European Journal of Heart Failure (2016)
doi:10.1002/ejhf.495

REVIEW

MicroRNAs in heart failure: from biomarker to target for therapy

Eline L. Vegter[1], Peter van der Meer[1], Leon J. de Windt[2], Yigal M. Pinto[3], and Adriaan A. Voors[1]*

[1]Department of Cardiology, University Medical Centre Groningen, Hanzeplein 1, 9713 GZ, Groningen, the Netherlands; [2]Department of Cardiology, CARIM School for Cardiovascular Diseases, Maastricht University, Maastricht, the Netherlands; and [3]Department of Cardiology, Heart Failure Research Centre, Academic Medical Centre, Amsterdam, the Netherlands

Received 28 August 2015; revised 8 December 2015; accepted 14 December 2015

MicroRNAs (miRNAs) are increasingly recognized to play important roles in cardiovascular diseases, including heart failure. These small, non-coding RNAs have been identified in tissue and are involved in several pathophysiological processes related to heart failure, such as cardiac fibrosis and hypertrophy. As a result, miRNAs have become interesting novel drug targets, leading to the development of miRNA mimics and antimirs. MicroRNAs are also detected in the circulation, and are proposed as potential diagnostic and prognostic biomarkers in heart failure. However, their role and function in the circulation remains to be resolved. Here, we review the potential roles of miRNAs as circulating biomarkers and as targets for therapy.

Keywords MicroRNAs • Heart failure • Circulation • Antimir

Introduction

MicroRNAs (miRNAs) have been studied intensively since their discovery more than two decades ago, which led to a drastic change in our understanding of regulatory epigenetic processes. MicroRNAs (~22 nucleotides in length) are involved in several cell processes by repressing messenger RNA (mRNA) translation mainly via binding at the complementary 3′-untranslated region, thus modulating gene expression at the post-transcriptional level. In cardiac development, miRNAs are needed for the formation of normal, functional heart tissue and a variety of miRNAs have been discovered as important regulators of several phases in cardiac development.[1–3]

The importance of miRNAs was originally demonstrated in embryonic development by modifications in Dicer, an enzyme involved in miRNA processing. Dicer1 gene targeting in mice resulted in early embryonic death.[4] Furthermore, cardiac-specific deletion of Dicer shortly after embryonic heart formation resulted in cardiac malformations, heart failure and eventually death.[1,5] Deletion of Dicer in the postnatal myocardium induced cardiac remodelling, increased atrial size, and resulted in early lethality.[2] These results led to an increasing number of studies identifying specific miRNAs associated with several phases of cardiac development, including miR-1 and miR-133, which will be discussed in this review in more detail. Interestingly, the miRNAs associated with the pathophysiology of heart failure were found to be closely similar to the miRNAs involved in the fetal gene program.[6] The activation of the fetal gene program results in adaptive processes in the heart, which eventually lead to heart failure.

Functional miRNA studies reported that a variety of miRNAs play a role in pathogenic mechanisms leading to heart failure, such as remodelling, hypertrophy, apoptosis, and hypoxia.[7,8] Furthermore, in response to the progression of heart failure, miRNAs were shown to behave in a dynamic and stage-specific way. For example, decreased levels of miRNAs (including miR-1 and miR-133a) were found in transgenic hypertrophic cardiomyopathy (HCM) mice before development of the disease, while an increasing number of miRNAs exhibited a dysregulated and mainly upregulated pattern in the later stages towards end-stage heart failure.[9] Others demonstrated that the aetiology of heart failure (ischaemic, aortic stenosis, or idiopathic cardiomyopathy) was associated with differentially expressed miRNA patterns.[10] Thus, compelling evidence suggests that miRNAs play an active role in the onset and progression of heart failure.

*Corresponding author: Tel: +31 50 361 2355; Fax: +31 0 50 361 4391; E-mail: a.a.voors@umcg.nl

Circulating microRNAs

Based on current knowledge, the effects of miRNAs on repressing mRNA translation take place inside cells. However, in 2008, miRNAs were discovered outside cells and in circulating blood.[11–13] These extracellular circulating miRNAs are remarkably stable, even under several extreme conditions such as repeated freeze–thaw cycles, boiling, and long-term storage.[11–13] This is because of the protective effect of their carriers against RNases in the circulation, including protein complexes, exosomes, apoptotic bodies, and other microvesicles. These findings led to an increasing number of studies investigating the potential of miRNAs as circulating biomarkers for disease, including heart failure. In the following section, the potential roles of miRNAs as biomarkers in the diagnosis, prognosis and treatment of heart failure will be discussed.

Potential clinical application for microRNAs as biomarkers in heart failure

Biomarkers are used in heart failure for several purposes. They play an important role in the diagnosis of heart failure and are used to determine the cause of heart failure. Further, many biomarkers can be used as prognostic markers and in some circumstances guide the choice, intensity, and the response to therapy. Finally, biomarkers may provide additional insight about specific pathophysiological mechanisms in heart failure.[14] As there is strong evidence that miRNAs play a role in the onset and progression of heart failure, and because of their stability in plasma, miRNAs are interesting potential novel biomarkers in heart failure.

MicroRNAs as diagnostic biomarkers

Although B-type natriuretic peptide (BNP) and *N*-terminal pro-brain natriuretic peptide (NT-proBNP) are currently used as the golden standard in ruling out and confirming the diagnosis of heart failure,[15] circulating miRNAs have been increasingly studied as potential diagnostic biomarkers. However, in order to be used as biomarkers for the diagnosis of heart failure, they should either outperform natriuretic peptides, or have an additive value. For the diagnosis of heart failure, the sensitivity of natriuretic peptides is high, but their specificity to detect heart failure leaves room for improvement. Several studies have investigated the potential of circulating miRNAs for the diagnosis of heart failure (*Table 1*). In chronic heart failure, multiple miRNAs have been described as candidates for future diagnostic biomarkers in heart failure.[16–19] A few studies have reported on circulating miRNAs that were able to distinguish patients with breathlessness due to heart failure and other causes of dyspnoea. Tijsen *et al.*[20] found miR-423-5p to be differentially expressed between heart failure patients and healthy controls, and patients with other causes of dyspnoea. Other studies also described differentially expressed circulating miRNAs in acute heart failure, including low levels of miR-103, miR-142-3p, miR-30b, and miR-342-3p,[21] and high levels of miR-499.[22] A recent study by our group identified a panel of acute heart failure-specific miRNAs, in which decreased miRNA levels were observed in acute

Table 1 Circulating microRNAs (miRNAs) associated with the diagnosis of heart failure

miRNA Diagnosis	Regulation in heart failure Compared with controls		References
Acute heart failure			
miR-18a-5p	Decreased		23
miR-26b-5p	Decreased		23
miR-27a-3p	Decreased		23
miR-30b	Decreased		21
miR-30e-5p	Decreased		23
miR-103	Decreased		21
miR-106a-5p	Decreased		23
miR-142-3p	Decreased		21
miR-199a-3p	Decreased		23
miR-342-3p	Decreased		21
miR-423-5p	Increased		20
miR-499	Increased		22
miR-652-3p	Decreased		23
Chronic heart failure			
miR-22	Increased		17
miR-30c	Decreased		26
miR-92b	Increased		17
miR-107	Decreased		18
miR-122*	Increased		19
miR-139	Decreased		18
miR-142-5p	Decreased		18
miR-146a	Decreased		26
miR-183-3p	Decreased		27
miR-190a	Decreased		27
miR-193b-3p	Decreased		27
miR-193b-5p	Decreased		27
miR-203	Decreased		16
miR-210	Increased		16
miR-211-5p	Decreased		27
miR-221	Decreased		26
miR-320a	Increased		17
miR-328	Decreased		26
miR-375	Decreased		26
	Increased		16
miR-423-5p	Increased		17
miR-494	Decreased		27
miR-520d-5p	Increased		19
miR-558	Decreased		19
miR-671-5p	Increased		27
miR-1180	Increased		16
miR-1233	Increased		27
miR-1908	Increased		16
HFpEF	*Compared with controls*	*Compared with HFrEF*	
miR-30c	Decreased	Decreased	26
miR-125a-5p	Increased	Increased	27
miR-146a	Decreased	Decreased	26
miR-190a	Decreased	Decreased	27
miR-221	Decreased	Increased	26
miR-328	Decreased	Increased	26
miR-375	Decreased	Increased	26
miR-550a-5p	Increased	Decreased	27
miR-638	Decreased	Increased	27

HFpEF, heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction.

Table 2 Circulating microRNAs (miRNAs) associated with the prognosis and response to therapy in heart failure patients

miRNA Prognosis	Regulation in heart failure Compared with controls		Endpoint	References
miR-18a-5p	Decreased		180-day all-cause mortality	23
miR-126	Increased		Cardiovascular death after 2 years	34
miR-508-5p	Increased		Cardiovascular death after 2 years	34
miR-652-3p	Decreased		180-day all-cause mortality	23
Response to therapy	*Compared with controls*	*After successful therapy*		
miR-1	Increased	Decreased	3 months after LVAD	16
miR-26b-5p	Decreased	Increased	12 months after CRT	36
miR-29a-3p	Decreased	Increased		36
miR-30d	Increased	Decreased	6 months after CRT	37
miR-30e-5p	Decreased	Increased	12 months after CRT	36
miR-92a-3p	Decreased	Increased		36
miR-145-5p	Decreased	Increased		
miR-208a/208b	Increased	Decreased	3 months after LVAD	16
miR-483-3p	Increased	Decreased	3, 6, 9, and 12 months after LVAD, change in NT-proBNP	35
miR-499	Increased	Decreased	3 months after LVAD	16
miR-1202	Increased	Decreased	3 months after LVAD, change in NT-proBNP	35

CRT, cardiac resynchronization therapy; LVAD, left ventricular assist device; NT-proBNP, *N*-terminal pro-brain natriuretic peptide.

heart failure patients compared with healthy controls and patients with an acute exacerbation of chronic obstructive pulmonary disease.[23]

In plasma of patients diagnosed with HCM without heart failure symptoms, miR-29a, among others, was found to be significantly upregulated and the only miRNA to correlate with both LV hypertrophy and fibrosis.[24] These results suggest that this miRNA may function as a biomarker for remodelling processes in HCM. The specificity of miR-29a to HCM was confirmed by Derda *et al.*,[25] demonstrating that miR-29a was able to differentiate between hypertrophic obstructive cardiomyopathy (HOCM), hypertrophic non-obstructive cardiomyopathy (HNCM), senile amyloidosis, and aortic stenosis. MiR-29a was positively correlated with the interventricular septum size, which is a parameter for remodelling processes including hypertrophy and fibrosis.

Recent evidence suggests that miRNAs might discriminate between heart failure with a reduced ejection fraction (HFrEF) and heart failure with a preserved ejection fraction (HFpEF). To date, three studies have reported differential levels of several circulating miRNAs in HFrEF and HFpEF, with only a few similarities.[21,26,27] Differentially expressed miRNAs between HFpEF and HFrEF might not only be relevant for diagnostic purposes, but might provide a better insight in their differential pathophysiology as well.

Prognostic value of microRNAs

In heart failure, large numbers of biomarkers are predictors of outcome. However, a limited number of studies have focused on the prognostic value of circulating miRNAs in patients with acute and chronic heart failure; these are summarized in *Table 2*. Circulating miRNAs have been studied more extensively in relation to the prognosis after myocardial infarction. Several circulating miRNAs or a combination of miRNAs in patients with acute myocardial infarction were associated with impaired LV contractility,[28] remodelling,[29,30] and risk of (cardiovascular) death or heart failure.[29,31–33]

Qiang *et al.*[34] measured miRNAs in endothelial progenitor cells (derived from mononuclear cells from the circulation) in 106 heart failure patients and found that low levels of miR-126 were associated with cardiovascular death in ischaemic heart failure patients, while high levels of miR-508a-5p were associated with cardiovascular death in non-ischaemic heart failure. We recently found that decreases in miR-18a-5p and miR-652-3p during a hospitalization for heart failure was predictive for 180-day mortality.[23]

MicroRNAs as biomarkers for response to therapy

Patients with severe end-stage heart failure who receive a left ventricular assist device (LVAD), undergo a sudden unloading of the heart. Morley-Smith *et al.*[35] showed that circulating and myocardial miR-483-3p was increased in patients after LVAD support. Interestingly, levels of circulating miR-1202 before LVAD implantation were able to distinguish responders from non-responders. Another study also showed a change in several circulating miRNA levels after LVAD implantation, with decreased levels of the myomirs miR-208a/208b, miR-499 and miR-1 after 3 months.[16]

In response to cardiac resynchronization therapy (CRT), Marfella *et al.*[36] found five miRNAs (miR-26b-5p, miR-145-5p, miR-92a-3p, miR-30e-5p, and miR-29a-3p) in higher levels in the circulation of responding patients, compared with non-responders. In a recent study comprising 61 patients receiving CRT, higher levels of miR-30d were found in responders to CRT therapy (LVEF increase of >10%) after 6 months.[37]

An animal study identified increased levels of miR-16, miR-20b, miR-93, miR-106b, miR-223, and miR-423-5p in plasma from rats with hypertension-induced heart failure compared with controls. After treatment with an antimir for miR-208a and/or an angiotensin-converting enzyme (ACE) inhibitor, these miRNAs (except for miR-19b) normalized partly or completely after 8 weeks, suggesting that circulating miRNAs can react in a dynamic way in response to therapy, potentially indicating efficacy of treatment.[38]

Together, several studies have shown the prognostic capacities of circulating miRNAs in heart failure (*Table 2*) and indicated a potential role for predicting response to LVAD, CRT, and pharmacological therapy. Although these studies need to be validated in larger independent cohorts, this potential role of circulating miRNAs is of interest and might lead to a more individualized approach to treating patients with heart failure.

Limitations of circulating microRNA studies and future considerations

However, the studies presently available do not yet provide sufficient evidence for a clinical use of miRNAs as biomarkers in heart failure. First, not all studies started with a large miRNA panel screen in order to select the most differentially expressed miRNAs. An unbiased approach is needed to identify the miRNAs of interest for further testing in extended cohorts. Second, it is important to compare the predictive diagnostic and prognostic value of the miRNAs to established heart failure biomarkers such as NT-proBNP and/or BNP to assess the individual predictive value and determine the additional value of the miRNA on top of natriuretic peptides. Some studies indeed report on miRNAs which (in combination with natriuretic peptides) outperform NT-proBNP or BNP alone in discriminating heart failure patients from non-heart failure patients,[19,21] but the lack of validation of their diagnostic predictive value in other, independent cohorts make these results difficult to interpret. Third, most studies have relatively small patient numbers, which decreases statistical power, therefore larger studies should be conducted to verify the diagnostic and prognostic potential of miRNAs.

The lack of consistency in the available studies regarding the most differential circulating miRNAs in heart failure is intriguing. Several factors may be contributing to these differences. Anticoagulants in blood collection tubes can be of influence, as heparin has been shown to cause difficulties in polymerase chain reaction (PCR) amplification, in contrast to ethylenediaminetetraacetic acid (EDTA) or citrate collection tubes.[39,40] Moreover, the material analysed (whole blood, serum or plasma) may contain different miRNA levels, as was demonstrated by several groups.[11,41,42] Further, the presence of blood cells in plasma and serum may contribute to higher levels of certain miRNAs, therefore appropriate plasma handling (e.g. centrifuging steps) is crucial. As there is currently no golden standard for measuring circulating miRNAs, variability resulting from the isolation protocol is therefore a plausible explanation. Among the available techniques to measure miRNAs in plasma such as microarrays, RNA sequencing and quantitative reverse transcription polymerase chain reaction (qRT-PCR), qRT-PCR is most commonly used. Several companies have developed qRT-PCR kits to measure miRNAs in blood, plasma and other body fluids, in which differences in the miRNA extraction techniques may lead to different findings.[43] Furthermore, different methods of internal normalization with housekeeping or reference genes were reported, although, to date, no global and standardized method has been proposed. Exogenous, synthetic miRNAs are commonly used as stable reference miRNAs to normalize the PCR data obtained from the miRNAs of interest. For example, synthetic *Caenorhabditis elegans*-derived miRNAs can be spiked-in before miRNA extraction to control for sample quality and extraction efficiency. Endogenous miRNAs and other small non-coding RNAs as data normalizers require stable expression levels under various conditions in the cohorts investigated. Owing to variability in clinical characteristics and comorbidities, well performing endogenous references in certain patient populations might not be suitable for miRNA profiling studies in other patient cohorts. Therefore, general accepted standardized protocols, techniques and a well-performing normalization method are needed before clinical use of miRNAs as biomarkers can be considered.

Circulating microRNAs and their potential role and function

Although there is increasing interest in circulating miRNAs in heart failure, there are still major uncertainties about their origin and function in the circulation. Some speculate that in heart failure, cells die and release miRNAs into the circulation, which could lead to higher levels of circulating miRNAs compared with controls. However, lower miRNA levels are frequently found in the circulation of patients with heart failure, arguing against this speculation. This might be explained by the possibility that cells take up miRNAs from the circulation to restore deleterious intracellular mechanisms related to the progression of heart failure. On the other hand, it is also likely that several deregulated circulating miRNAs in heart failure might not even come directly from the heart.

Therefore, before using miRNAs as circulating biomarkers, it is highly desirable to understand (i) how these miRNAs are released into the circulation, (ii) if they play an active role in the circulation and (iii) whether these circulating miRNAs reflect tissue levels.

How are microRNAs released into the circulation?

Primary miRNAs are transcribed in the nucleus after which several processing steps follow (*Figure 1*). The mature miRNA is formed after processing by Dicer in the cytoplasm of the cell. In the circulation, miRNAs were discovered in various ways; in conjunction with exosomes and other microvesicles,[44] apoptotic bodies,[45] HDL particles,[46] and other RNA-binding proteins.[47] The exact mechanisms underpinning release of miRNAs into the extracellular space are not known, although some mechanisms involving extracellular vesicles have been described. Precursor and mature miRNAs packaged into microvesicles can leave the cells by blebbing of the plasma membrane. Exosomes containing miRNAs can be formed in the cell after which they are transported to

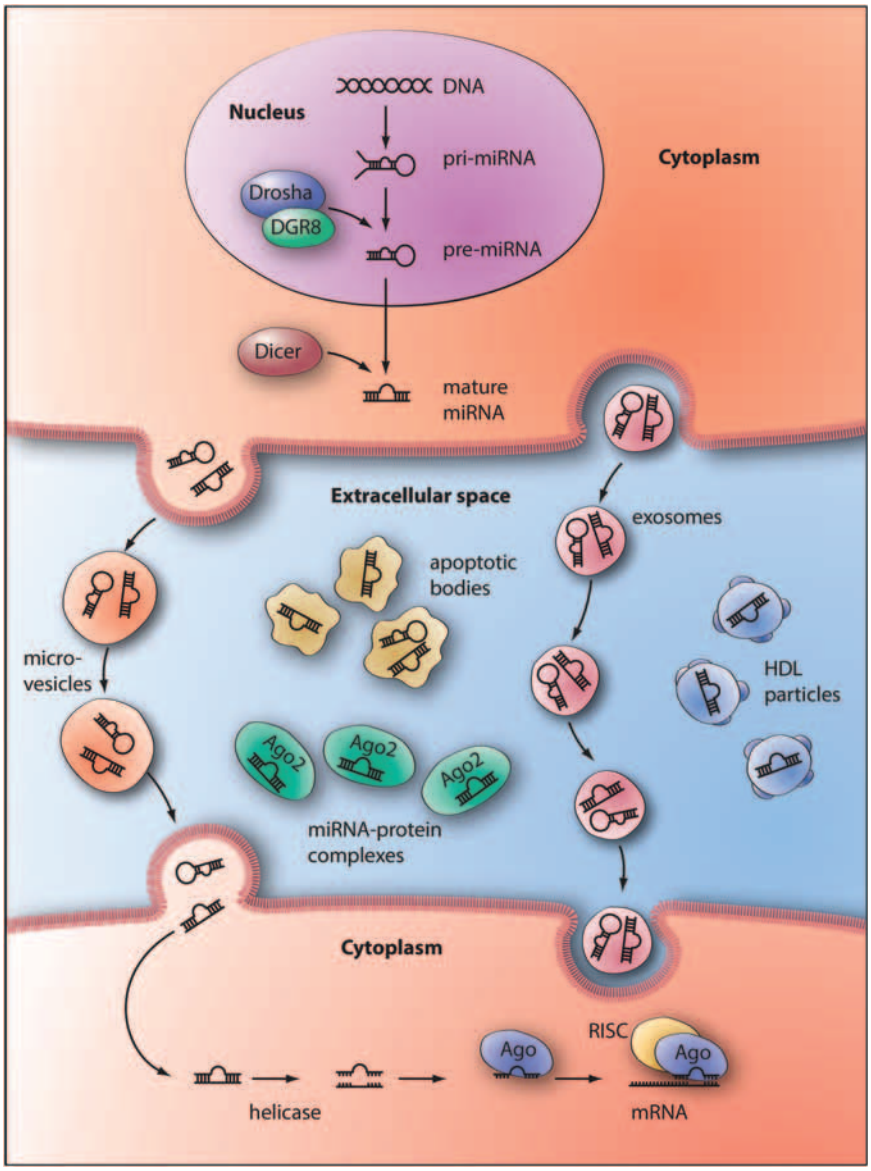

Figure 1 Mechanisms of microRNA (miRNA) processing and transportation. Primary miRNAs (pri-miRNAs) are transcribed from DNA, after which Drosha and DiGeorge syndrome chromosomal region 8 (DGR8) cleave the pri-miRNAs into precursor hairpin miRNAs (pre-miRNAs). Outside the nucleus, the pre-miRNAs are further processed by Dicer, which results in the loss of the hairpin structure and the formation of the mature miRNA. MiRNAs can act directly on mRNA targets in the cytoplasm or are released into the extracellular space and circulation by the shedding of exosomes and other microvesicles. Recipient cells can engulf the exosomes and the microvesicles can fuse with the recipient cell membrane to establish intracellular communication. Other carriers of miRNAs in the circulation are high-density lipoprotein (HDL) particles, apoptotic bodies and miRNA-protein complexes, mostly with Argonaute2 (Ago2). In the recipient cell, miRNAs can be cleaved by helicase after which an Ago protein binds to the active miRNA strand. The miRNA–Ago complex will be loaded into a RNA-induced silencing complex (RISC) which can bind to the target mRNA. With perfect complementary sequences, the mRNA transcript will degrade, while with incomplete binding the mRNA will be repressed translationally.

the cell membrane and released into the extracellular compartments and circulation. The ceramide-dependent pathway, which is under the influence of neutral sphingomyelinase 2 (nSMase2), has been described to be involved in the release of miRNAs via exomes outside the cell.[48] The precise sorting mechanisms that determine when and how miRNAs are selected to leave the cell have not yet been established. However, the membrane-associated RNA-induced silencing complex (RISC) was found to regulate miRNA loading into exosomes.[49] Further, the heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) has been reported to recognize miRNAs by sequence motifs which then control the loading of miRNAs into exosomes.[50] RNA-binding proteins such

as Argonaut 2 (Ago2), nucleophosmin 1 (NPM1) and high-density lipoprotein (HDL) are also capable of transporting miRNAs outside the cell, although the exact mechanisms are still unclear.[46,47]

Do microRNAs play an active role in the circulation?

An increasing number of studies have shown that miRNAs can be secreted into the circulation by one cell and taken up by another cell, suggesting a role in cell-to-cell communication. Exosomes, microvesicles and other extracellular vesicles can transfer miRNAs to recipient cells by fusion with the recipient cell membrane (*Figure 1*). In some cases, the recipient cells contain higher levels of specific miRNAs than the cells from which the exosomes originated, suggesting a process that actively stimulates specific miRNA transfer via exosomes. Interestingly, protein synthesis in recipient cells was found to change, suggesting a regulating role of miRNAs in these cells.[51–53] Heart failure-related research on miRNA communication through exosomes was conducted by Bang *et al.*[54] They found that cardiac fibroblasts excreted exosomes containing several miRNA passenger strands, of which miR-21-3p was able to induce hypertrophy in recipient cardiomyocytes.

Although increasing evidence has emerged regarding the mechanistic processes of circulating miRNAs in extracellular vesicles, several critical notes can be made. It is proposed that in order to exert an effect in a cell, a substantial amount of miRNA should be transferred, which means numerous exosomes should fuse with the recipient cell in order to obtain miRNA levels that can regulate protein synthesis.[55,56] As exosomes also carry other particles within their membranes, the change in gene expression of the target cell might not exclusively be related to miRNA function.[57–59] Furthermore, because these studies are conducted in a controlled, experimental setting, this might not accurately reflect the (patho)physiology of miRNA transport in the human body.[60]

Other extracellular vesicles reported as transporting miRNAs are apoptotic bodies; vesicles derived from apoptotic cells. Zernecke *et al.*[45] investigated miRNAs in apoptotic bodies derived from endothelial cells in atherosclerosis and found a set of miRNAs in apoptotic bodies similar to those found in their cells of origin, suggesting a paracrine function. MiR-126 was enriched in these apoptotic bodies and was found to function as regulator of vascular endothelial growth factor (VEGF).

High-density lipoproteins are also able to transport miRNAs between cells. Vickers *et al.*[46] found miRNAs bound to HDL particles which are not found within exosomes, suggesting that the mode of export of miRNAs might be specific to cell types. Furthermore, HDL particles from patients with hypercholesterolemia contained increased levels of miR-105, compared with healthy subjects. Adding these miR-105-enriched particles to cultured hepatocytes led to a change in gene expression of multiple genes, mostly putative targets of miR-105.

Although the transport of miRNAs within extracellular vesicles and HDL is being increasingly studied, it is believed that the majority of the miRNAs in the circulation (95–99%) are transported as miRNA-protein complexes, mostly bound to Argonaute (Ago) proteins, protecting them from degradation by RNase in the circulation.[61–63] It is hypothesized that these miRNA-Ago complexes are byproducts of cell death, which makes potential paracrine miRNA signalling less convincing.[61,62] However, the exact transport of miRNA–protein complexes is largely unknown and it is unclear whether cells are able to engulf these miRNAs from the circulation in order to function as post-transcriptional regulators in recipient cells. Thus, more extensive research, especially involving these miRNA–protein complexes would be valuable.

Are circulating microRNA levels reflecting tissue levels?

In heart failure, similar responses in miRNA levels in the circulation and in myocardial tissue have been observed. For example, Tijsen *et al.*[20] found higher levels of miR-423-5p in both the circulation of heart failure patients and in post-mortem cardiac tissue of patients with dilated cardiomyopathy. To determine whether differentially expressed circulating miRNAs in heart failure might be derived from the heart, several groups investigated the transcoronary gradients of miRNA levels. The transcoronary gradient of miR-423-5p was found to be higher in heart failure patients than in controls, suggesting that this miRNA may be predominantly derived from the heart.[64] In contrast, miR-423-5p levels did not differ in the femoral arteries, veins, and coronary sinus between the two groups, which may be due to the influence of pharmacotherapy on miRNA levels or the small population size. In patients with an acute coronary syndrome, De Rosa *et al.*[65] showed increased transcoronary concentration gradients of miR-133a and miR-499, which suggests that these miRNAs were likely released by the heart. Further, these miRNA levels correlated with myocardial injury biomarker high-sensitivity troponin T. A recent study in patients receiving CRT reported significantly increased miR-30d levels in coronary sinus blood compared with peripheral blood, suggesting a cardiac origin of this miRNA.[37]

However, the current concept is that blood cells are the major contributors to the circulating miRNA pool, which was demonstrated by Pritchard *et al.*[41] They showed that different types of blood cells are the main origin of previously described circulating miRNAs in cancer. Akat *et al.*[16] showed that the differences in miRNA expression in myocardial tissue in severe heart failure patients compared with healthy controls are, in general, not reflected in circulating miRNA levels, suggesting that the heart may be an unlikely origin of the most abundant circulating miRNAs. Moreover, most circulating miRNAs in this study were highly abundant in haemopoietic cells and endothelial cells; only 0.1% consisted of muscle and cardiac specific miRNAs. However, these circulating myomirs changed comparably with the miRNA expression in myocardial tissue, which confirms that the origin of these cardiac-specific miRNAs probably lies in the heart. Higher levels of these circulating myomirs were found in severe heart failure patients compared with controls, suggesting these miRNAs could still be potential biomarker candidates in heart failure, despite their low abundance in the circulating miRNA pool. These results indicate that although certain circulating miRNAs such as the myomirs can be tissue specific, the majority of the abundant circulating miRNAs originate from blood or endothelial cells. Consequently, the search for cardiac-derived circulating miRNAs as novel biomarkers in heart failure might be challenging

because of their low concentrations in the circulation. However, we hypothesize that the most differentially expressed circulating miRNAs in heart failure may be a consequence of a systemic (vascular) response to an overloaded heart along with an impaired perfusion of multiple organs, which may lead to a differential miRNA response. These miRNAs may therefore serve as potential biomarkers in heart failure.

Therapeutic microRNA-based strategies in heart failure

The discovery of circulating miRNAs has opened new windows for novel drug development by the administration of extracellular miRNAs. Several studies have shed light on the role of miRNAs in the maladaptive processes involved in heart failure, such as hypertrophy and fibrosis. Here, we discuss the most intensely studied miRNAs in cardiac hypertrophy and fibrosis and the potential of miRNA-based therapies to inhibit or reverse these processes. Further, we elaborate on the difficulties in moving forward towards clinical application of miRNA mimics and antimirs in heart failure patients.

MicroRNA mimics and antimirs

In general, low expression levels of miRNAs can be restored with miRNA mimics, which are synthetic double-stranded oligonucleotides resembling precursor miRNAs. In target cells they are cleaved into functional single-stranded miRNAs where they are able to bind to the 3′ untranslated region of the mRNA, leading to unsuccessful translation into proteins. MiRNA mimics can be administered exogenously using adeno-associated viruses (AAVs), subcutaneously and directly into the circulation. MiRNA mimics have to undergo the same processes as double-stranded pre-miRNA, therefore chemical modifications to optimize their specific delivery, uptake in the recipient cell and regulating function are more challenging for miRNA mimics than for antimirs.[66]

Antimirs are single-stranded antisense oligonucleotide molecules with varying chemical modifications to resist ectonucleases and endonucleases. They directly block the miRNA function by binding to single-stranded mature miRNAs thus preventing their binding to target mRNAs. Antimirs can be administered intravenously, subcutaneously and in the intraperitoneal space. In the circulation, antimirs remain stable owing to chemical modification processes.[67,68] Enhanced uptake by target cells has been achieved by adding cholesterol particles to the antimirs.[68,69] Other modification processes to promote stability and uptake such as 2′-O-methyl modifications, locked nucleic acid (LNA) modifications, non-nucleotide ZEN (N,N-diethyl-4-(4-nitronaphthalen-1-ylazo)-phenylamine) modifications, conjugation to *N*-acetylgalactosamine sugars and the use of miRNA sponges have been recently reviewed by Philippen *et al.*[70] The majority of antimirs can be taken up into tissue within hours and animal models have shown that antimirs remain stable in the intracellular space for months.[71] However, functional effects of the antimirs may be expected only after several days or weeks.[72]

Although, to date, no clinical trials with antimirs or miRNA mimics in the cardiovascular field are ongoing, preclinical trials have been conducted using miRNA-based therapies in animal heart failure models.

Hypertrophy

A variety of miRNAs have been associated with cardiac hypertrophy, of which miR-1 has been described as one of the key regulating miRNAs in this process (*Figure 2*). MiR-1 is highly abundant in the heart and deletion of this miRNA causes serious cardiac defects.[1,73,74] Sayed *et al.*[75] demonstrated that in hearts of mice undergoing transverse aortic constriction (TAC), miR-1 is downregulated even before development of hypertrophy. Elia *et al.*[76] identified insulin-like growth factor-1 (IGF-1) and IGF-1 receptor as targets of miR-1, controlling cell growth and differentiation. Further, twinfilin-1, a cytoskeleton regulatory protein, was also identified as miR-1 target.[77] The heart-type fatty acid-binding protein-3 (FABP3) was also found to be targeted by miR-1, and plasma FABP3 levels functioned as an indirect biomarker of miR-1 expression.[78]

The use of an antimir for miR-1-induced cardiac hypertrophy in neonatal rat ventricular cardiomyocytes, while overexpression of miR-1 was capable of inhibiting cardiac hypertrophy by regulating calmodulin-encoding genes and genes implicated in calcium handling mechanisms.[79] Interestingly, intravenous administration of a miRNA mimic using an AAV expressing miR-1 in rats with left ventricular hypertrophy induced by pressure overload resulted in regression of cardiac hypertrophy, reduction of fibrosis and apoptosis, and improved calcium signalling.[80] These results suggest that miR-1 might play a role in the development of hypertrophy and that this process may be reversed with a miR-1 mimic.

The myomir miR-133 exists within the same transcriptional unit as miR-1 and is also highly abundant in the myocardium. In both animal and human models, miR-133 was identified as regulator of cardiac hypertrophy, with lower levels in heart failure and cardiac hypertrophy compared with controls (*Figure 2*).[74,77,81–83] The pro-hypertrophic phosphatase calcineurin was found to regulate this miRNA, resulting in a parallel increase of calcineurin and decrease of miR-133 in both *in vivo* and *in vitro* cardiac hypertrophy.[83] This miRNA also plays an important role in β-adrenergic receptor signalling with its ability to target multiple effectors of this pathway.[84] *In vitro* and *in vivo* overexpression of miR-133 was shown to be cardioprotective by inhibiting apoptosis. Disturbances in the β-adrenergic signalling pathway can lead to a longQT phenotype in single miR-1/133 cluster knockout mice. Moreover, prolonged action potentials were found in cardiomyocytes with a deletion of miR-1/133a, suggesting that this miRNA cluster is essential for normal electrophysiology in the heart.[85] Matkovich *et al.*[86] demonstrated that miR-133 had protective effects in miR-133 transgenic mice undergoing TAC. They observed stable levels of miR-133, a significant decrease in myocardial fibrosis, improvement in diastolic function, and no development of hypertrophy. In another murine TAC-induced hypertrophy model, inhibition of miR-133 by an antimir resulted in myocardial hypertrophy and left ventricular dilatation, whereas overexpression induced by an

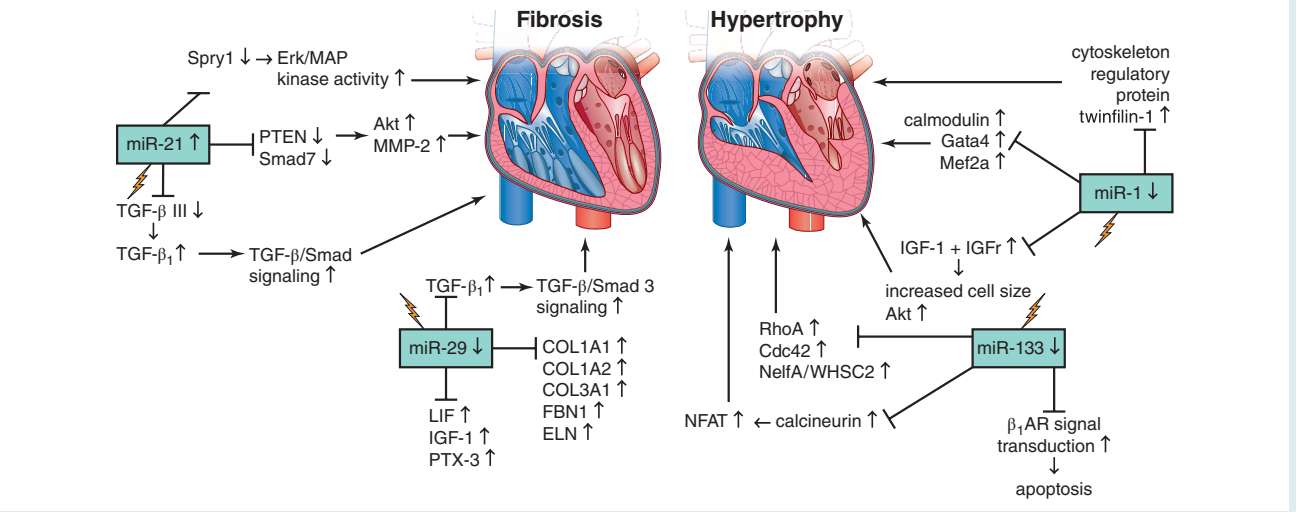

Figure 2 MicroRNAs (miRNAs) with key roles in cardiac fibrosis and hypertrophy in response to cardiac injury or overload. MiR-1, miR-21, miR-29, and miR-133 are presented with their known targets. The expression of miR-1, miR-29, and miR-133 is downregulated in cardiac tissue in response to cardiac injury or overload, leading to a decreased negative regulation of their mRNA targets. MiR-21 is upregulated in response to cardiac injury or overload, resulting in increased negative regulation of the corresponding targets. This in turn activates pathways contributing to cardiac fibrosis and hypertrophy. β1AR, beta-1 adrenergic receptor; Cdc42, cell division control protein 42 homologue; COL1A1, collagen type I, alpha 1; COL1A2, collagen type I, alpha 2; COL3A1, collagen type III, alpha 1; ELN, elastin; ERK, extracellular signal-regulated kinase; FBN1, fibrillin 1; Gata4, GATA binding protein 4; IGF-1; insulin-like growth factor-1; IGFr, insulin-like growth factor receptor; LIF, leukaemia inhibitory factor; MAP, mitogen-activated protein; Mef2a, myocyte enhancer factor 2a; MMP-2, matrix metalloproteinase-2; NelfA/WHSC2, negative elongation factor complex member A/Wolf–Hirschhorn syndrome candidate 2; NFAT, nuclear factor of activated T-cells; PTEN, phosphatase and tensin homologue; PTX-3, pentraxin-3; RhoA, ras homolog gene family member A; Smad 3, Smad family member 3; Smad 7, Smad family member 7; Spry1, sprouty homolog 1; TGF-β, transforming growth factor beta.

adenovirus containing a miR-133 mimic led to a decrease in hypertrophy by means of Akt activation.[74] A recent study confirmed the regulatory role of miR-133a in the Akt pathway and showed a beneficial effect of a miR-133a mimic on cardiac function in heart failure rats.[87]

In 2011, Ucar *et al.*[88] reported on the miR-212/132 family which are known to regulate cardiac hypertrophy by targeting the FoxO3 transcription factor. Overexpression of miR-212 and miR-132 in mice led to a phenotype with an increase in cardiac hypertrophy and heart failure; however, after administration of a miR-132 antimir, cardiac hypertrophy and heart failure development were attenuated. Another study demonstrated that an antimir for mir-208a, a well-described and cardiac-specific miRNA involved in heart failure and cardiac remodelling, improved cardiac function and increased survival rates in a pressure overload rat heart failure model.[71]

In addition to the above-described miRNAs, several other miRNAs have been related to cardiac hypertrophy, such as miR-23a, miR-27b, miR-132, miR-199b, miR-378, and miR-499, which are well reviewed elsewhere.[89,90]

Fibrosis

Non-myocyte myocardial cells, such as endothelial cells and fibroblasts, play a key role in remodelling processes, contributing to the development of heart failure. Among others, miR-21 and miR-29 have been studied most intensively in relation to cardiac fibrosis (*Figure 2*).

The miR-29 family has been associated with several extracellular matrix (ECM)-mediating encoding genes for fibrillin, elastin, and collagens. MiR-29 was downregulated in failing hearts compared with control hearts, under the regulation of TGF-β.[91] Proteomic analysis of mouse cardiac fibroblasts revealed that miR-29b targeted multiple fibrosis related genes, such as IGF-1, leukaemia inhibitory factor, and pentraxin-3.[92] Zhang *et al.*[93] proposed miR-29b as drug target in an angiotensin II induced hypertensive mouse model. *In vitro* knockdown of miR-29 resulted in increased cardiac fibrosis whereas overexpression resulted in reduced cardiac fibrosis. *In vivo*, miR-29b overexpression by miR-29b transfection into the heart attenuated the progression of fibrosis and led to an improvement of cardiac function. The TGF-β/Smad3 signalling pathway has been described as playing a crucial role in cardiac fibrosis as target of miR-29.[93,94]

MiR-21 was also associated with the TGF-β pathway and TGF-β receptor,[95] in which overexpression of miR-21 led to increased cardiac fibrosis due to the TGF-β mediated endothelial-to-mesenchymal transition.[96] This miRNA was upregulated in the mouse myocardium after cardiac stress[97] and in the myocardial tissue of patients with aortic stenosis.[81] In these patients, miR-21 was only present in interstitial cells and correlated with collagen expression in the heart.[81] Another study showed that miR-21 was specifically increased in fibroblasts of

failing hearts and capable of enhancing fibrosis by stimulating the extracellular signal-regulated kinase (ERK)–mitogen-activated protein (MAP) kinase signalling pathway.[72]. By using an antimir to silence miR-21 in mouse hearts, cardiac ERK-MAP kinase activity was reduced, leading to less cardiac fibrosis and preserved cardiac function. Roy *et al.*[98] demonstrated that metalloprotease-2 (MMP-2) expression is elevated in murine cardiac fibroblasts under the regulation of miR-21 via the phosphatase and tensin homologue (PTEN)–AKT phosphorylation-dependent pathway. A recent study found that the cytokine osteopontin (OPN) activated miR-21 thereby promoting cardiac fibrosis.[99] This process was inhibited *in vivo* by LNA-mediated silencing of miR-21, which led to restored expression levels of *PTEN* and *SMAD7*, both genes involved in cardiac fibrosis. These results propose miR-21 as potential promising drug target.

In contrast to miR-21, it has been shown that miR-24 regulates heart failure-related processes in both cardiomyocytes and fibroblasts. In a post-myocardial infarction (MI) model, miR-24 was downregulated shortly after MI, correlating with factors of ECM remodelling such as collagen, fibronectin, and TGF-β.[100] Transfection of miR-24 to the mouse myocardium before inducing MI led to a reduced infarct size and improved cardiac function. MiR-24 regulated cardiac fibrosis by targeting furin, which is necessary for TGF-β excretion.[100] In cardiomyocytes, miR-24 was mainly described as regulating apoptosis and cell survival via intrinsic apoptotic pathways.[101] In transgenic miR-24 mice undergoing MI, significantly less apoptosis and improved cardiac function was found compared with wild-type mice.[102] MiR-24 was also found to have a regulatory role in excitation–contraction uncoupling of the sarcoplasmic reticulum and T-tubules via the junctophilin-2 protein.[103] The effect of miR-24 on cardiomyocytes and on fibroblasts emphasizes the broad range of regulating functions miRNAs can exert, which may challenge the development of targeted miRNA therapies.

Towards clinical application of microRNA-based therapies

As described above, experimental and animal data already suggest a future role for miRNA-based therapies in the treatment of patients with heart failure. The use of antimirs has also been successful in larger animals. For example, in pigs undergoing percutaneous ischaemia and reperfusion, the LNA-modified antisense miR-92a led to a reduced miR-92a expression after both intravenous and percutaneous administration.[104] However, only regional administration additionally resulted in a reduced infarct size and improved recovery of cardiac function.

Despite several difficulties regarding miRNA-based therapies, the first phase 1 and 2 trials have been conducted investigating the potential therapeutic application of miRNA-based drugs intended for indications other than heart failure. The administration of MRX34 (miR-34) is currently under investigation in a phase 1 clinical trial in patients with primary liver cancer, other solid tumours, and haematological malignancies (NCT01829971). Another phase 1 trial studies the safety and efficacy of TargomiRs containing a mir-16-based mimic in patients with malignant pleural mesothelioma and non-small cell lung cancer who are not responding to standard therapy (NCT02369198). To date, the most rapidly developing miRNA-based drug is miravirsen, a subcutaneously administrated antisense oligonucleotide for miR-122, which is under investigation in chronic hepatitis C patients. The first phase 1 and 2a results have been promising, showing reduced RNA levels of hepatitis C virus with no important side-effects.[105] The success of this drug without off-target effects mainly results from the fact that miR-122 is a tissue-specific miRNA that is only expressed in the liver, while most miRNAs involved in heart failure processes are not restricted to the heart.

Before clinical application of miRNA-based therapy becomes widely available, some important issues need to be addressed. The pharmacodynamic and pharmacokinetic effects of antimirs and miRNA mimics are not yet fully established but are crucial for future clinical application. It is currently unknown by which mechanisms antimirs remain functional, stable and how they are released, even after a substantial amount of time. The effect of antimirs can last for months, as demonstrated by studies in mice and monkeys.[69,106] These results raise concerns about potential toxicity and irreversibility. Moreover, long-term follow-up studies are necessary to identify any side-effects that may develop after months or years. Organ-specific delivery of antimirs is currently challenging and techniques to establish targeted delivery to the heart are needed. Therefore, focusing on miRNA targets that are mainly expressed in the heart, such as the previously described miR-1, miR-133, and miR-208 would limit these unwanted side-effects. Furthermore, as one miRNA has multiple target genes, miRNA-based therapy may lead to unfavourable off-target effects in tissues other than the target tissue. For example, the use of miRNA mimics leads to intracellular competition with endogenous miRNAs in terms of binding to the RISC machinery, which in turn results in disturbances in normal miRNA functioning and thereby gene regulation.[107]

Despite these concerns, promising data have been published in which miRNA mimics and antimirs were able to modify miRNA function in heart failure models, and this might pave the way to the first studies in humans.

Conclusions

Our knowledge of miRNAs and their potential role in heart failure has greatly increased over recent years. Although many uncertainties remain regarding the purpose and origin of circulating miRNAs, an increasing number of studies have provided important clues about their transport and function in the circulation. Furthermore, circulating miRNAs are promising new biomarkers in heart failure for diagnostic and prognostic purposes, and to identify a patient's response to therapy. Several miRNAs have been related to important mechanisms leading to heart failure, such as hypertrophy and fibrosis. An increasing number of miRNAs and miRNA targets have been reported in heart failure models, increasing our insight into the pathophysiology of this syndrome. Loss- and gain-of-function experiments revealed an important role

for miRNA mimics and antimirs—an interesting development that might broaden the treatment options for patients with heart failure. Together, miRNAs and miRNA-based therapies comprise one of the most innovative advancements of the last years and hold great promise for future clinical application in heart failure.

Acknowledgements

We acknowledge the support from the Netherlands CardioVascular Research Initiative: the Dutch Heart Foundation, Dutch Federation of University Medical Centres, the Netherlands Organization for Health Research and Development and the Royal Netherlands Academy of Sciences.

Funding

This work was supported by a grant from the Dutch Heart Foundation: Approaching Heart Failure By Translational Research Of RNA Mechanisms (ARENA), [grant number CVON 2011–11].

Conflicts of interest; E.L.V. and L.J.D.W. have no conflicts to report. Y.M.P has a minor stake (<5%) in a University spin-off which commercializes IP in the field of biomarkers and microRNAs. P.V.D.M. and A.A.V. are patent holders of a panel of acute heart failure related circulating microRNAs.

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