Review

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microRNAs in cardiovascular disease – clinical application

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Abstract: microRNAs (miRNAs) are well-known, powerful regulators of gene expression, and their potential to serve as circulating biomarkers is widely accepted. In cardiovascular disease (CVD), numerous studies have suggested miRNAs as strong circulating biomarkers with high diagnostic as well as prognostic power. In coronary artery disease (CAD) and heart failure (HF), miRNAs have been suggested as reliable biomarkers matching up to established protein-based such as cardiac troponins (cT) or natriuretic peptides. Also, in other CVD entities, miRNAs were identified as surprisingly specific biomarkers – with great potential for clinical applicability, especially in those entities that lack specific protein-based biomarkers such as atrial fibrillation (AF) and acute pulmonary embolism (APE). In this regard, miRNA signatures, comprising a set of miRNAs, yield high sensitivity and specificity. Attempts to utilize miRNAs as therapeutic agents have led to promising results. In this article, we review the clinical applicability of circulating miRNAs in CVD. We are giving an overview of miRNAs as biomarkers in numerous CVD entities to depict the variety of their potential clinical deployment. We illustrate the function of miRNAs by means of single miRNA examples in CVD.

Keywords: biomarker; cardiovascular disease; diagnostic; microRNA; prognostic.

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Introduction

microRNAs (miRNAs) are short non-coding RNAs that bind messenger RNAs (mRNAs) and either initiate mRNA degradation or translational repression and consecutively regulate gene expression on a post-transcriptional level [1–4]. From a functional point of view, miRNAs are crucial regulators of cellular pathways in proliferation and differentiation as well as apoptosis, stress response, and tumorgenesis [5]. miRNAs mediate gene expression and regulate phenotypic control in the cell of origin and serve as key regulators of metabolism [6–8] – their expression patterns are specific for different organs and cell types [9]. miRNAs are also secreted into different types of body fluid such as blood and urine [10], which allows them to transmit their signal to different cells and tissues [11]. This has led to their exploration as emerging circulating biomarkers for a variety of diseases [12–14]. Furthermore, miRNAs are intensively explored as therapeutic agents and promising targets of disease modeling by specifically altering miRNA levels in particular diseases [15, 16].

In cardiovascular disease (CVD), the utilization of miRNAs as diagnostic biomarkers for specific disease entities such as coronary artery disease (CAD), myocardial infarction (MI) and heart failure (HF) has been explored in numerous experimental studies and patient cohorts, putting them on the verge of implementation in clinical disease evaluation [8, 17]. The exploration of miRNAs as therapeutic CVD agents has taken major steps forward and encouraging results from in vitro and in vivo experiments give rise for promising future application in human trials [18, 19].

Meanwhile, the implementation of miRNA-based diagnostics and therapy in clinical routine is hampered by technical and analytical difficulties such as normalization problems, unexplored effects of influencing factors in quantification analyses, and suboptimal time-effectiveness [17].

This review article summarizes the latest knowledge of miRNA research in CVD, focusing on CAD and HF in particular. We will describe the current scientific state of miRNA exploration as circulating biomarkers and give an overview to what extend miRNAs might influence

therapeutic strategies. Covering all miRNAs involved in different CVD entities is beyond the scope of this article; therefore defined examples of important miRNAs will be addressed. An overview of the selected miRNA examples is given in Table 1.

miRNAs in cardiovascular disease

The clinical relevance of miRNAs in CVD becomes clear when we look at the numerous pathways that are affected by miRNA regulation. For example, miRNAs function as regulators of metabolic pathways such as lipid metabolism and glucose homeostasis, which are highly involved in vascular disease [51]. Besides their role in metabolism, miRNAs are also key regulators in vascular cells and endothelial cells (EC) [51]. A decade ago, the first in vivo description of the relevance of miRNAs in heart disease was made in cardiac hypertrophy and HF of mice and humans [52]. Shortly afterward, Ikeda et al. for the first time showed that miRNA expression profiles are specifically altered in different entities of heart disease comprising distinct changes of single miRNA expressions [53]. These results reflect the tissue- and cell type-specific regulation of miRNAs and gave rise to screening projects to identify single miRNAs in CVD, their mode of function, and importantly, their potential for clinical application [54].

miRNAs in vascular integrity and Endothelial cell function

During the process of atherosclerotic plaque development, defined sets of miRNAs have been found in different stages of plaque progression [55]. Additionally, miRNA dysregulation plays an essential role in the destabilization and rupture of atherosclerotic plaques [56]. miRNAs specifically alter a diversity of signaling pathways that affect proliferation, differentiation, migration, and cell survival in EC and smooth muscle cells. Zampetaki and Mayr correlated miRNAs with angiogenesis and the development of vascular disease and interlinked their altered expression profiles with proteomic and metabolomic changes [51].

Reduced function of EC significantly contributes to vascular inflammation, which in turn facilitates the development of atherosclerosis and CVD. A group of miRNAs, termed angiomiRs, plays a major role in the mediation and regulation of angiogenic processes. Among them are miR-210, miR-221, miR-222, miR-126-3p, mir-92a, and miR-132 [57]. Especially, miR-126-3p is a key regulator in the maintenance of vascular integrity and serves as a pro-angiogenic factor, which is specific for the vascular system [58]. MiR-126-3p regulates EC gene expression and is involved in EC dysfunction as well as atherosclerosis triggered by alterations of blood flow conditions [59] as they occur in CAD. MiR-126-3p$^{-/-}$ (miR-126-3p knock-out) mouse embryos suffered from systemic edema multifocal hemorrhages, and ruptured blood vessels throughout embryogenesis [58]. From a clinical point of view, miR-126-3p overexpression was shown to reduce atherosclerosis [60]. Thus, miRNAs might be important regulators during angiogenesis after ischemic and hypoxic events due to diminished angiogenic response. Moreover, the current state of knowledge suggests miR-126-3p as a promising therapeutic target to enhance neo-angiogenesis and cardiac repair with a focus on ischemic myocardium and thus in CAD [54]. The modulating effect of miR-126-3p on EC strongly suggests the potential for clinical applicability in CVD as diagnostic and/or prognostic biomarker on the one hand and as potential therapeutic targets on the other.

Besides EC, it was recently shown that miR-126-3p is present in platelets and modulates platelet aggregation [61]. Inhibition of miR-126-3p in mice attenuates platelet aggregation and levels of circulating miR-126-3p show positive correlations with platelet function tests in mice and men [62]. These findings interlink miR-126-3p with platelet-associated vascular and EC function and raise further questions as to what extend pharmacological platelet inhibition might interfere with accurate quantification measures in miRNA analyses in general (see below).

Further efforts are needed to explore the gaps in the knowledge of miRNAs in CVD pathophysiology and progression in a translational fashion – linking clinical findings with molecular understanding.

miRNAs in coronary artery disease and myocardial infarction

Atherosclerosis is the major contributor to CAD and MI and thus leads to CVD. The complex balance of pro- and anti-angiogenic signaling pathways is dysregulated, resulting in pathological angiogenesis and vascular inflammation, which contribute to the development and progression of CAD and consecutively MI. Ischemia-reperfusion can serve as a model of unstable CAD. In such a model, dysregulated levels of specific miRNAs were identified compared with healthy controls [63]. While miR-1, miR-126-3p, and miR-208 were increased, miR-21, miR-133 and miR-195

Table 1: miRNAs dysregulated in different CVD entities.

Disease entity	Value as biomarker	microRNA	Biomaterial	Direction of alteration	ROC Analysis	Comparison with established biomaker	Reference
ACS/AMI/UAP	Diagnostic	miR-1	Plasma	Up-regulated in AMI vs. UAP	No	Independently associated with hs-TnT	[20]
	Diagnostic	miR-92a	Serum	Up-regulated in UAP vs. SAP	No	No correlation to cTnI levels	[21]
	Diagnostic	miR-133a	Plasma	Up-regulated in AMI vs. UAP	No	Independently associated with hs-TnT	[20]
	Diagnostic	miR-208b	Plasma	Up-regulated in AMI vs. HC	Yes	Inferior to cTnT	[22]
	Diagnostic	miR-208b	Plasma	Up-regulated in AMI vs. UAP	No	Independently associated with hs-TnT	[20]
	Diagnostic	miR-486	Serum	Up-regulated in UAP vs. SAP	No	No correlation to cTnI levels	[21]
	Diagnostic	miR-499-5p	Plasma	Up-regulated in AMI vs. HC	Yes	Inferior to cTnT	[22]
	Diagnostic	miR-499-5p	Plasma	Down-regulated in NSTEMI vs. HC	Yes	Diagnosis of NSTEMI:Similar to cTnT Differentiation of NSTEMI vs. CHF superior to cTnT and hs-cTnt	[23]
	Diagnostic	Signature: miR-1 miR-21 miR-499	Serum	Up-regulated in ACS vs. HC	Yes	Superior to hs-cTnT	[24]
	Diagnostic	Signature: miR-134 miR-198 miR-370	PBMC	Up-regulated in UAP vs. SAP	No	No	[25]
	Diagnostic	Signature: miR-132 miR-150 miR-186	Serum	Down-regulated in UAP vs. NCCP	Yes	Superior to hs-cTNI in differentiating UAP from NCCP	[26]
	Diagnostic	Signature: miR-1 miR-134 miR-186 miR-208 miR-223 miR-499	Serum	Up-regulated in AMI vs. HC	Yes	Superior to cTnT and CK-MB in differentiating AMI from UAP	[27]
	Diagnostic	Signature: miR-142-5p miR-498 miR-492 miR-1281, miR-497* miR-151-5p miR-802 miR-23b* miR-455-3p miR-1250 miR-380* miR-135b* miR-345 miR-566 miR-631 miR-1254 miR-139-5p miR-892b miR-146b-3p	Whole blood	Differentially dysregulated in AMI	Yes	Earlier diagnosis of AMI compared with cTnT	[28]

Table 1 (continued)

Disease entity	Value as biomarker	microRNA	Biomaterial	Direction of alteration	ROC Analysis	Comparison with established biomaker	Reference
	Prognostic	miR-106a-5p miR-424-5p let-7g-5p miR-144-3p miR-660-5p	Serum	Differentially dysregulated in future fatal AMI	Yes	No	[29]
	Prognostic	miR-126 miR-197 miR-223	Plasma	Differentially dysregulated in future AMI	No	No	[30]
	Prognostic	miR-126 miR-197 miR-223	Serum	Upregulated in future cardiovascular death	No	No	[31]
	Prognostic	miR-19a miR-19b miR-132 miR-140-3p miR-150 miR-186 miR-210	Serum	Up-regulated in future cardiovascular death	No	No	[32]
HF	Diagnostic	miR-22	Serum	Up-regulated in HF vs. HC	No	Association with NT-proBNP	[33]
	Diagnostic	miRNA-26b-5p	Plasma	Down-regulated in HF vs. HC	Yes	Inverse correlation with NT-proBNP	[19]
	Diagnostic	miRNA-29a-3p	Plasma	Down-regulated in HF vs. HC	Yes	Inverse correlation with NT-proBNP	[19]
	Diagnostic	miRNA-30e-5p	Plasma	Down-regulated in HF vs. HC	Yes	Inverse correlation with NT-proBNP	[19]
	Diagnostic	miR-30b	Plasma	NA	Yes	Inferior to NT-proBNP and hs-cTnT	[34]
	Diagnostic	miRNA-92a-3p	Plasma	Down-regulated in HF vs. HC	Yes	Inverse correlation with NT-proBNP	[19]
	Diagnostic	miR-92b	Serum	Up-regulated in HF vs. HC	No	Association with NT-proBNP	[33]
	Diagnostic	miR-103	Plasma	no	Yes	Inferior to NT-proBNP and hs-cTnT	[34]
	Diagnostic	miR-142-3p	Plasma	No	Yes	Inferior to NT-proBNP and hs-cTnT	[34]
	Diagnostic	miRNA-145-5p	Plasma	Down-regulated in HF vs. HC	Yes	Inverse correlation with NT-proBNP	[19]
	Diagnostic	miR-208b	Plasma	Elevated after AMI	Yes	Correlating with cTnT	[35]
	Diagnostic	miR-320a	Serum	Up-regulated in HF vs. HC	No	Association with NT-proBNP	[33]
	Diagnostic	miR-342-3p	Plasma	No	Yes	Inferior to NT-proBNP and hs-cTnT	[34]
	Diagnostic	miR-423-5p	Serum	Up-regulated in HF vs. HC	No	Association with NT-proBNP	[33]
	Diagnostic	miR-423-5p	Plasma	No	Yes	Improvement of diagnostic accuracy of NT-proBNP in HF	[34]
	Diagnostic	miR-423-5p	Plasma	Associated with HF	No	Associated with proBNP levels	[36]
	Diagnostic	miR-499	Plasma	Up-regulated after AMI	Yes	Correlating with cTnT	[35]
	Diagnostic	Signature: miR-1 miR-21 miR133a miR-208	Plasma	Time-dependent changes after AMI	No	No	[37]

Table 1 (continued)

Disease entity	Value as biomarker	microRNA	Biomaterial	Direction of alteration	ROC Analysis	Comparison with established biomaker	Reference
	Diagnostic	Signature: miR-520d-5p miR-558 miR-122* miR-200b* miR-622 miR-519e* miR-1231 miR-1228*	Whole blood	Differentially dysregulated in HFREF	Yes	No	[38]
	Prognostic	miR-182	Serum	Up-regulated in future cardiovascular death	Yes	Superior to NT-proBNP in the prediction of future cardiovascular mortality	[39]
HFPEF	Diagnostic	Numerous miRNAs	Plasma, Serum, Tissue	NA	No	No	Review: [8]
ISR	Diagnostic	miR-21	Plasma	Up-regulated in ISR	Yes	No	[40]
	Diagnostic	miR-100	Plasma	Down-regulated in ISR	Yes	No	[40]
	Diagnostic	miR-143	Plasma	Down-regulated in ISR	Yes	No	[40]
	Diagnostic	miR-145	Plasma	Down-regulated in ISR	Yes	No	[40]
AF	Diagnostic	miR-126	Serum	Downregulated in AF vs. HC	No	Inversely correlated with NT-proBNP	[41]
	Diagnostic	miR-150	Plasma	Down-regulated in AF vs. HC	No	Negatively associated with CRP	[42]
	Diagnostic	miR-150	Serum and Platelets	Down-regulated in AF vs. HC	No	No	[43]
	Diagnostic	miR-328	Whole blood	Down-regulated in prevalent AF vs. HC	No	No	[44]
	Diagnostic	miR-328	Plasma	Up-regulated in AF vs. HC	No	No	[45]
	Diganostic	miR-409-3p	Plasma	Downregulated in AF vs. HC	No	No	[46]
	Diagnostic	miR-432	Plasma	Downregulated in AF vs. HC	No	No	[46]
Infective carditis	Diagnostic	miR-208b	Plasma	Up-regulated in infective carditis vs. HC	No	Correlating with cTnT and severety of disease	[35]
	Diagnostic	miR-499	Plasma	Up-regulated in infective carditis vs. HC	No	Correlating with cTnT and severety of disease	[35]
APE	Diagnostic	miR-28-3p	Plasma	Up-regulated in APE vs. HC	Yes	No	[47]
	Diagnostic	miR-134	Plasma	Up-regulated in APE vs. HC	Yes	No	[48]
	Diagnostic	miR-1233	Serum	Up-regulated in APE vs. HC and NSTEMI	Yes	No	[49]

Table 1 (continued)

Disease entity	Value as biomarker	microRNA	Biomaterial	Direction of alteration	ROC Analysis	Comparison with established biomaker	Reference
TTC	Diagnostic	Signature: miR-1 miR-16 miR-26a miR-133a	Plasma	Up-regulated in TTC vs. HC; Up-reglated in STEMI vs. TTC	Yes	No	[50]

ACS, acute coronary syndrome; AMI, acute myocardial infarction; UAP, unstable angina pectoris; SAP, stable angina pectoris; HC, healthy control; NCCP, non-coronary chest pain; NSTEMI, non-ST elevation myocardial infarction; cTnT, cardiac troponin T; hs-cTnT, high sensitive cardiac troponin T; CHF, congestive heart failure; NT-proBNP, n terminal prohormone of brain natruiuretic peptide; HF, heart failure; HFREF, heart failure with reduced ejection fraction, HFPEF, heart failure with preserved ejection fraction; NA, not applicable; ISR, in-stent restenosis; AF, atrial fibrillation, CRP, c-reactive protein; APE, acute pulmonary embolism; TTC, takotsubo cardiomyopathy; STEMI, ST-elevation myocardial infarction.

levels were decreased. Not surprisingly, miR-126-3p was found among these miRNAs. Additionally, miR-126-3p was found up-regulated in non-infarcted areas of rat hearts after induced MI [64] and diminished survival rates were observed in miR-126-3p knock-out mice after coronary artery occlusion compared with wild-type mice [65]. Thus, miR-126-3p is thought to play a role in myocardial recovery after MI [66].

Another interesting miRNA in this respect is miR-208, which was up-regulated in infarcted tissue of patients who had died from MI [67]. Importantly, miR-208 is solely expressed in cardiomyocytes and therefore differentially released during cardiac cell death in MI [68]. This observation is somewhat analogous to cardiac specific troponins, which makes it appear as a miRNA example of a potential target for diagnostic purposes with respect to biomarkers in MI.

miRNAs in heart failure

On the cutting edge between cardiac fibrosis and HF increased levels of a signature of five miRNAs (miR-24, miR-125b, miR-195, miR-199a, and miR-214) were observed in failing hearts of mice and humans [52]. These miRNAs were identified as significant contributors to adverse cardiac remodeling [52], interlinking cardiac fibrosis and HF on an RNA level. Looking at enzymatic miRNA regulation, the RNase III endonuclease Dicer catalyzes the key enzymatic step of cleaving the hairpin-structured precursor miRNAs. Dicer knock-out led to the development of dilatative cardiomyopathy (DCM) [69]. Additionally, 28 miRNAs were identified as elevated in cardiac tissue of HF patients [70]. Importantly, 20 of these miRNAs returned to near normal levels after cardiac recovery in left ventricular assist device (LVAD) patients [70]. These results interlink the clinical phenotype of HF in general with dysregulated miRNAs in cardiac tissue. A more differentiated approach in human biopsy samples revealed dysregulations of miRNAs in distinct HF disease entities such as ischemic cardiomyopathy (ICM) and DCM [53]. For example, miR-19 was strongly down-regulated in DCM but not in ICM [53]. Importantly, miR-133 and miR-208, which are strongly associated with cardiac hypertrophy and fibrosis, were unchanged in both HF entities. This observation puts a question mark behind the robustness of clinical miRNA quantification, especially, when looking at results of a different working group: the authors reported elevated miR-208 levels in cardiac tissue of DCM patients and identified miR-208 to be a strong predictor of clinical outcome [71]. Nevertheless, the reported data suggest a specific dysregulation of distinct miRNAs in disease entities of CVD. Recent data report differentiated dysregulations of miRNAs in right ventricular (RV) heart failure (RVHF) compared to left ventricular (LV) heart failure (LVHF) [72, 73], exploring a potential way to develop the first RV-specific HF therapies [74]. The differentiated applicability of miRNAs in anatomically separated structures of the heart is underlined by recent data revealing chamber-specific expression of miR-208 and their host genes α-MHC and β-MHC (see above) in the human heart [75]. From this point of view, the application of miRNAs could allow for differentiated diagnostic purposes in separate types of heart disease. As the availability of tissue material is limited, necessitating other sources of biomaterial for miRNA analyses.

miRNAs as circulating biomarkers

The release of miRNAs into extracellular compartments, and especially into the blood stream, has presented the

possibility to non-invasively detect circulating miRNAs and to use them as disease biomarkers. Opposed to their cellular origin, miRNAs are well-known as extracellular messengers and detectable in peripheral blood [76]. They are furthermore characterized by a remarkably stable structure, which prevents them from early degradation [77–79].

Diagnostic biomarkers

Single miRNAs in the diagnosis of acute coronary syndrome and acute myocardial infarction

In acute myocardial infarction (AMI), miRNAs are released into the blood [80]. miRNAs with high myocardial expression (i.e. myomiRs, see above) are increased in peripheral blood of patients with AMI [81, 82]. To evaluate the usefulness of miRNA quantification for AMI diagnostic purposes, their comparison with established biomarkers, such as cardiac troponins (cT), is essential. In a mouse model of induced MI miR-208, plasma levels were identified to be significantly increased compared with healthy controls [83]. The authors found a comparable time course with cT with respect to the detection limit [83], giving a hint to their potential as useful diagnostic biomarkers. These findings were verified in a clinical study involving 424 AMI patients with suspected ACS where plasma levels of miR-208b and miR-499-5p successfully discriminated MI [22]. Meanwhile, the discriminating power of the two miRNAs was significantly lower than troponin T (cTnT) [area under the operating receiver curve (AUC): cTNT 0.95; miR-208b 0.82; miR-499-5p 0.79] [22]. Despite the continuous improvement of troponin assays and the introduction of high sensitive troponins, there is still a considerable percentage of false positive results [84]. In this respect, miRNAs might help to improve the diagnostic accuracy of blood-based biomarkers in AMI. For example, miR-499-5p was identified as highly increased in patients with non-ST segment elevation MI (NSTEMI) [23]. Analogous to the above results concerning miR-208, the power of miR-499-5p to discriminate NSTEMI from healthy controls was similar to cTnT [23]. Even more important is the observation that the accuracy to differentiate NSTEMI from acute heart failure (AHF) was higher for miR-499-5p compared with cT [23]. On the basis of these findings, a meta-analysis including 15 studies of diagnostic miRNA quantification for MI discovered an overall sensitivity of 0.78, specificity of 0.82, and an AUC for overall miRNA analysis regarding discrimination of MI [85], comparable with cTNT. At the same time, a high correlation between miRNAs and cTnT, high sensitive (hs-) cTnT as well as CK and CK-MB was identified [85]. Finally, miR-1, miR-499, and miR-21 were identified to significantly increase the diagnostic value in combination with hs-TnT in ACS patients [24], and the combinatory utilization of these three miRNAs improved the AUC to significantly higher values (0.94) than hs-TnT alone (0.89) [24].

As an example for the high sensitivity of miRNAs in ACS, miR-133a was differentially detected in the serum of unstable angina pectoris (UAP) patients compared with healthy controls [20] – a remarkable ability considering that UAP is defined to be cTNT-negative.

A further quality of miRNAs in the diagnosis of ACS seems to be their discriminating ability between AMI and UAP. In 444 ACS patients, miR-1, miR-133a, and miR-208b were elevated in MI as opposed to UAP patients [86]. Again, miRNA levels were associated with troponin levels [86].

Taken together, these data suggest circulating miRNAs as interesting new biomarkers in the diagnosis of AMI – complementing established, protein-based biomarkers.

miRNAs in the diagnosis of unstable and stable angina pectoris and acute myocardial infarction

With normal values of cT, the diagnosis of UAP is made upon clinical evaluation with no laboratory parameters available. At the same time, the diagnosis of UAP is crucial because the risk for MI is elevated in these patients [21]. In this clinically important field of CAD, there are few results with respect to single miRNAs.

MiR-486 and miR-92a serum levels were recently identified to discriminate between UAP and SAP [87]. The data were derived from 95 CAD patients consisting of 30 SAP patients, 39 UAP patients, 26 post-MI patients, and 16 healthy controls. Apart from this study, there are more data identifying single miRNAs as circulating biomarkers for UAP [25, 88]. Meanwhile, results for the differentiated diagnosis of UAP as opposed to SAP or non-coronary chest pain (NCCP) based on circulating miRNA analyses exclusively involve miRNA signatures – as opposed to single miRNAs. A cluster of the three miRNAs miR-134, miR-198, and miR-370 was identified overexpressed in peripheral blood mononuclear cells (PBMC) of 25 UAP patients compared with 25 SAP patients. Importantly, this miRNA signature was able to differentiate UAP from SAP in this patient collective [89]. Similar results were reported when

a set of microparticle-derived pro-inflammatory miRNAs from plasma of 10 patients was observed to discriminate between SAP and UAP [90]. Although a similar approach could not validate these results [26], promising results were reported in a clinical screening-validation setting comprising 46 UAP patients compared with 63 NCCP patients [37]. The authors found a miRNA signature consisting of miR-132, miR-150, and miR-186, which strongly discriminated UAP and NCCP [37].

The fact that merely miRNA signatures, as opposed to single miRNAs are reported to be able to discriminate between UAP and SAP on the one hand and NCCP on the other, might be a reflection of the subtle metabolic changes in affected cardiac tissue and circulating biomarkers when cardiac infarction has not taken place yet. It puts emphasis on the importance to further evaluate miRNAs' potential as discriminating circulating biomarker in CAD.

miRNAs as circulating diagnostic biomarkers in heart failure

Ischemic cardiac events such as MI can consecutively cause myocardial remodeling and fibrosis and might finally lead to HF. miRNAs that have previously been reported to affect myocardial growth, hypertrophy, fibrosis and viability were also shown to be dysregulated in plasma of 12 post-MI patients compared to 12 healthy controls [35]. The authors identified a signature of miR-1, miR-21, miR-133a, and miR-208 altered in the time course after MI [35]. A comparable approach led to the identification of miR-208b and miR-499 as correlating with myocardial damage after AMI [91]. At the same time, miR-499 was significantly up-regulated in a subgroup of patients who developed acute HF [91]. Since most of the above miRNAs are reported to be involved in both CAD and HF, these data mirror the pathophysiological processes on a molecular level of miRNA research and the clinical cause of HF disease.

Numerous studies have evaluated the potential of miRNAs as circulating diagnostic biomarkers in HF and have been reviewed before (8, 19). For example, a set of 24 miRNAs was significantly down-regulated in a group of HF patients compared with healthy controls [92]. Important from a clinical point of view is the diagnostic power of miRNAs as potential novel circulating biomarkers for HF in terms of sensitivity and specificity. The most established classical, protein-based biomarker for HF is N-terminal pro B-type natriuretic peptide (NT-proBNP) [33]. Analogous to cT, NT-proBNP has a high sensitivity but brings along a considerable false-positive rate, which illustrates the usefulness of novel HF biomarkers. Five miRNAs down-regulated in HF patients were inversely correlated with NT-proBNP and directly correlated with EF [92]. Receiver operating characteristics (ROC) analysis revealed strong AUC values between 0.84 and 0.91 for predicting the diagnosis of HF, suggesting these miRNAs as strong circulating biomarkers for HF [92]. In an interesting study, Goren and co-workers performed a screening of circulating miRNAs in patients with HF and identified a set of four circulating miRNAs [93]. The authors reported miR-423-5p, miR-320a, miR-22, and miR-92b up-regulated in HF patients compared with healthy controls [93]. MiR-320 and miR-423-5p had previously already been associated with HF [34, 94, 95]. The authors further successfully developed a score from these miRNAs that can discriminate HF patients from healthy controls and found a significant association between this miRNA score and several established HF parameters such as NT-proBNP, a wide QRS complex, and LV dilatation [93]. Another clinically important study analyzed plasma of 44 HF patients compared to 32 chronic obstructive pulmonary disease (COPD) patients, 59 patients with breathlessness for other diagnoses, and 15 healthy controls with respect to miRNA levels [36]. The authors performed regression and ROC analyses and reported seven miRNAs associated with HF diagnosis (miR-103, AUC 0.642; miR-142-3p, AUC 0.668; miR-199a-3p, AUC 0.668; miR-23a, AUC 0.637; miR-27b, AUC 0.642; miR-324-5p, AUC 0.621; miR-342-3p, AUC 0.644) [36]. Interestingly, from a clinical point of view, plasma levels of four of these miRNAs were suitable to distinguish between HF and exacerbation of COPD on the one hand and other causes of dyspnea on the other [36]. Compared with the previously mentioned studies, miR-423-5p could not be identified to predict HF diagnosis, but in a combinatory approach the addition of miR-423-5p to NT-proBNP led to a significant improvement of the AUC for diagnosing HF [36]. Interestingly, miR-423-5p was recently identified to target O-GlcNAc transferase (OGT) [96] and to be associated with HF and the expression levels of proBNP [96]. A following report found that miR-423-5p silencing protected cardiomyocytes from H2O2-induced apoptosis [27].

The repetitive identification of miR-320 and miR-423-5p in multiple diagnostic HF trials as well as the connection to established diagnostic and prognostic measures of HF in combination with the exploration of the underlying molecular pathways refer to the promising and clinically applicable role of circulating miRNAs as diagnostic biomarkers in HF.

miRNA signatures

miRNA signatures in the diagnosis of acute coronary syndrome and acute myocardial infarction

Sensitivity and specificity with diagnostic purposes is supposed to improve in combinatory approaches when a set of multiple miRNAs – termed miRNA signature – is quantified at the same time. A signature of six miRNAs (miR-1, miR-134, miR-186, miR-208, miR-223, and miR-499) was identified to reliably predict the diagnosis AMI [28]. Remarkably, the AUC of this miRNA signature significantly exceeded cTnT and CK-MB (0.83 vs. 0.768 and 0.709, respectively) when comparing MI and UAP [28]. Meanwhile, the AUC value for the diagnosis of MI of this miRNA signature was higher compared with each of the single miRNAs – indicating the potential of miRNA signatures to improve accuracy in the diagnosis of MI. Comparable data were reported in a clinical evaluation of AMI patients where the authors described a signature of 20 miRNAs identifying AMI with a higher predictive power as well as a better specificity and sensitivity than each of the single miRNAs [38]. Relating to classic protein-based biomarkers, the identified signature even allowed for an earlier diagnosis of AMI than cTnT [38].

miRNA signatures in the diagnosis of heart failure

Analogous to CAD, the combination of two or more miRNAs as a defined set is supposed to enhance the discriminatory power in HF as well [8]. Such results were published in a cohort of 53 patients with non-ischemic heart failure and reduced ejection fraction (HFREF) compared to 39 healthy controls [29]. The authors performed a two-step screening-validation study in which they identified eight miRNAs (miR-520d-5p, miR-558, miR-122*, miR-200b*, miR-622, miR-519e*, miR-1231, and miR-1228*) that reliably predicted the diagnosis of HFREF (AUC 0.81) [29]. A stepwise comparison revealed a further improvement of discrimination of HFREF patients from controls when the signature was used compared with the most powerful single miRNAs miR-558, miR-122*, and miR-520-d-5p (AUC between 0.7 and 0.71) [29]. This is another example of the potential superiority of miRNA signatures compared with quantification of single miRNA for diagnostic purposes.

It is important to note that the approach of 'miRNA signatures' seems only useful in cases with low correlations of single miRNAs among each other and where statistical analyses prove a predictive superiority of miRNA signatures compared with single miRNAs.

miRNAs as prognostic biomarkers

Apart from their diagnostic potential to identify CAD and MI in the acute phase, miRNAs have been analyzed for their ability to predict future cardiovascular adverse events in the general population and in patients with known CAD.

miRNAs in prediction and prognosis of coronary artery disease and myocardial infarction

Latest results with respect to prognostic miRNA evaluation in MI report a set of five miRNAs associated with future MI in the HUNT study [32], while a set of seven miRNAs was identified to reliably predict cardiovascular death in patients with CAD in the AtheroGene study [30]. The first data from a large-scale study were reported by Zampetaki et al. in 2012 [97]. The group identified miR-126, miR-197, and miR-223 to reliably predict future MI in 820 individuals from the Bruneck study [97] (for further details on the Bruneck study, see [31]). These results were recently validated [98] by identifiying miR-197 and miR-223, as well as miR-126, as strong predictors of cardiovascular death in a large cohort of 873 patients with invasively diagnosed CAD [98]. These results not only transfer the diagnostic potential of miRNAs in CVD to a prognostic level, they also indicate the promising applicability of miRNAs as useful biomarkers in primary and secondary prevention in CVD. These are the largest clinical miRNA studies with respect to the included amount of individuals, and intriguingly, they represent a validation of data that is rare in clinical miRNA research.

miRNAs in prediction and prognosis of heart failure

Several trials have identified miRNAs as potential prognostic circulating biomarkers in HF [36, 39, 71, 93]. In a clinical study comprising 42 HF patients and 15 healthy controls, a large number of miRNAs was dysregulated in HF compared to controls [99]. More importantly, miR-182 was identified to predict cardiovascular mortality and, interestingly, miR-182's prognostic power was even greater than NT-proBNP and high-sensitive C-reactive

protein [99]. Thus, there is great potential of circulating miRNAs in their utilization as prognostic biomarkers and in risk evaluation for future cardiovascular events in HF patients. Meanwhile, sample sizes in the reported studies were small and so far no confirmation studies were reported validating any of the results.

miRNAs as circulating biomarkers for secondary diseases in heart failure

HF can be the origin for failure of numerous other organs such as renal failure. Decreased plasma levels of miR-199a-3p strongly predicted future renal failure in a group of patients admitted to hospital for acute HF [100]. This example is supposed to depict the potential applicability of circulating miRNAs within different entities of specific diseases.

miRNAs as circulating biomarkers in the differentiated diagnosis of HF with preserved ejection fraction (HFPEF)

The highly promising results of circulating miRNAs as diagnostic biomarkers in HF have led to their exploration in the differentiated observation of HFREF and HFPEF.

Molecular miRNA signaling pathways suggests miR-21 as a well-suited biomarker for early stages of fibrosis and remodeling on the one hand and as a promising target for disease treatment on the other [101–107]. Especially, in HFPEF, an early diagnosis of the disease is hard to make since functional assessment of the heart often reveals near normal parameters and clinical symptoms usually appear at later stages of disease progression. Therefore, circulating miRNAs could help to improve secondary prevention at an early stage. To evaluate the suitability of miR-21 as a biomarker for the differentiated diagnosis of HFPEF, Dong and co-workers analyzed cellular levels of miR-21 in rat cardiomyocytes in a HFPEF model and found significantly higher miR-21 levels compared with healthy controls [101].

The important next step is to transfer these findings to a clinical stage. There are three recent clinical studies evaluating circulating miRNAs as biomarkers in HFPEF [108–110]. All of these trials succeeded to report single miRNAs or miRNA signatures that were able to predict the differentiated diagnosis of HFPEF vs. HFREF as reviewed elsewhere before [8]. The reported data suggest a potential for miRNAs as new biomarkers for the diagnosis of HFPEF. However, none of the identified miRNAs was reported more than once and no validating results could be presented so far. This emphasizes the need for more and larger clinical trials evaluating miRNAs in this disease entity. Moreover, miR-21 (with its plausible molecular signaling pathways and its promising preliminary results from cellular analyses) was not among the identified circulating miRNAs. This observation exemplifies the need for translational approaches in miRNA analyses, combining clinical findings with molecular analyses. Especially, the exploration of signaling pathways and validation analyses in further clinical cohorts are of utmost importance.

miRNAs as biomarkers in additional entities of cardiovascular disease

miRNAs in in-stent-restenosis (ISR)

In CAD, in-stent restenosis (ISR) affects the long-term outcome after percutaneous coronary intervention (PCI) [111]. miRNAs are aberrantly expressed in the vascular walls after balloon injury [112] and thus could serve as biomarker in disease progression after PCI. Molecular aspects gave rise to analyses investigating miRNAs as biomarkers for in-stent restenosis [40, 112–114]. Indeed, circulating miR-21, miR-143, miR-145, and miR-100 were reported to be dysregulated in ISR patients compared with control patients without ISR after drug eluting stent implantation [115]. A recent study confirmed dysregulated circulating miR-21 levels in pig and mouse models after coronary stenting [116]. Analogous to the above findings miR-21 [23, 117, 118] and miR-145 [119, 120] have been identified as potential diagnostic circulating biomarkers for CAD, strengthening the assumption of their applicability in disease monitoring. However, there are no data available on miR-143 with respect to clinical utilization as circulating biomarker in CAD.

Atrial fibrillation (AF)

The regulatory function of miRNAs at the post-transcriptional level and their gene silencing effect have been evaluated regarding their association with AF. There is evidence for the involvement of miRNAs in mediating cardiac excitability and arrhythmogenesis [121–123]. Atrial remodeling as an important factor in the genesis of AF was shown to

be associated with miR-328 dysregulation through L-type Ca(2+) targeting [124]. Not surprisingly, atrial fibrosis – a downstream result of remodeling – was associated with miR-21 dysregulation [42]. Cellular analyses from atria of AF patients showed significantly elevated levels of miR-21, which were linked to an involvement of its target *Sprouty 1* (Spry1) [42]. In one of the first clinical evaluations, Liu et al. reported reduced miR-150 plasma levels in AF patients compared with healthy controls and a significant association of miR-150 with AF [125], indicating the potential use of miRNAs as circulating biomarkers for AF. Further clinical studies followed, predominantly undertaken in atrial tissue and analyzing cellular miRNAs. Compared with CAD and HF there is much less data from clinical trials reported on circulating miRNAs in diagnostic approaches for AF. Some clinical trials, however, identified circulating miRNAs including miR-499 [44], miR-328 [43, 45], miR-150 [46], miR-409-3p, and miR-432 [41], miR-126 [126]. None of the reported miRNAs were validated and all trials refer to rather small patients cohorts. Therefore, in miRNA-based diagnosis of AF, more data from clinical trials evaluating circulating miRNAs are needed.

Infective carditis

For infective carditis such as myocarditis or pericarditis, there is no specific biomarker. Therefore, its diagnosis is primarily made via clinical evaluation and by combinatory approaches of known protein-based biomarkers that reflect myocardial damage. miRNAs have been assessed for their ability to be utilized as diagnostic biomarkers for infective carditis and cardiac myocyte associated miR-208b and miR-499 were reported to be elevated in plasma of patients with diagnosed viral myocarditis [91]. Interestingly, plasma levels of leucocyte-expressed microRNAs were not significantly increased, despite elevated white blood cell counts [91]. Limiting the unbiased interpretation of these results is the fact, that these two miRNAs are detectable in general myocardial damage rather than specifically in carditis and were also identified as dysregulated in MI (correlating with cTnI elevation) [91]. Nevertheless, the quantification of miR-208b and miR-499 in myocarditis can potentially be used to determine the severity of the disease [91, 127]. Pointing into the same direction are findings that describe miR-21 as an indicator for the transition of chronic myocarditis into dilated cardiomyopathy [128]. These results allow for a disease monitoring in known myocarditis – again only results from cellular analyses of cardiac tissue were reported. Results with a diagnostic character were reported in miRNA analyses from RV septal biopsies from patients with acute myocarditis [129]. The authors reported a disease-specific up-regulation of inflammatory miR-155 during acute myocarditis [129]. Again, no circulating miRNAs were evaluated, limiting the meaningfulness of the results from a clinical perspective. The same miRNA was confirmed to play a major role in viral myocarditis, when miR-155 levels in mouse cardiac tissue were identified to increase during carditis in a macrophage dependent manner [130]. Another recent study found miR-221 and miR-222 as key regulators of the cardiac response to viral myocarditis [131]. This study was performed in cardiac tissue samples of myocarditis patients, as well. Taken together, there is only one study that reported circulating human miRNAs as potential biomarkers in infective carditis.

Pulmonary embolism

The susceptibility to chronic thromboembolic pulmonary hypertension (CTEPH) has been suggested to be associated with genetic polymorphisms and in this respect with miR-759 [132]. More recently, anti-oncogenic miRNA Let-7b was identified to be down-regulated in plasma of CTEPH patients [48]. These results gave rise for the exploration of miRNAs in the diagnosis of acute pulmonary embolism (APE) – a diagnosis for which there is no specific biomarker. In the first clinical approach, plasma levels of 32 APE patients, 32 healthy controls and 22 non-APE patients with potentially APE-associated symptoms revealed miR-134 as a specific diagnostic predictor of APE [47]. The authors stated the need for large-scale investigations to enable the clinical utilization of miRNAs in this diagnostic field of miRNA research [47]. Results from comparable analyzes did not confirm these findings of miR-134, putting a question mark behind its suitability in diagnosing APE [49]. In the same study, circulating miR-28-3p was identified to predict the diagnosis of APE in plasma of 37 APE patents [49]. Latest data identified circulating miR-1233 as a sensitive and specific biomarker for the diagnosis of APE [133]. The authors describe that dysregulation of this miRNA was able to discriminate APE from NSTEMI [133], which is clinically relevant since symptoms of these two disease entities can be alike. Importantly, miR-1233 has not been described associated with other entities of CVD – such as CAD or MI – before.

Deep vein thrombosis (DVT) is an important diagnosis relevant for the formation of APE. Recently, serum levels miR-582, miR-195, and miR-532 were identified to strongly discriminate DVT from controls with impressive AUC

values of 0.959, 1.0, and 1.0, respectively. The authors suggested these miRNAs as potential circulating biomarkers for the diagnosis of DVT, which is closely related to APE [50]. Comparable to the above results concerning infective carditis, there is an indication for miRNAs to be successfully deployable in the diagnosis of APE.

Tako-Tsubo cardiomyopathy

Tako-Tsubo cardiomyopathy (TTC) is a relatively rare disease closely related to acute MI. The diagnostic means are based on morphological analyzes comprising echocardiography and ventriculography and there is no biomarker specific for TTC – especially in the differentiation from MI. Jaguszewski and co-workers analyzed circulating miRNA levels in plasma of TTC and MI patients and found a signature of miR-1, miR-16, miR-26a, and miR-133a not only up-regulated in TTC compared with healthy controls but also to differentiate between TTC and MI [134]. One other study confirmed miR-133's diagnostic potential for the detection of TTC [20]. More data is needed to evaluate the clinical applicability of miRNAs in TTC.

miRNAs – therapeutic application

miRNAs are currently analyzed for their abilities as molecular therapeutics in CVD. miRNA treatment options focus on the specific artificial elevation or suppression of selected miRNA levels. Detailed explanations on the mode of function of the diverse approaches on how to alter miRNA levels can be found elsewhere [17, 135, 136]. Briefly, a specific elevation of target miRNAs can be achieved by miRmimics [137], while miRNA suppression can be obtained by anti-miRNA antisense oligomers – so-called antagomiRs [138, 139], sponges [140–142], masking [143] and erasers [139]. At the moment, there is no data available from clinical trials evaluating the therapeutic clinical deployment of miRNAs in CVD. In vitro studies and animal models, though, have produced promising results with potential for a future clinical application [144–148]. Here, we provide an example on how miRNAs could effectively and useful be applied in a clinical setting:

miRNA therapeutics via drug eluting stents

Recently, miR-125 as anti-vascular-proliferative agent in drug eluting stents was shown to reduce in-stent restenosis rate [149]. Also, a knock-down of miR-21 and miR-221 may reduce neointima formation after vessel injury [150]. Systemically administered antagomiRs and miRmimics have been the method of choice to explore their effect, but had substantial off-target effects and were primarily targeting the kidney, liver, lung and spleen with considerable side effects [150, 151]. Therefore, the local administration of miRNAs as therapeutic agents in the prevention of ISR was evaluated [151]. The authors successfully implanted antimiR-21 coated vascular stents into human left internal mammarian arteries, grafted those arteries into mice in abdominal aortal position and found a significantly reduced myointima formation compared with a control group treated with bare metal stents (BMS) [151]. This example summarizes the molecular usefulness of miRNA therapeutics as well as the need for and applicability of a sophisticated mode of application to minimize systemic undesirable effects.

miRNAs as biomarkers in monitoring of CVD disease treatment

Besides their exploration as therapeutic agents, miRNAs have been evaluated for their ability to monitor disease treatment in CVD. The inhibition of platelets is widely used for therapeutic as well as preventive indications – especially in patients with CAD, and while miRNAs were identified as biomarkers for platelet activation [152] a meta-analysis has revealed that approximately 25% of aspirin treated patients have an insufficient platelet inhibition [153]. Therefore, recently, analyses were conducted revealing that circulating miR-92a levels in plasma of aspirin treated patients showed a decreasing slope according to increasing aspirin response and, intriguingly, miR-92a was able to identify aspirin responders from non-responders with high sensitivity and specificity [154]. Regarding the same mechanism, Kaudewitz et al. have proven a correlation of miRNAs with established platelet function tests in patients with ACS and platelet activation markers in the general population and identified miR-126 to affect platelet reactivity [62]. Interestingly, the miR-126 pathway modulating platelet function impacts on P2Y12 expression [62] – the target of modern thrombocyte aggregation inhibitors such as clopidogrel, prasugrel, and ticagrelor, which are widely used in patients with CAD. These results may improve the diagnostic means available in monitoring therapy effectiveness of patients receiving P2Y12 receptor antagonists on the one hand and help to identify non-responders on the other. These are examples

of possible applications of miRNAs in the monitoring of disease treatment in one filed of CVD.

Analytical considerations in miRNA quantification

There are confounding factors influencing the accuracy of miRNA analyses. Critical consideration of such influencing factors is of utmost importance to avoid misleading results and to enable comparability of the various analyses. In RNA isolation methods for example, a maximum of efficiency, reproducibility and reliability is of tremendous importance since i.e. even minimal co-extraction of inhibiting factors can significantly influence measurement results. Different methods of detection (high-throughput sequencing, real-time PCR, microarrays) are used to quantify miRNA levels. Their application needs to be defined for specific indications to minimize deviations between studies. Importantly, the method of normalization needs to be standardized and reproducible isolation and detection protocols should be applied; otherwise, comparability is considerably hampered [155]. Apart from these analytical aspects, there are influencing parameters affecting miRNA levels in the analyzed biomaterial. Medication such as statins, antiplatelet drugs and heparin can have a confounding effect on miRNA measurements. For example, statins decrease circulating miR-122 levels, antiplatelet drugs alleviate the amount of freely circulating thrombocyte-derived miRNAs, and heparin influences the polymerase chain reaction during the quantification process [156]. Finally, there are differences in the results from miRNA quantification analyses between cellular and extracellular sources of biomaterial [157].

Summary

miRNAs emerged as promising new biomarkers in CVD more than a decade ago, and their clinical applicability has been evaluated numerous times since then. Especially in CAD and HF, miRNAs have been reported to serve as potential strong biomarkers. miRNAs show high diagnostic power, comparable with or even superior to established protein-based biomarkers, whereas miRNA signatures usually improve diagnostic accuracy. A few large-scale clinical studies in CAD have identified miRNA signatures that strongly predict future cardiovascular events. In other different CVD entities such as AF, a few studies have identified single miRNAs as potential future diagnostic biomarkers, but consistent replication studies are needed to confirm first findings. A promising approach to clinically utilize miRNAs as diagnostic biomarkers in CVD seems to be a complementary use in combination with established, protein-based biomarkers. The prognostic potential of miRNAs seems to be optimally addressed when utilizing miRNA signatures as opposed to single miRNAs. The evaluation of miRNA applicability as therapeutic agents in CVD has led to promising results in in vitro and in vivo studies, but no clinical trials have been reported so far. Analytical considerations play an important role in miRNA analyses with an emphasize on the need for harmonized methods (i.e. normalization) and to minimize influencing factors (i.e. drug induced).

Conclusions

In CVD, miRNAs yield tremendous potential to serve as clinically applicable diagnostic as well as prognostic biomarkers. Their organ- and cell-specific regulation allows for differential applicability in a variety of disease entities within CVD. Meanwhile, validation of reported results are rare, and therefore, large-scale clinical trials are urgently needed, especially with respect to a potential clinical applicability of miRNAs as therapeutic agents.

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