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Circulating miRNAs: Reflecting or Affecting Cardiovascular Disease?

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Abstract MicroRNAs are a class of small, noncoding RNAs encoded by the metazoan genome that regulate protein expression. A collection of studies point to vital roles for microRNAs in the onset and development of cardiovascular diseases. So far, microRNAs have been considered as important intracellular mediators in maintaining proper cardiac function and hemostasis, and have been proposed as potential therapeutic targets in cardiovascular disease. The recent discovery that microRNAs circulate in a stable form in many body fluids, including blood, suggests that circulating microRNAs can serve as a new generation of biomarkers for cardiovascular diseases. In this review, we summarize the findings of studies focusing on circulating microRNAs present in human blood cells or plasma/serum, where they potentially could serve as diagnostic or prognostic markers for a variety of cardiovascular pathologies, including acute myocardial infarction, heart failure, coronary artery disease, stroke, diabetes and hypertension. The significance and limitations of microRNAs as the new biomarker generation for cardiovascular disease are also discussed.

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Introduction

MicroRNAs (miRNAs) are a class of small, noncoding RNAs that target and regulate the expression of complementary mRNAs. By affecting protein translation, miRNAs are well-established regulators of most, if not all, biological processes. Deregulation of intracellular miRNA expression and function has been described in many clinical conditions, including several cardiovascular diseases, where it plays a role in cardiac hypertrophy [1, 2]. Fibrosis [3, 4], angiogenesis [5, 6], and heart failure (HF) [2, 7, 8].

Because miRNAs were initially found in intracellular locations, most studies have assessed the expression levels of miRNAs in original tissue samples. Recently, extracellular miRNAs that are likely of placental origin have been found circulating in plasma of pregnant women [9], and in serum/plasma of cancer patients, that may derive from the tumor [10]. The existence of circulating miRNAs indicates resistance to digestion by exonucleases present in serum and other body fluids. In fact, circulating miRNAs remain stable even after exposure to severe conditions that would induce prompt degradation of free RNA, such as extreme pH, high temperatures and prolonged storage [11, 12], likely because circulating miRNAs are non-membrane bound and/or integrated into lipoprotein complexes such as microvesicles and exosomes [13•, 14•, 15•].

While the levels of miRNAs in serum are stable and can be reproducibly detected among healthy individuals, variations in those levels are associated with a variety of clinical conditions, including cardiovascular diseases (CVD). In this review, we highlight the most recent findings indicative of circulating miRNAs as potential clinical biomarkers to monitor CVD.

Circulating microRNAs: Release and Stabilization

Circulating miRNAs are not, per se, intrinsically resistant to RNase activity by exonucleases and endonucleases, but dependent on the formation of a protein-miRNA complex or incorporation into lipidic carriers. From the total circulating miRNA content, 80 % are by-products of dead cells and exist in a non-vesicle form, protein-bound. Proteins shown to be associated with circulating miRNAs that aid in the formation of highly stable complexes are nucleophosmin 1 (NPM1), a nuclear protein implicated in ribosomal processing [16], and Argonaute 2 (Ago2), a component of the RNA-induced silencing complex (RISC) [13•]. It is also known that nucleic acids can bind lipids within lipoproteins [17–19]. High-density lipoproteins (HDL) have been described as carriers in drug delivery to human hepatocellular carcinoma cells in vitro [20]. Recently, lipid proteins were also suggested to bind and transport miRNAs, since HDL from human plasma contains small RNA molecules and miRNAs [21•]. In fact, HDL isolated from humans with familial hypercholesterolemia was enriched in miR-223, mi-105 and mi-206a [21•], suggesting that variations in HDL levels will affect the transport and cell transfer of specific miRNAs.

A smaller proportion of miRNAs is stored in lipid vesicles known as microvesicles, of which several types have been described. Microparticles are shed from the cell membrane of most cell types, either under physiological or pathological conditions, and are relatively large (100 nm to 1 μm) [14•, 22, 23]; exosomes are small vesicles (50–100 nm) derived from the endosomal compartment [15•, 24]; and apoptotic bodies are larger (<4 μm) vesicles that are released from cells undergoing apoptosis [25••]. Incorporation of miRNAs into microvesicles and exosomes occurs selectively. For example, members of the Let-7 family are selectively released into the extracellular milieu through exosomes in metastic gastric cancer, most likely to maintain the cellular oncogenic and metastic properties [26]. Similarly, several human cell lines only secrete certain miRNAs upon serum deprivation, also suggesting that this secretion process occurs as part of a stress response [16]. Moreover, 121 miRNAs were described to associate with exosomes, several of them being more abundant in exosomes compared to the cells of origin [15•], which suggests the existence of mechanisms that regulate packaging. The presence of microRNAs in microvesicles suggests that circulating miRNAs may act as a new intercellular communication system potentially contributing to disease progression [25••, 27]. However, examples of mechanisms by which miRNA paracrine signaling contributes to CVD remain scarce.

Circulating microRNAs: Reflection of the Cardiovascular System?

The interest of the scientific community to study circulating miRNAs in peripheral blood and their potential use as clinical biomarkers of CVD has recently increased. In the past three years, several studies have reported the use of miRNAs as circulating biomarkers for diagnosis/prognosis of cardiovascular diseases such as ischemic heart disease (IHD), stroke, HF, hypertension, atherosclerosis and diabetes mellitus (DM), (Fig. 1 and Table 1).

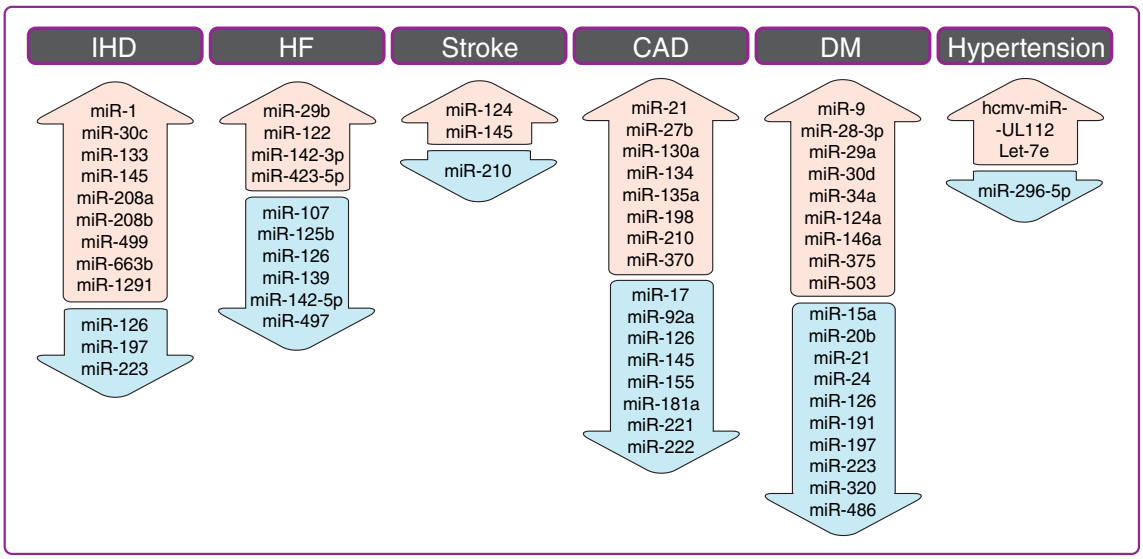

Fig. 1 Overview of Circulating miRNAs in various Cardiovascular Diseases. Each cardiovascular condition has a specific circulating miRNA signature. Results from several relevant studies were combined to give an overview of which miRNAs are elevated in plasma and which ones are decreased in specific cardiovascular diseases. IHD indicates Ischemic Heart Disease; HF, Heart Failure; CAD, Coronary Artery Disease; DM, Diabetes Mellitus

Table 1 Circulating microRNAs as biomarkers for cardiovascular diseases

Group size	Source	miRNAs	Normalization	Multivariate data analysis	Reference
Ischemic Heart Disease					
36 atypical chest pain; 32 MI	Citrate plasma	miR-1, miR-133, miR-208b, miR-499	3 c. elegans spike-in	TnT	[35]
33 STEMI, 17 healthy	EDTA plasma	miR-1, miR-133a, miR-133b, miR-499-5p, miR-208a	miR-17-5p	Age-corrected	[36]
14 AMI, 10 healthy	EDTA plasma	miR-499	synthetic miRNA	No	[38]
31 AMI, 20 healthy	Serum	miR-1	No	CK-MB	[40••]
93 MI, 66 healthy	Citrate plasma	miR-1	RNU6	QRS widening	[43••]
20 MI, 20 healthy	Whole blood	miR-30c, miR-145, miR-663b, miR-1291	No	hsTnT	[44••]
9 MI, 11 healthy	Plasma	miR-1, miR-133, miR-208b, miR-499-5p	No	TnT	[45••]
327 MI, 117 unstable AP	Plasma	miR-1, miR-133, miR-208b	cel-miR-54 spike-in	Age, gender, hsTnT	[63••]
Heart Failure					
33 acute HF, 34 healthy	EDTA plasma	miR-122, miR-499	3 c. elegans spike-in	No	[35]
10 HF, 17 controls (asymptomatic)	EDTA plasma	miR-126	Synthetic spike-in	No	[37]
9 MI, 5 unstable AP, 15 HF, 10 healthy	EDTA plasma	miR-499	Synthetic miRNA	CK-MB	[38]
20 HF, 20 non-HF with dyspnea, 39 healthy	Citrate plasma	miR-18b*, miR-129-5p, miR-423-5p, miR-622, miR-654-3p, miR-1254	miR-1249	Gender and age	[48•]
15 ICM, 19 NIDCM, 19 healthy	PBMC	miR-19b, miR-107, miR-125b, miR-139, miR-142-3p, miR142-5p, miR-497	miR-16	No	[51]
Stroke					
112 stroke patients, 60 healthy	Whole blood	miR-210	No	No	[57]
32 ischemic stroke patients, 14 healthy	Whole blood	miR-145	RNU18	No	[61]
Coronary Artery Disease					
67 CAD, 31 healthy	EDTA plasma	miR-17, miR-92a, miR-126, miR-145, miR-155	cel-miR-39 spike-in	No	[39]
25 unstable AP, 25 stable AP, 20 healthy	PBMC	miR-135, miR-147	let-7a, miR-16	No	[66••]
Diabetes Mellitus					
80 DM, 80 healthy	Plasma	miR-15a, miR-28-3p, miR-29b, miR-126, miR-223	miR-454, RNU6b	BMI, smoking, social status, alcohol, physical activity, C-reactive protein	[46••]
162 impaired glucose tolerance, 580 controls	Plasma	miR-126	miR-454, RNU6b	BMI, smoking, social status, alcohol, physical activity, C-reactive protein	[46••]
19 susceptible, 19 pre-diabetic, 18 DM	Plasma	miR-9, miR-29, miR-30d, miR-34a, miR-124, miR-146a, miR-375	RNU6b	No	[55]
Hypertension					
151 hypertensive patients, 89 healthy	EDTA plasma	HCMV-miR-UL112, let-7e, miR-296-5p	No	No	[78]

MI—myocardial infarction; STEMI—ST elevation myocardial infarction; AMI—acute myocardial infarction; AP—angina pectoris; ICM—ischemic cardiomyopathy; NIDCM—non-ischemic dilated cardiomyopathy; CAD—coronary artery disease; DM—diabetes mellitus; PBMC—peripheral blood mononuclear cell

Ischemic Heart Disease

Acute myocardial infarction (AMI) is characterized by cardiac cell death as a result of exposure to prolonged ischemia after occlusion of a coronary artery [28]. Cell damage induces the release of different proteins into circulation, which, when detected in peripheral blood, can serve as markers of myocardial injury [28, 29]. Because a timely and accurate diagnosis of AMI may possibly decrease the mortality rate [30], proteins such cardiac troponins (Tn), I and T, myoglobin and creatine kinase-MB (CK-MB) have been used extensively in the clinic as standard biomarkers to diagnose AMI [28, 30, 31]. The foremost shortcoming of the currently available diagnostic assays of AMI relies on the fact that a variety of other clinical conditions, including heart and renal failure, can enhance the levels of biomarkers in peripheral blood in the absence of MI [32, 33].

Because miRNAs are expressed in a cell-type and tissue-specific manner, initial studies tested the hypothesis that AMI induces the release of cardiac-specific miRNAs from injured cardiomyocytes into circulation. These studies identified miR-208 as an exclusive cardiac miRNA [34] that is not detectable at baseline, but rapidly increases in circulation after MI [35••, 36••]. In plasma of AMI patients, miR-208b was the most upregulated miRNA (~3000 times more), compared to healthy subjects [36••]. Furthermore, elevated miR-208b levels correlated with the presence of TnT, indicating myocardial injury [35••, 37]. In a larger population study (n=444) comparing patients with non-ST-elevation myocardial infarction (NSTEMI), ST-elevation myocardial infarction (STEMI), and unstable angina [37], miR-208b was only detectable in NSTEMI and STEMI patients. While mortality at 6 months after MI directly correlated with elevated circulating levels of miR-208b, subjects with undetectable miR-208b had the best prognosis. However, after adjustment for TnT, the association of circulating miR-208b levels with the patient outcome was no longer present [37], which questions the prognostic value of measuring circulating miR-208b levels at this time point.

The use of miR-208a as a biomarker for AMI remains controversial. A single study described a significant increase in circulating miR-208a in AMI patients [38], and showed high sensitivity and specificity of this miRNA for diagnosing MI, based on the receiver operating characteristic (ROC) curve analysis (area under the curve, AUC=0.97). However, others could not detect or could only show very low levels of miR-208a in AMI patients [36••, 39, 40••]. These differences might relate to the timing of sampling, since miR-208a peaks 3 hours after MI and is restored to baseline after 24 hours [39]. A different explanation could be the baseline expression differences for miR-208 between human and mouse. miR-208 belongs to the myomiR family of miRNAs, with miR-208a being encoded within an intron of a-cardiac muscle myosin heavy chain (α-MHC) while miR-208b is encoded within an intron of β-MHC [41]. β-MHC is the main isoform in adult human hearts, while in rodents it is the α-MHC [42]. As expression levels of these miRNAs normally follow their host genes, their expression levels in rodent and human are likely to be different. Altogether, this could potentially explain why independent studies were not able to detect miR-208a, even when collecting blood samples at suitable time points [39, 40••, 43••]. Finally, threshold cycle values obtained by TaqMan PCR showed that miR-208 is difficult to detect compared to other miRNAs [43••].

The previously mentioned large population study, where specific miRNAs (heart-associated, fibrosis-associated or leukocyte-associated) were measured in plasma of patients with different cardiac conditions, including AMI, viral myocarditis, diastolic dysfunction and acute HF, demonstrated select increases in circulating miR-499 only in AMI patients, and expression levels relapsed to normal by time of hospital discharge [38]. Similar to miR-208b, miR-499 is not detected in plasma of healthy subjects, but is elevated after AMI [40••]. ROC curve analysis revealed an AUC of 0.92 for miR-499, indicating this miRNA as an ideal biomarker for AMI. Furthermore, miR-499 was confirmed as a sensitive marker of cardiac damage, increasing simultaneously with troponin I (TnI) after coronary occlusion, in patients, but also in animal models, of AMI [36••].

In AMI patients, miR-1 and miR-133 are also increased [35••], although to a lesser extent as miR-499. The advantage of miRNAs compared to already well established cardiac biomarkers could be in their rapid timing of release upon myocardial damage. Two independent studies analyzed the kinetics of miRNA release in patients with or without AMI, and determined the potential of miR-1 as a biomarker of this cardiac condition [40••, 43••]. MiR-1 was elevated in plasma and serum 6 hours after infarction in patients with AMI, and correlated well with TnT [39], serum creatine kinase-M levels [43••], and QRS widening [44••]. In an earlier time point (156 minutes), miR-1 and miR-133 levels displayed a higher peak than TnI, indicative of their earlier release after MI [39]. In animal models of AMI, levels of circulating miR-1 and miR-133 rapidly increased after infarction and returned to baseline 3 days after occlusion [36••, 43••]. Both miRNAs are known to be anti-hypertrophic, and their expression levels were decreased 6 hours after coronary artery occlusion (CAO) in the infarcted and border zone areas of the heart. The observed increase in systemic levels of miR-1 and miR-133 after AMI indicates release of these miRNAs into circulation after myocardial injury. A larger cohort with 159 subjects, with and without AMI, confirmed increased levels of miR-1 in circulation, but those were not associated with age, gender, or the established AMI biomarkers, which further was reflected by

a ROC curve of 0.74 for separating AMI from non-AMI individuals [44••]. Additionally, plasma levels of miR-1 might be affected by renal elimination, since they strongly correlate with the renal glomerular filtration rate [45••]. Because AUC for miR-1 varies from 0.74 [44••] to 0.98 [38, 45••] in the different studies, miR-1 could be considered as fair to excellent biomarker for AMI.

Other circulating miRNAs reported to be increased after AMI are miR-1291 and miR-663b [44••]. miRNA-1291 is able to predict the presence of AMI with specificity and a sensitivity of 85 % (17 of the 20 disease samples), while miRNA-663b shows a specificity of 95 % (19 of the 20 disease samples) and a sensitivity of 90 % [46••]. Although systemic levels of miR-1291 and miR-663b correlated with TnT release, the highest correlation, with correlation coefficients up to 0.71, was observed for miR-145 and miR-30c [46••]. Recently, a prospective study on the association of circulating miRNAs with incidence of MI showed negative associations for miR-197 and miR-223, but showed a positive association between miR-126 and development of MI [47] Moreover, this study emphasizes the contribution of platelets in maintaining the circulating miRNA pool [47].

Altogether, and despite some discrepancies, miRNAs that are released as a response to myocardial damage may represent an appropriate alternative to the established biomarkers in early detection of AMI.

Heart Failure

Recent research has demonstrated that specific plasma miRNA expression profiles exist in patients with chronic heart failure (CHF). In a study where classification of CHF subjects was based on circulating NT-proBNP levels above 1000 ng/L, a microarray approach comparing plasma from 12 CHF patients and 12 healthy subjects described differential expression of 108 miRNAs in CHF patients [48•]. Patients with recent history of cardiac ischemia or infarction were excluded in order to rule out the influence of cardiac cell death on circulating miRNAs levels. From those differentially expressed miRNAs, miR-423-5p was specifically enriched in CHF and with an AUC of 0.91, the ROC curve analysis further established this miRNA as a predictor of HF. One could, however, question the array-based approach taken for the detection of miRNAs in plasma in this study, since, in general, low detection rates were observed [49•]. Nevertheless, increased levels of circulating miR-423-5p were associated with increased N-terminal prohormone brain natriuretic peptide (NT-proBNP) and lower left ventricular ejection fraction. Although miR-423-5p is threefold upregulated in human failing myocardium [7], suggesting that increased plasma levels are derived from the myocardium, it is still uncertain whether it originates from the failing heart or from other organs through different release mechanisms. In healthy subjects, miR-423-5p was shown to specifically associate with Ago2 complexes, independent of vesicles. However, it is not clear whether this is also true for HF patients. According to the exclusion of patients with recent ischemic episodes, no elevated levels of miR-1, miR-208a/b and miR-499 were observed in the group of HF patients selected [50]. This is, to some extent, in contrast with previous results showing that only circulating miR-499 levels were found in patients with acute HF, but miR-208 and miR-1, also predictive of AMI, were not elevated in CHF [35••]. On the one hand, this discrepancy could reflect a specific circulating miRNA response to different pathological conditions as elevated levels of circulating miR-208b and miR-499 were observed in patients with viral myocarditis [35••], while on the other hand, it may also reflect a technical failure in detecting major changes in circulating muscle-specific miRNAs in CHF. Whether improved assays may aid in a more reliable assessment of the low levels of myocardial enriched myomiRs remains to be determined.

The endothelial-enriched miR-126 was reported to inversely correlate with age (R^2=0.52, P<0.001) and severity of HF in patients, based on brain natriuretic peptide levels (R^2=0.25, P<0.0005) and New York Heart Association (NYHA) classification [37]. Although this could suggest miR-126 as a useful biomarker for HF, the same inverse correlation for miR-126b was also observed in patients with coronary artery disease (CAD) [39] and in diabetic patients [46••], questioning the potential of miR-126 to be used as a specific biomarker for heart failure, as this could be secondary to worsened endothelial function and angiogenesis. A different study compared miRNA expression profiles in peripheral blood mononuclear cells PBMCs from HF patients either with ischemic (ICM) or non-ischemic dilated cardiomyopathy (NIDCM) [51]. Both HF conditions displayed decreased levels of miR-107, miR-139 and miR-142-5p. Additionally, ICM fractions displayed decreased miR-125b and miR-497 levels and NIDCM fractions displayed increased miR-142-3p and miR-29b levels. Altogether, the fact that miRNA expression is altered in PBMCs derived from both NIDCM and ICM patients versus control subjects suggests a way to discriminate between healthy individuals and CHF patients, by measuring a small number of specific miRNAs. Further studies are necessary to test whether the identified miRNAs may constitute a useful tool for patient diagnosis and treatment. Interestingly, miR-122, a liver-specific miRNA associated with hepatic damage, is also increased in HF patients most likely reflecting hepatic venous congestion [35••].

Overall, it is clear that more conclusive studies are needed to select the miRNAs with the highest potential to become biomarkers of HF.

Stroke

Strokes are mostly caused by obstructions in brain blood vessels (ischemic stroke, ~ 85 % of cases) and, less often, by a disruption (hemorrhagic stroke) of a brain blood vessel. While 10 % of the subjects die after ischemic stroke, in general, the prognosis for hemorrhagic stroke is even worse, with 38 % of the cases resulting in death within 30 days [52]. One of the critical steps within the first minutes following initial symptoms is to distinguish between both types of stroke. At present, stroke is diagnosed based on the patient examination by a clinician, complemented by brain imaging. However, clinical assessment of stroke is not always straightforward, since computerized tomography images are often not conclusive and do not detect mild ischemic strokes. Furthermore specific biological markers to distinguish between different types of stroke are not yet available.

Because miRNA expression can be tissue-specific and miRNAs are remarkably stable in plasma, it is very tempting to consider miRNAs as potential biomarkers in stroke diagnosis. Initial studies investigated the use of miR-124, a brain-specific miRNA, as a circulating marker of cerebral ischemia in rats where ischemic conditions were induced by middle cerebral artery occlusion (MCAO). miR-124 levels were greatly increased in plasma after MCAO and peaked at 24 hours (up to 150-fold compared to sham-operated animals), indicating its ability to serve as a systemic biomarker of cerebral ischemia and opening the possibility to probe for brain-specific miRNAs as biomarkers of tissue injury [49•].

Other attempts to profile miRNA expression patterns in both blood and brain of a rat MCAO model revealed specific regulation patterns of seven miRNA clusters, suggesting miRNAs as potential useful biomarkers in stroke and associated pathologies [50]. Analysis of miRNA expression profiles in peripheral blood of young patients with ischemic stroke revealed a set of miRNAs that were commonly modulated both in patients and in the MCAO rat model (miR-124, let-7c, miR-16, miR-19b, miR-23a, miR-103, miR-106b, miR-185, miR-191, miR-320, miR-451, and miR-298). Differential miRNA expression among experimental samples correlated not only with different stroke causes (large vessel atherosclerosis, cardioembolism, small vessel disease or unknown cause) [53], but also with the cellular processes involved (endothelial, vascular and neural function, erythropoiesis and angiogenesis) [54, 55]. Furthermore, in another comparative study, blood and brain miRNA expression profiles of rat models of ischemic and hemorrhagic stroke revealed unique miRNA patterns for each experimental condition. both in blood and brain [56]. Among the regulated miRNAs, miR-210 was decreased after brain ischemia. These results were validated by the observation of decreased miRNA-210 in blood samples of stroke patients, with a minimum at 7 days and 14 days after stroke onset [57], and were further confirmed in a mouse model of stroke [57]. These results are intriguing because miR-210 upregulation is considered a hallmark of the hypoxic response in many cell types [58]. Similarly, high miR-210 expression is also observed in ischemic myocardium and in biopsies and plasma samples of cancer patients [59]. Because miR-210 expression was higher in stroke patients with beneficial outcome, the decreased miR-210 may be a reflection of damage severity, which would also be in line with the previously described anti-apoptotic role of miR-210 [59, 60]. Notwithstanding, the specificity of the brain response to ischemia needs further investigation, not only regarding miR-210, but also other brain-specific miRNAs that might be of interest as potential biomarkers of human acute ischemic stroke [49•].

Finally, a recent study has proposed miR-145 as a potential biomarker for ischemic stroke [61]. Circulatory miR-145 levels are increased in peripheral blood of ischemic stroke patients, as compared to control. Although this finding may have implications for the development of suitable markers for ischemic stroke, no ROC curve analysis has been performed and the source of circulating miR-145 remains to be identified. So far, miR-145 is speculated to derive from circulating progenitor cells that are prone to differentiate into vascular smooth muscle cells (VSMCs) [61], because expression of miR-145 in neural crest stem cells is enough to induce differentiation into VSMCs [62] by targeting KLF4/5 proteins [63••] and myocardin [64].

To conclude, while the previous studies suggest several circulating miRNAs as genomic biomarkers to rapidly identify stroke, stroke subtypes, and possibly therapy outcome, so far, no relationship between miRNA levels and other promising markers of stroke have been established. Additionally, no brain imaging or ROC/AUC levels were used to supplement the described observations.

Coronary Artery Disease

Atherosclerosis, the major cause of Coronary Artery Disease (CAD), is characterized by endothelial activation, lipid accumulation and macrophage infiltration resulting in plaque formation, narrowing of the arterial lumen and in hardening and thickening of the arterial walls. As plaque formation induces thrombus formation, the risk of myocardial infarction and arrhythmias also increases once partial arterial blockage rapidly progresses to complete occlusion [65]. Moreover, CAD occurrence and progression are highly variable and are driven by both environmental factors and genetic determinants. Because CAD is a major cause of death in developed nations [39, 66••], the identification of individuals with unstable plaques and, therefore, at risk of acute coronary syndromes, has great clinical relevance. To

this end, specific plasma proteins such as fibrinogen, von Willebrand factor and C-reactive protein (CRP) have been established as markers of CAD [65]. However, the value of these markers in the diagnosis of cardiovascular conditions is still limited, since they can be affected by CAD-unrelated environmental factors and disease backgrounds [65]. Moreover, the available imaging techniques mainly detect disease at end stages, with limited value for the early diagnose of CAD. Overall, there is a need for consistent and innovative markers of plaque stability and atherosclerosis.

miRNAs may also be used to identify patients at risk for clinical conditions related to CAD, and a few studies have addressed their potential role as such. A first study that followed a microarray approach to assess the circulating miRNA signatures in plasma from eight CAD patients and eight healthy subjects [43••] identified 20 significantly upregulated and 46 downregulated miRNAs. Validation of the results in a larger patient cohort by qPCR revealed that most of the downmodulated miRNAs were highly expressed in endothelial cells and, subsequently, in the vessel wall. These included the vascular miRNAs, miR17, miR-92a and miR-126, the inflammation-associated miR-155 and the smooth-muscle miRNA, miR-145. Cardiac miRNAs, such as miR-133 and miR-208a [39], were confirmed not to be upregulated in the larger validation cohort, which is in line with the idea that these miRNAs are specific markers of AMI. Since the onset of atherosclerotic lesions directly correlates with endothelial cell activation and microvesicle release, the fact that circulating vascular miRNAs are decreased in CAD may suggest lower numbers of circulating progenitor endothelial cells, as described previously [67]. Age differences between the disease and control groups may also account not only for the differences observed in miR-126 levels, but also the decreased circulating levels of miR-155. miR-155 is mainly released by inflammatory cells, and since atherosclerosis is closely related to inflammation, one would expect increased levels of circulating miR-155 in CAD patients. Similarly, decreased miR-92a in patients with CAD is still not well understood, since recent data also suggests a proatherosclerotic function, with endothelial cell impairment, for miR-92a [5, 54]. In a different study, a qRT-PCR based approach revealed a consistent increase (fivefold) in miR-135a and a decrease (fourfold) in miR-147 in PBMCs from CAD patients versus healthy controls, suggesting that the miR-135a/miR-147 ratio could serve as a promising biomarker for CAD [66••] Furthermore, patients with unstable angina pectoris could be discerned from stable patients, due to increased levels of three miRNAs: miR-134, miR-198 and miR-370, further exemplifying the use of specific miRNA signatures in the identification of patients at risk for acute coronary syndromes [66••]. Besides having included relatively small cohorts, RNA pools of each patient group were used, and no validation of results was performed in larger cohorts.

Patients with peripheral arterial disease displayed increased levels of miR-21, miR-27b, miR-130a, and miR-210, while miR-221 and miR-222 were decreased [68•]. Remarkably, the expression pattern of circulating miRNAs was similar to the one obtained from the intima of the same patients and the increase in miR-27b and miR-130-a correlated with disease severity [68•]. Finally, a recent study including two independent cohorts of 21 morbidly obese and 125 high-risk obese and non-obese patients, showed decreased levels of miR-181a in blood samples of obese patients, suggesting miR-181a as a putative biomarker of CAD and metabolic syndrome [69]. Although automation and lab-on-a-chips are needed to fully characterize the potential of monocytic miRNAs as biomarkers, this study is the first one to propose a monocytic miRNA as probe for obesity and associated clinical conditions.

Diabetes Mellitus

Type 2 diabetes mellitus (T2DM), a major risk factor of CVD, is characterized by increased systemic glucose levels and insulin resistance, often causing endothelial dysfunction and vascular complications [70, 71]. Although many risk factors are known, among which are age, sex, triglycerides, HDL, cholesterol, hypertension, impaired glucose tolerance, parental diabetes and ethnicity, it remains difficult to identify patients at risk for T2DM [72]. This is in part related to the fact that a considerable part of the population at risk does not display the standard risk factors, and score very low when using the current prediction models [72]. Many biomarkers have been established for diabetes diagnosis, such as CRP, triglycerides, HDL, adiponectin, insulin and different types of chemokines. Nevertheless, diabetes risk screenings have not yet been generally implemented and it is becoming clear that these should also account for the risk of CVD. Such a combinatorial approach would allow the identification of patient subgroups and the development of more exclusive therapies. For the reasons above, there is a need for the identification of novel biomarkers for the prediction of T2DM and its cardiovascular risks.

A first systematic analysis of circulating miRNAs in plasma of diabetes patients showed decreased levels of 10 miRNAs in those individuals (miR-15a, miR-20b, miR-21, miR-24, miR-126, miR-191, miR-197, miR-223, miR-320 and miR-486), and a mild increase in miR-28-3p [46••]. The authors suggest that the five most significant regulated miRNAs are both necessary and sufficient to distinguish DM patients (70 %) from controls (92 %) [46••]. Furthermore, subjects with normal glycemic levels were classified and predicted to develop DM in the following 10 years, and this was confirmed (52 %) in the 10-year follow-up [46••]. This study also revealed that a decrease in circulating miR-126 expression is associated with the risk for future

development of diabetes, which is also consistent with the vasculoprotective properties of miR-126 [25••, 55, 73]. These results were not only confirmed by in vitro experiments showing downregulation of miR-126 expression in microparticles derived from endothelial cells that were exposed to glucose, but also in vivo using in diabetic mouse models [46••]. miR-126 was recently shown to be downregulated in endothelial progenitor cells (EPCs) from diabetic patients, impairing EPC-mediated functions by targeting Spred1, an intracellular inhibitor of Ras/extracellular signal-regulated kinase (ERK) cascade [74]. Decreased miR-126 levels may inhibit EPC proliferation and migration while promoting apoptosis and thus impairing new blood vessel formation or repair of pre-existing vasculature. Altogether, these miRNAs could represent potential biomarkers for the diagnosis, and maybe more interesting, prediction of DM.

In serum samples of recently diagnosed T2DM patients compared to T2DM-susceptible subjects with normal glucose tolerance [55], seven miRNAs (miR-9, miR-29a, miR-30d, miR-34a, miR-124a, miR-146a and miR-375) were shown to be elevated. Because all these miRNAs have been previously related to insulin regulation [75], it is tempting to speculate that an elevated miRNA level in serum of DM patients mainly originates from β-cells. Finally, a recent study described release of miR-503 from endothelial cells after exposure to high glucose and low concentration of growth factors as an in vitro model of DM with hyperglycemia accompanied by tissue starvation [76••]. Overexpression of miR-503 in endothelial cells impaired endothelial functions such as proliferation, migration and adhesion, which were again restored in loss-of-function in vitro experiments. Elevated circulating miR-503 levels were also present in serum samples of diabetic patients with severe ischemia, compared to control subjects. Furthermore, downregulation of miR-503 by a gene therapy approach based on adenoviral-mediated delivery of a 3′-UTR decoy for miR-503, to downregulate miR-503 activity in ischemic muscles, improved neovascularization in a mouse model of diabetes [76••]. These results not only imply miRNA-503 as a diagnostic tool, but also as potential therapeutic target in DM.

Idiopathic Hypertension

Idiopathic hypertension, also known as primary or essential hypertension, is a major risk factor for AMI, HF, stroke and chronic renal failure [77]. Microarray-based miRNA expression profiling identified 27 differentially regulated miRNAs in plasma samples from hypertensive patients and healthy subjects [78]. One of those was the human cytomegalovirus (HCMV)-encoded miRNA, hcmv-miR-UL112. Because this suggests an association between HCMV infection and risk for hypertension, the authors measured HCMV titers and observed that these were higher in hypertensive patients as compared to healthy subjects [79]. Additionally, HCMV titers strongly correlated with hcmv-miR-UL112 in hypertensive patients ($P=0.003$). While this study is the first to use a genome-wide expression array platform in the discovery of possible regulatory roles for miRNAs in hypertension, it remains unclear whether hcmv-miR-UL112 might become a biomarker in the diagnosis of hypertension.

Circulating microRNAs: Effects on the Cardiovascular System?

Besides of their potential role as biomarkers, circulating miRNAs may also have a role in influencing gene expression at intracellular locations. This would, however, implicate cellular uptake of circulating miRNAs, which as already been shown for RNA [7, 25••]. In fact, several studies have suggested that circulating miRNAs can be transferred to target cells and regulate gene expression. Administration of miR-126 in apolipoprotein $E^{-/-}$ mice resulted in miRNA delivery to atherosclerotic lesions through apoptotic bodies, and resulted in reduced lesion formation [27]. Another study, besides showing transfer of HDL-associated miRNAs to cultured hepatocytes [24], also isolated HDL from individuals with familial hypercholesterolemia. This HDL, enriched in miR-105, was able to deliver the miRNA to target cells, which very rapidly accumulated increased levels of miR-105. So far, most of the studies showed that microvesicles and lipids are able to modulate miRNA levels in recipient cells. However, the possibility that the observed effects are due to miRNA-unrelated signaling pathways could not be excluded. Therefore, more properly controlled research is necessary to conclude that circulating miRNAs can be delivered to and modulate specific processes in the recipient cells.

Conclusions: Significance and Limitations of Circulating microRNAs

In general, the scientific community has great expectations for the use of circulating miRNA as noninvasive biomarkers for the diagnosis, prognosis and therapeutic appraisal of CVD. The fact that miRNA levels in serum plasma are detectable and very stable, blood collection is a common clinical procedure, different individuals within the same species display similar levels of circulating miRNAs, expression profiles are tissue-specific, and differential expression levels of circulating miRNAs have been associated with several disease processes, suggests the potential for using circulating miRNAs as a new generation of biomarkers for CVD. Moreover, most studies indicate that

miRNAs are more sensitive and specific when compared to well-established, conventional, blood-based markers. In fact, inclusion of circulating miRNAs will likely enhance the currently available diagnostic tools and increase the ability to diagnose specific CVDs. Importantly, because many studies have reported regression of circulating miRNA levels to baseline following recovery from AMI [40••, 43••] and after chemotherapy in different types of cancer [80, 81], levels of circulating miRNAs may also aid in assessing treatment efficacy.

Despite all scientific observations so far, several limiting aspects need to be addressed before establishing miRNAs as new clinical diagnostic tools for CVD. To date, no standardized method for detection and quantification of circulating miRNAs in plasma and serum samples has been established, making it difficult to compare miRNA expression profiles generated by different academic laboratories throughout the world. While the most used system of miRNA detection is qRT-PCR, standardization of measurement protocols is still required. Second, the choice of normalization is critical for evaluating circulating miRNA levels by qRT-PCR strategies. Most studies use housekeeping genes or miRNAs as normalizers, while others use equal amounts of serum among all samples, both approaches including spike-in normalization. While housekeeping genes and miRNAs have been suggested to vary in their expression levels within serum samples [11, 82], and equal volumes of serum may generate different amounts of total RNA [83], spike-in normalization does not account for internal variations in circulating miRNA levels between distinct individuals nor for stability of synthetic miRNAs in plasma. Altogether, combined methods should always be applied to assure robustness of findings independent of the normalization approach. Third, the majority of studies thus far included relatively small patient cohorts and should, therefore, be considered as pilot studies prior to confirmation and validation of results in larger sample groups. Moreover, it is important to relate levels of circulating miRNAs with previously established markers of CVD; so far, only a few studies have included these controls in their approach. Finally, efforts to better understand the biological processes controlling miRNA release and stability are needed. Although the correlation between circulating and tissue miRNAs is still controversial, increasingly more arguments are generated against the hypothesis that miRNA levels in blood are a direct reflection of the changes that took place in the tissues of origin, as miRNAs could also derive from immune cells. While miRNA-based diagnostic assays have been developed and fully validated for diseases such as lung cancer and pancreatic adenocarcinoma [84–86], circulating miRNAs are only emerging as potential diagnostic and prognostic biomarkers in CVD and future research will be needed to establish their role as paracrine signaling molecules.

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