-2 DM, and this was the opposite for miR-182 and miR-30d (25). MiR-126, the miRNA that was most strongly related to DM type 2 in the Bruneck population, (56) was not changed, which might be explained by the different sample characteristics (plasma vs. whole blood) between the two studies.

Caporali et al. (6) identified miR-503, a miRNA involved in diabetic endothelial dysfunction, to be enriched in plasma of 11 patients with DM with critical ischemia compared with 11 control subjects. That this miRNA is not detected in the other studies (25, 26, 56) on circulating miRNAs in DM suggests that miR-503 might be a biomarker for ongoing ischemia in DM.

In conclusion, several circulating miRNAs are reported to show aberrant levels in patients with type-2 DM, of which three miRNAs (miR-29a, miR-30d, and miR-146a) were shared between the studies by Kong et al. (26) and Karolina et al. (25). The diagnostic ability of miR-126 was successfully validated in a large prospective cohort of 822 individuals. Furthermore, this miRNA was already regulated years before manifestation of the disease and therefore possibly useful for risk prediction.

Future Perspective

Challenges for circulating miRNAs as biomarker. Circulating miRNAs are emerging as blood-based biomarkers for cardiovascular diseases, since they offer many attractive features of biomarkers. They are stable in the circulation, their sequences are evolutionarily conserved, their expression is often tissue or pathology specific, and their detection is based on sequence-specific amplification, features that are helpful in the development of sensitive and specific assays. As discussed in this review, initial candidate miRNAs have been proposed as biomarker for CAD, MI, hypertension, HF, VM, and type-2 DM. However, there are also challenges associated with the

discovery-validation pipeline for circulating miRNAs as biomarkers for disease. First, most studies performed to date are evaluated in populations with <100 subjects. Although several candidate miRNAs are confirmed in more than one study, further validation in larger patient cohorts is needed. In these larger studies one should not only focus on the diagnostic performance of these miRNAs but also investigate the usefulness of the miRNAs in predicting the prognosis of patients. For instance, it will be interesting to identify a set of miRNAs that may be able to predict which patients with CAD are at risk for developing MI. Furthermore, future studies should also clarify whether miRNAs are useful to monitor the response to therapy, as suggested for miR-499-5p and miR-423-5p, which are reduced in rats with HF upon treatment with anti-miR-208 (36).

Validation of candidate miRNAs in larger patient cohorts is not the only challenge for circulating miRNAs to become clinically useful as biomarker. The other challenges mainly relate to the low amount of total RNA in plasma or serum, which makes miRNA amplification often necessary to measure circulating miRNAs. The low amount of RNA in plasma and serum makes it virtually impossible to measure concentration and quality of the isolated RNA, and as a consequence, variances based on the amount of starting material and miRNA extraction might occur. Therefore normalization is an important aspect in the measurement of circulating miRNAs, but at the moment no satisfying "housekeeping" circulating miRNA has been identified. Several reports use the small nucleolar RNA U6 or other miRNAs as an internal control, and although these miRNAs may be stable in some studies, they may change in other pathological conditions and are therefore not suitable as internal control in all studies. Another widely used method for normalization is the addition of synthetic spike-in miRNAs, mainly *Caenorhabditis elegans* miRNAs without homology to human miRNAs, during the purification process. This method worked well for Mitchell et al. (35), who reported it first, but they also found these synthetic miRNAs to be unstable in crude plasma. Therefore, the moment of adding the spike-in miRNAs to the plasma is of crucial importance since plasma RNase activity should be fully inactivated before the synthetic spike-in RNAs are added to the sample. Cheng et al. (9)

reported that correcting for plasma volume is the best method of normalization, as volume of plasma is clinically standard for other biomarkers. Future studies are needed to compare these different methods and identify the most reliable method of normalization, which might be specific for the release route of the miRNAs (microparticles or protein bound).

Clinical application. At the moment most studies are investigating the usefulness of individual miRNAs as biomarker for disease, but it is expected that a combination of multiple miRNAs may provide greater accuracy. For example, a combination of miR-208a, miR-133, miR-1, and miR-499-5p in one test will result in a test able to identify patients with MI in a broader time range after onset of complaints, as the first three miRNAs peak at 3 h after MI and miR-499 at 12 h (12, 49). Furthermore, a combination of miRNAs in a diagnostic test may provide better diagnostic accuracy since different causes for the disease might result in different levels of plasma miRNAs. This might be the case in atherosclerotic and non-atherosclerotic forms of HF and in right and left ventricular HF. An indication that a panel of miRNAs provides better diagnostic accuracy is described in type-2 DM by Zampetaki et al. (56), where a combination of five miRNAs resulted in a diagnostic test with high sensitivity and specificity.

In conclusion, the identification of stable circulating miRNAs launches a new generation of potential biomarkers, for which assays can be developed with relative ease, at a relatively low expense, but with potentially unrivaled specificity and sensitivity. These assays could easily be designed to combine a large number of circulating miRNAs, which could drastically change the use and interpretation of circulating biomarkers as we know them.

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DISCLOSURES

Y. M. Pinto is a cofounder of and holds less than 5% equity in ACS Biomarker BV, a company that commercializes cardiovascular biomarkers.

AUTHOR CONTRIBUTIONS

A.J.T. and E.E.C. drafted manuscript; A.J.T., Y.M.P., and E.E.C. edited and revised manuscript; A.J.T., Y.M.P., and E.E.C. approved final version of manuscript.

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