Chapter 8

General discussion and future perspectives

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Heart failure is a complex syndrome unfortunately accompanied by high morbidity and mortality rates. As such, there is a continuous need for more insight into pathophysiological mechanisms and eventually new therapeutic options. Since the discovery of miRNAs in 1993,\textsuperscript{1,2} researchers began to explore the involvement of these small, non-coding RNAs in the development and progression of several different diseases. With their gene regulating function, miRNAs were shown to be required for normal cardiac development\textsuperscript{3-5} and able to modulate important physiological processes involved in cardiovascular disease and heart failure.\textsuperscript{6} Consequently, the search for new agents targeting miRNA function and thereby altering deleterious disease mechanisms in heart failure has moved on apace in recent years. More than a decade later, miRNAs were also discovered outside cells and proven to be present in blood, urine and other body fluids.\textsuperscript{7,8} This opened new windows in miRNA research, especially the role of miRNAs as biomarkers was increasingly investigated. In this thesis, we addressed the role of circulating miRNAs in heart failure and we additionally tried to increase our understanding of their potential meaning and function.

In Chapter 2, we reviewed the current knowledge regarding the role of miRNAs in hypertrophy and fibrosis, and reported new developments in miRNA-based therapies. We also specifically focused on circulating miRNAs, their potential as biomarker and the general understanding on the release and uptake in the circulation and role in cell-to-cell communication.

We described key miRNAs known to be involved in the development of cardiac hypertrophy, including miR-1 and miR-133. A downregulation of miR-1 and miR-133 was consistently found in hypertrophic hearts and can lead to a cascade of processes involved in the development of cardiac hypertrophy,\textsuperscript{9,10} while the miRNAs miR-21, miR-24 and miR-29 have been shown to regulate cardiac fibrosis inducing mechanisms. Pre-clinical in vitro and in vivo studies with antimiRs (inhibitors of miRNA function) or miRNA mimics (stimulating miRNA function) were able to demonstrate inhibition or reversal of these pathophysiological mechanisms.\textsuperscript{11-15} These studies proposed that these miRNAs with crucial functions in cardiac pathophysiology could function as promising new drug targets. Interestingly, in the circulation, these or other cardiac-enriched miRNAs were often not among the miRNAs found to be most differentially expressed between heart failure patients and healthy subjects. However, this does not exclude the ability of other miRNAs to function as biomarkers aiding in the diagnosis, prognosis and evaluation of therapy in heart failure patients. We described several studies which have shown diagnostic potential of circulating miRNAs and reviewed the few studies in which the associations with clinical outcome and response to therapy were investigated. To better understand their potential role as biomarkers, information on the miRNA biology in the circulation is highly desirable, but unfortunately scarce. Growing evidence suggests a
paracrine function of miRNAs in the circulation.\textsuperscript{16,17} In these studies, it was shown that miRNAs were excreted into the circulation and transported within extracellular vesicles to be taken up by target cells in order to regulate gene expression. However, as these data were all derived from controlled \textit{in vitro} studies, it remains to be proven that this process also occurs \textit{in vivo}. Not only extracellular vesicles but also apoptotic bodies, HDL particles and protein complexes are capable of transporting miRNAs throughout the circulation, in which the latter comprises the majority of all miRNA transporters in the circulation. Currently, it is unknown whether these miRNA-protein complexes can be absorbed by target cells in order to regulate gene expression.

**IDENTIFICATION OF A CIRCULATING MICRONA PROFILE IN PATIENTS WITH HEART FAILURE**

In heart failure, some studies reported the associations between circulating miRNAs and heart failure, however mostly in small populations and/or without proper validation.\textsuperscript{18-21} Further, data on differentially expressed circulating miRNAs in acute heart failure are scarce. Therefore, in \textbf{Chapter 3} of this thesis we investigated circulating miRNAs in acute heart failure patients and report an unique signature of circulating miRNAs, including let-7i-5p, miR-16-5p, miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, miR-223-3p, miR-423-3p, miR-423-5p and miR-652-3p. These miRNAs were identified in the discovery phase from a large panel of 375 miRNAs, and showed a fold increase of $>4$ in acute heart failure patients at time of admission compared to healthy control subjects. All these miRNAs were downregulated in patients with acute heart failure, and slightly higher in patients with less acute stages of heart failure and chronic heart failure, gradually increasing towards the highest levels in healthy control subjects. These lower miRNA concentrations in heart failure patients were consistently found in multiple different heart failure cohorts and validated in other, independent cohorts. Interestingly, in patients with dyspnea due to an acute exacerbation of chronic obstructive pulmonary disease no significant circulating miRNA differences could be found compared to controls, suggesting that these miRNAs are not solely reflecting breathlessness or hypoxia, but have some specificity for heart failure. Further, a decrease of a subset of these miRNAs during the first 48 hours of an acute heart failure hospitalization was related to 180-day mortality. Our results suggest that decreasing levels of the identified circulating miRNAs are related to an increasing acuity of heart failure and that a further drop in circulating miRNA levels early during hospitalization is associated with a worse outcome.

Although we presented very robust and consistent results, suggesting some diagnostic and prognostic biomarker potential for the identified miRNAs, future prospective
studies are needed to truly investigate not only diagnostic and prognostic abilities of these miRNAs, but also their role in the evaluation of response to therapy. In addition, more knowledge about these circulating miRNAs and the pathophysiological processes they might reflect could lead to a better understanding of their role in the development and progression of acute heart failure.

CIRCULATING MICRONAS AND THEIR POTENTIAL ROLE AND FUNCTION

Not much is known about this set of circulating miRNAs and to better understand their function we investigated several hypotheses potentially contributing to the current information on circulating miRNAs and their role in heart failure. First, we tried to associate the circulating miRNAs to heart failure-related disease processes. Well-known biomarkers in heart failure have previously been linked to cardiac-related mechanisms such as inflammation, cardiac stretch and remodeling,\textsuperscript{22-24} and therefore we sought to make use of these biomarkers to learn more about the role of miRNAs in the circulation. In Chapter 4 we studied the previously identified circulating miRNAs in relation to other, established and novel heart failure biomarkers in 100 patients hospitalized for heart failure. These biomarkers from different pathophysiological domains reflecting disease processes such as fibrosis, inflammation and renal dysfunction were selected based on their predictive value for a worse outcome (180-day mortality). We investigated these biomarkers and miRNAs in relation to severity of disease, by dividing the acute heart failure population in 4 groups based on clinical parameters during hospitalization. At hospital admission, no significant correlations could be identified between circulating miRNAs and other biomarkers, whereas after 48 hours, several significant negative correlations could be found (between miR-16-5p and c-reactive protein (CRP), miR-106a-5p and creatinine, miR-223-3p and growth differentiation factor 15 (GDF-15), miR-652-3p and soluble ST2, miR-199a-3p and procalcitonin (PCT), miR-18a-5p and PCT and miR-199a-3p and galectin-3). These associations were specifically found in those patients with the most unfavorable clinical course during hospitalization and the worst outcome. To gain more insight into potentially involved pathways and mechanisms, we performed miRNA target prediction and pathway analyses. This resulted in the identification of several predicted targets functioning in top-enriched pathways all previously related to cardiac disease. Furthermore, it was shown that some of the miRNA-biomarker correlations could be linked to the predicted target genes of the investigated miRNAs. As the significant miRNA-biomarker correlations were specifically identified after 48 hours of hospitalization and mainly in patients with the worst in-hospital course, our results suggest that these relations are highly time-dependent and related to clinical disease status. Moreover, the acute heart failure population is very heterogeneous owing to dif-
ferences in aetiology, volume status, medication use and comorbidities and it remains challenging to mechanistically position the previously identified heart failure-related circulating miRNAs with use of known functions of other biomarkers. It is therefore important that these results are regarded to as hypothesis generating and further in vitro and in vivo experiments are needed to investigate potential underlying heart failure-related processes.

A standout feature of the identified set of heart failure-related circulating miRNAs is their consistent downregulation in heart failure compared to controls. Although several other studies also have shown downregulated miRNA levels in plasma of heart failure patients compared to healthy subjects, the consistent findings are striking and prompted the further investigation of possible explanations of this phenomenon. Different hypotheses include the increased uptake of miRNAs from the circulation by target cells or the reduced production and secretion into the circulation, although none of these theories have been confirmed to date.

A critical aspect of acute heart failure is volume overload, exemplified by signs and symptoms such as dyspnea, orthopnea, edema, jugular venous dilatation and ascites. Excessive intravasal fluid in heart failure patients can be reflected by lower levels of haemoglobin, also called haemodilution. Similarly, when patients lose fluid due to diuretic treatment, an increase in haemoglobin levels is often seen, which is commonly referred to as haemoconcentration. As the concentration of circulating miRNAs was found to be the lowest in patients with acute heart failure and gradually increased in patients with chronic heart failure towards the highest levels in healthy individuals, the question arose whether fluid overload and hypervolemia could influence circulating miRNA levels. We therefore investigated in Chapter 5 the effect of volume status on circulating miRNA levels and hypothesized that fluid overload may contribute to lower circulating miRNA levels in plasma of acute heart failure patients. This relation was demonstrated by significant associations between change in haemoglobin levels and change in miRNA levels. We found that in patients with haemoconcentration (defined as an increase in haemoglobin levels on day 7 compared with hospital admission) miRNA levels increased, whereas in patients without haemoconcentration (no change or a decrease in haemoglobin levels on day 7 compared with baseline), miRNA levels decreased. As it has been shown that patients without haemoconcentration have a worse outcome compared to patients who do haemoconcentrate, we also corrected for prognostically important clinical and laboratory parameters in acute heart failure patients. The majority of the associations between miRNAs and change in haemoglobin levels remained significant, suggesting they do not solely depend on disease status. Thus, our results suggest that low miRNAs levels are associated with hypervolemia and that these levels increase with fluid loss. This coincides with the previous results in
Chapter 3 which demonstrate a clear increase in miRNA levels in patients with less acute and more stable forms of heart failure and healthy controls. Despite the consistency, absolute miRNA changes in this cohort are subtle, indicating that other underlying pathophysiologic processes besides volumes status are also likely contributors to the downregulation of the previously found circulating miRNAs in heart failure patients.

As detailed in Chapter 2, several studies have tried to relate cardiac miRNA expression to circulating miRNA levels in heart failure. By measuring the transcoronary miRNA concentration gradient, some groups were able to show an increased miRNA gradient in patients with myocardial infarction and heart failure, suggesting that these miRNAs might be secreted by the heart. However, most studies did not compare these levels with peripheral venous levels or were not able to show miRNA concentration differences between heart failure patients and controls without structural heart disease, challenging the extrapolation to readily available venous circulating miRNA levels. Further, it has been shown that the most abundantly circulating miRNAs known to be differentially expressed in cancer originate from blood cells, and in heart failure it was found that the miRNA expression in tissue does not correspond to the miRNA profile in the circulation. In addition, most circulating miRNAs were highly expressed in haemopoietic cells and endothelial cells, and only 0.1% in healthy individuals to 1% in heart failure patients was found to be cardiac or muscle-enriched. Although these myocardrials were only expressed in low levels, significant changes between heart failure patients and controls were found, suggesting that changes in disease status can be reflected in the circulation (when detected). In our previous study, no cardiac-specific or cardiac-enriched miRNAs (including miR-1, miR-133, miR-208 and miR-499) were measurable or differentially expressed. Our results and previous data therefore suggest that blood and endothelial cells might be the source of the detected circulating miRNAs in our miRNA profiling study. Although some of the heart failure-related circulating miRNAs identified in Chapter 3 were already described in relation to cardiovascular disease processes (in addition to the results of Chapter 4), we also found literature on potential involvement in vascular-related processes such as inflammation, endothelial dysfunction and angiogenesis. We therefore aimed in Chapter 6 to study the associations between the set of heart failure-related miRNAs and vascular-related disease processes in patients with heart failure and additionally atherosclerotic disease. We were able to associate decreasing miRNA levels with an increasing number of manifestations of atherosclerotic disease (coronary artery disease, transient ischaemic attack/stroke and peripheral arterial disease) and show that the large majority of the investigated miRNAs were specifically downregulated in peripheral arterial disease. In addition, multiple significant associations were found between these miRNAs and biomarkers reflecting vascular disease processes including inflammation, endothelial dysfunction and angio-
genesis, in which lower miRNA levels were related to higher biomarker levels. Finally, we were able to show that low levels of 6 miRNAs were associated to cardiovascular-related rehospitalization, whereas this was not the case for other clinical endpoints including mortality and heart failure rehospitalization. These results suggest a potential involvement of the previously identified miRNAs in vascular disease and related pathophysiological processes. Similar to the results in Chapter 3, 4 and 5, we again show that low miRNA levels seem to be related to an unfavorable clinical status and a worse outcome in patients with heart failure. In this chapter we were able to additionally provide data supporting the hypothesis that these low miRNAs levels are mainly related to vascular disease processes as reflected in heart failure patients with atherosclerosis.

Investigation of a suitable animal heart failure model for mechanistic follow-up studies

In order to get mechanistic insight into the complex nature of miRNAs in heart failure, animal heart failure models are necessary for experimental follow-up studies. Therefore, in Chapter 7 we investigated the miRNAs previously identified in human heart failure in 3 rodent models of heart failure, including homozygous TGR(mREN2)27 rats (characterized by overexpression of the renin-2 gene), mice with angiotensin II (AngII)-induced heart failure and mice with permanent MI-induced heart failure. Ren2 rats develop severe hypertension and as a result left ventricular hypertrophy and cardiac fibrosis, eventually resulting in heart failure. AngII mice also develop cardiac hypertrophy and fibrosis and reflect mainly diastolic dysfunction. To fully capture a broad spectrum of different heart failure mechanisms, we also included a myocardial infarction-induced heart failure model (caused by permanent ligation of the left coronary artery). Interestingly, in none of these well-known, established heart failure models we were able to identify differences in circulating miRNA expression compared to control animals. In the ischaemic heart failure mice we additionally measured 2 cardiac-specific miRNAs (miR-499a-5p and miR-208a-3p), however only miR-499a-5p was detectable (in low levels) in the circulation of both control and heart failure mice and did not differ in plasma concentrations. Further, in kidney and left ventricle tissue of these animals, the cardiac-specific miRNAs (among 5 other miRNAs from our panel) showed clear expression differences between the kidney and left ventricle, with higher levels in the heart compared to kidney tissue. With the relatively small animal numbers we were not able to report differences between miRNA expression levels in the hearts of ischaemic heart failure mice and control animals.

These results can be of major importance for future follow-up studies in animals based on circulating miRNA data in humans, as we could not replicate our findings of
low circulating miRNA levels in human heart failure in 3 different rodent heart failure models. Although several explanatory factors can be contributable, such as the method of measuring and conservation of miRNA function and regulation, it is conceivable that these heart failure models do not reflect the heterogeneous clinical human heart failure syndrome, especially in relation to comorbidities and medication use. This is not the only study addressing the discordance between multiple experimental models and human data. Schlosser et al. reported that miRNAs with known differential expression in pulmonary hypertension showed inconsistent changes in tissue and circulating plasma in different experimental models and human pulmonary hypertension.44

Our findings challenge the understanding of circulating miRNAs and their role in heart failure. Also, the human situation can not be directly translated to animal data, which complicates the set-up of experimental studies to investigate the function and regulation of circulating miRNAs in more detail.
In this thesis we addressed the identification of a circulating miRNA signature in patients with (acute) heart failure and placed these findings in perspective by investigating several hypotheses regarding their potential role and function. All of the identified miRNAs were found in the lowest levels in patients with the most acute state of heart failure and showed an increase towards more stable forms of heart failure and healthy control subjects. A further in-hospital miRNA level decrease was found to be related to an increased risk of mortality. Moreover, several associations between miRNAs and other, well-known and novel heart failure-related biomarkers were identified in patients with worsening heart failure, and target prediction complemented by pathway analyses resulted in potential mechanisms implicated in cardiac disease. Our results indicate that circulating miRNAs and their correlation with biomarkers are highly dependent on timing of measurement and clinical disease status. It was additionally shown that changes in volume status were associated with changes in circulating miRNA levels, suggesting that fluid overload may contribute to downregulated miRNA levels in acute heart failure. Furthermore, these miRNAs were associated to vascular dysfunction as reflected by the presence of atherosclerosis and related disease processes including inflammation, angiogenesis and endothelial dysfunction, and also have predictive value for the risk of specifically cardiovascular-related rehospitalization. In our attempt to identify a suitable heart failure animal model for mechanistic miRNA studies we were not able to replicate the previously found downregulated miRNA levels in human heart failure, as we did not observe any miRNA level differences in plasma of heart failure animals in comparisons to controls. These results place emphasis on the difficulty of translation between the human heart failure setting and animal data, and underline the complex biology of circulating miRNAs.
Future perspectives

CAN MICRORNAS BECOME CLINICAL APPLICABLE BIOMARKERS IN HEART FAILURE?

In 1998, the National Institutes of Health defined a biomarker as a biological marker that is objectively measured and evaluated as indicator of normal biological processes, pathological processes, or pharmacological responses to therapeutic interventions. Although this definition also captures physiological measurements and other clinical assessments, the term biomarker is most often used for a biochemical marker measurable in blood. In heart failure, biomarkers can be useful diagnostic and prognostic tools, or serve as evaluators of initiated therapy. As discussed in this thesis, several miRNAs have been investigated as potential biomarkers in heart failure. However, there is very little overlap of candidate miRNAs between studies, and the variability in methodology, lack of validation and small population sizes are possible explanations for this. To this day, the natriuretic peptides B-type natriuretic peptide (BNP) and N-terminal pro-BNP (NT-proBNP) are known as the most established biomarkers in heart failure and only few studies have examined the additional value of potential miRNA candidates compared to and/or on top of natriuretic peptides and clinical parameters. As natriuretic peptides only reflect a small part of all pathophysiological mechanisms in heart failure, it is conceivable that miRNAs may add to this spectrum and provide additional value to conventional biomarkers. However, several crucial steps are necessary towards clinical application of miRNAs as biomarkers in heart failure. First, a standardized method of measuring should be developed, as several different techniques (including RNA-sequencing, qRT-PCR, microarrays) are currently available. To date, qRT-PCR is the most widely used method for miRNA analysis and a general accepted method for data normalization is a prerequisite for future (clinical) use of circulating miRNAs. Recently, de Ronde et al. proposed a protocol for qPCR data processing, addressing issues including technical errors, low detection and imputation. Second, as with most biomarker studies, population sizes are small and in order to truly bring miRNAs forward as biomarkers, population sizes should increase substantially. Third, data analysis should include statistical analyses assessing the (additional) value of miRNAs in comparison or on top of the predictive value of natriuretic peptides and important clinical information. Finally, ideally miRNAs (and biomarkers in general) should change clinical practice in terms of improvement of diagnosis, adjustment of therapeutics and better outcomes.
Another important feature of biomarkers is that they can reflect pathophysiological mechanisms. In heart failure, and especially in heart failure with a preserved ejection fraction, there is an unmet need for markers capable of not only improving diagnosis but also elucidating underlying biological mechanisms. Circulating miRNAs could contribute to this knowledge and it would therefore be valuable to know more about miRNA-related mechanisms in heart failure. This would also require a better understanding of their origin, release into the circulation, potential uptake by target organs and the associations with miRNA levels in tissue. Although some progress have been made in this area, further research is necessary to fully understand the biology of circulating miRNAs. As such, several hurdles have to be overcome in the process towards clinical application of circulating miRNAs and as it stands right now there is no place for miRNAs as biomarkers for the diagnosis, prognosis and evaluation of therapy in heart failure.

**MICRONOA-BASED THERAPIES**

MiRNA function can be inhibited or promoted by antimir and miRNA mimics. This ability to modulate miRNA function stimulated research on their therapeutic potential. Further, owing to the known sequences, the effect on the complementary mRNA and small size, development of suitable modulating chemistries is attractive. Pharmacological development of mainly antimir have made rapid progress in recent years, in both the cardiovascular and non-cardiovascular field. Although no clinical trials started yet in heart failure patients, pre-clinical work in rodents but also large animals have shown success. For example, miR-208-a cardiac-specific miRNA- was targeted by a locked nucleic acid (LNA) antimir in Dahl hypertensive heart failure rats, preventing cardiac remodeling and improving cardiac function. Further, antimir have been used to silence the function of miR-21 to inhibit cardiac fibrosis in a mouse pressure-overload model. Also in larger animal models, antimir have shown to be effective. In a porcine ischaemia/reperfusion model, antimir-15 was found to protect the heart from cardiomyocyte cell death after myocardial infarction and another group demonstrated that an antimir for miR-92 was able to reduce infarct size and consequently attenuation of myocardial dysfunction. Clinical trials have been initiated for several non-cardiovascular indications, including hepatitis C (with antimir-122), type 2 diabetes and non-alcoholic fatty liver disease (antimir-103/107), T cell lymphoma (antimir-155), scleroderma (miR-29 mimic), lung cancer (miR-16 mimic) and other multiple solid tumors (miR-34 mimic). Miravirsen, the antimir for the liver-specific miR-122 and potential new treatment of hepatitis C, has progressed substantially over the years and is, with additional phase II studies with long-term follow-up, the closest to application in clinical practice.
While the future for miRNAs as biomarkers in heart failure is rather unsure, owing to the promising pre-clinical results in cardiac pathology and successful trials in other diseases, it is not unthinkable that miRNA-based therapies eventually translate from bench to bedside, also in heart failure. Difficulties in the development of miRNA-based therapies in heart disease comprise several factors, including the selection of a potential, disease-specific miRNA candidate. As heart failure is a heterogeneous syndrome with several pathways and mechanisms involved, the identification of a suitable target is complex. Integration of miRNA(-sequencing) data with information on mRNA and proteomics would allow an in-depth network analysis using a bioinformatics approach, pin-pointing common regulators of a certain disease or disease processes, as demonstrated by Yang et al. in ovarian cancer. Novel high-throughput techniques including RNA-sequencing and crosslinking immunoprecipitation (CLIP-seq) enable the genome-wide mapping of miRNA-mRNA interactions as well as protein-RNA binding sites, facilitating the identification of a promising target. As shown in the previous chapter, translation of human data to animal models and vice versa is complex and it is known that although miRNA conservation is high, miRNA function may be different in humans and animals, limiting the usability of animal models for the identification of a suitable miRNA target. Moreover, stable delivery techniques, off-target effects and tissue-specific targeting are key issues which still need further improvement. Nevertheless, the rapidly developing field of miRNA-based therapeutics holds great promise for the initiation of clinical trials in heart failure and possible future clinical application of miRNA-based therapies.

**NEW KIDS ON THE BLOCK**

**Long non-coding RNAs**

The majority of non-coding RNAs consists of long non-coding RNAs (lncRNAs). These large (>200 nucleotides) transcripts do not encode for proteins, with some exceptions. Unlike miRNAs, lncRNAs are less conserved between species but quite cell- and organ specific. The gene regulating function of lncRNAs is diverse and takes place at both the transcriptional and translational level, where they can function as molecular scaffolds, decoys, guide lncRNAs or regulators of splicing. However, the precise mechanisms are not well-understood.

LncRNAs have been related to cardiac disease and heart failure. For instance, MIAT (myocardial infarction-associated transcript) was found to be associated with the risk of myocardial infarction, using genome-wide association methods. In addition, the lncRNA MYHEART (myosin heavy-chain-associated RNA transcript) was shown to be protective in terms of development of cardiac hypertrophy. In blood of patients with acute myocardial infarction patients this lncRNA was significantly elevated and a mouse
model demonstrated that MYHEART could prevent cardiomyocyte death. Some studies investigated circulating lncRNAs in regard to their diagnostic and prognostic potential. The first study reporting on the predictive value of a circulating lncRNAs in humans was by Kumarswamy et al. Although a large number of lncRNAs was not detectable in blood plasma, they present the circulating lncRNA LIPCAR (long intergenic ncRNA predicting cardiac remodeling) to be predictive of cardiac remodeling and risk of death after myocardial infarction. Further, serial measurements demonstrated an initial decrease after myocardial infarction while LIPCAR levels were elevated in plasma of patients developing heart failure. Another study measured known lncRNAs in plasma from heart failure patients and controls and identified NRON (non-coding repressor of NFAT) and MYHEART to be differentially expressed in heart failure compared to healthy subjects. Vausort et al. described that 4 out of 5 previously identified and well-known lncRNAs are differentially expressed in whole blood of patients with myocardial infarction. However, when compared to conventional biomarkers such as NT-proBNP, creatine kinase and clinical characteristics, the lncRNAs were only weakly predictive of left ventricular dysfunction. The above described approaches will unlikely yield circulating lncRNAs as potential biomarkers. The lack of knowledge about their mechanisms of action and the limited specificity for cardiac disease of the previous reported circulating lncRNAs are major drawbacks. Another limitation in the search for new lncRNA biomarkers is the poor evolutionary conservation, which complicates the translation of animal models to the human setting. Further, the amount of lncRNA in plasma is low, hampering the measurability of circulating lncRNAs. Full blood may provide a better source of lncRNAs, although the composition of blood components may drive the circulating lncRNA expression and hinder correct interpretation.

The time for clinical use of lncRNAs as biomarkers may not be in the near future. However, a previous study already demonstrated a role for lncRNAs in clinical decision making, by using known lncRNAs to predict response to anti-diabetic therapy. Further, as the knowledge of circulating lncRNAs is far from complete, there is much to learn in respect to their biogenesis and function. The exploration of lncRNAs as new drug targets is still in its infancy, however the specificity of lncRNAs for certain cell types/ organs and their abundant expression in tissue make them -in theory- attractive candidates for lncRNA-based therapies.

**Circular RNAs**

Circular RNAs (circRNAs) are closed, single-stranded, long non-coding transcripts located in the cytoplasm and nucleus of the cell and are >100 nucleotides or longer. They are formed from precursor mRNAs by back-splicing of exons from protein-coding genes, and although their biogenesis is not entirely clear, 3 different pathways of circularization have been proposed including intron sequence complementarity, interaction...
between RNA binding proteins or intralariat splicing. CircRNAs were discovered more than 2 decades ago, but at that time researchers thought they were non-functional and splicing byproducts. Now it is clear that they are present in many species and tissues as well as in the circulation, and some of them have demonstrated to regulate gene expression. The exact mechanisms of their influence on gene expression are unclear, although it has been proposed that the formation of circRNAs from mRNA would result in less processed mRNAs. It was also shown that circRNAs can function as competitors with mRNAs for miRNA binding. For example, HRCR (heart-related circRNA) in mice is downregulated in heart failure tissue and is able to act as a miR-223 sponge (thereby silencing miR-223 function) in order to inhibit cardiac hypertrophy and eventually heart failure. Another gene expression modulating mechanism includes the regulation of transcription in cis (nearby loci) of intron-containing circRNAs.

CircRNAs are also measurable in blood and the role of circRNAs as biomarkers in heart failure but also in other diseases is currently under investigation. Vausort et al. showed that the circRNA MICRA (myocardial infarction-associated circular RNA) was able to predict LV dysfunction 3-4 months after myocardial infarction in blood of patients from 2 independent cohorts. So far, this is the only study addressing the potential use of circRNAs as biomarkers in heart failure and may therefore be the stepping stone for future circulating circRNA studies. Naturally, the implementation of circRNAs as biomarkers in heart failure faces roughly the same challenges as circulating lncRNAs and miRNAs, however the fact that large numbers of circRNAs are cell-specific and their relatively high abundance in circulating blood make circRNAs interesting for further exploration.
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