Circulating microRNAs in heart failure
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Chapter 1

Introduction and aims

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HEART FAILURE

Heart failure is a major public health concern and affects 1-2% of the adult population. The European Society of Cardiology defined heart failure as a clinical syndrome characterized by typical symptoms (such as dyspnea and fatigue) and signs (pulmonary rales, ankle oedema, elevated jugular venous pressure), accompanied by structural and/or functional cardiac abnormality, resulting in a reduced cardiac output and/or elevated intracardiac pressures at rest or during stress. The aetiology of heart failure is diverse, including cardiac abnormalities caused by for example valve defects, congenital heart disease and myocarditis, as well as arrhythmias and systemic diseases such as hypertension, diabetes and anaemia. Although it is likely that multiple factors contribute to the development of heart failure, ischaemic heart disease is known as the most common cause of heart failure in the Western world. Ischaemic heart disease results from a reduced blood supply from the coronary arteries to the myocardium, most often caused by atherosclerosis. This in turn leads to a mismatch between oxygen demand and supply, which eventually can lead to ventricular remodeling and impaired cardiac function.

Acute heart failure is characterized by the acute onset of severe signs and symptoms of heart failure requiring immediate treatment, while physicians generally speak of chronic heart failure when patients are stable and treated for previously diagnosed heart failure. Heart failure can be further categorized in 3 main groups based on left ventricular ejection fraction (LVEF); heart failure with a reduced LVEF (<40%), also known as HFrEF, heart failure with a mid-range LVEF of 40-49% (HFmrEF) and heart failure with a preserved LVEF of ≥50% (HFpEF). These 3 groups represent patients with different underlying characteristics and do not respond in a similar way to therapy.

In the last decades several advancements in heart failure treatment have been made, such as the development of neurohormonal antagonists (beta-blockers, ACE inhibitors, angiotensin II receptor blockers and mineralocorticoid receptor antagonists), and more recently the new drug sacubitril/valsartan, an angiotensin receptor-neprilysin inhibitor. Besides medication, device therapy including the implantable cardioverter-defibrillator (ICD) and cardiac resynchronization therapy (CRT) has shown to improve survival in selected patient groups. Despite these much-needed improvements, the prognosis of heart failure is poor. Five-year mortality in heart failure ranges between 41-60%, while more recent data on 1-year mortality report 7-17%. When a patient is hospitalized with acute heart failure, the 5-year mortality rate increases to more than 75%.

As the prognosis of heart failure patients clearly remains unsatisfactory, new treatment strategies are needed. In search for new drug targets, it is important to understand the underlying pathophysiological mechanisms in heart failure. Several processes have
been discovered with crucial involvement in the development and progression of the disease, for example the neurohormonal adaptation system; the keystone of the presently available heart failure pharmacotherapy. These drugs are mainly focused on targeting cell-surface or intracellular mineralocorticoid receptors, eventually attenuating cardiac remodeling and cardiac dysfunction. Other approaches focusing on intracellular molecular signalling pathways may lead to additional benefit ideally resulting in reduced mortality and rehospitalisation rates. Currently there is a rapidly growing interest in the genetic and epigenetic background of heart failure patients, providing detailed information on the cellular level. Gene therapy can interfere with these molecular processes by modulating the production and/or activity of important regulatory proteins in heart failure. Pharmacogenomics may therefore be a promising new area of interest and may eventually emerge as new treatment strategy in heart failure.

**GENE EXPRESSION AND EPIGENETICS**

A large part of the human body consists of proteins and they play a central role in the function of important (patho)physiological processes. Proteins are molecules made of amino acids and the information to produce these proteins lies within portions of the DNA (deoxyribonucleic acid), called genes. DNA consists of two strands which are both formed of nucleotides. These nucleotides contain the nucleobase adenine (A), thymine (T), cytosine (C) or guanine (G), deoxyribose (a sugar) and a phosphate group, and form sequences holding the genes containing the biologically important information for the production of proteins. The nucleobases of the 2 strands join together according to base complementary (A with T and C with G). DNA gets transcribed into RNA (ribonucleic acid) and the mature transcript (messenger RNA, or mRNA) leaves the nucleus. This mRNA can then be translated into amino acids to form proteins. This process from gene to gene product (most often proteins) is called gene expression. Gene expression of specific genes can be quantified by measuring the mRNA transcripts in a particular cell type or tissue and can give an impression of the regulation of important (patho) physiological processes.

Gene expression can be regulated and this may have important consequences for protein synthesis and subsequently the development of diseases. These gene expression modifying mechanisms are all collectively known as epigenetics. The Oxford dictionary defines epigenetics as “the study of changes in organisms caused by modification of gene expression rather than alteration of the genetic code itself”, or in other words; a change in phenotype without a change in genotype. At the moment, several different gene expression modifying mechanisms have been described including DNA methylation, chromatin remodeling, histone modifications and non-coding RNA mechanisms.
Non-coding RNAs are transcribed from DNA, but are not translated into proteins. This is the large majority of all transcripts, as only 2% is translated into proteins. In the early days, these non-coding transcripts were regarded to as non-functional, however it is now clear that non-coding RNAs have several functions in regulatory mechanisms of gene expression and function. Non-coding RNAs can be divided into short non-coding RNAs (< 30 nucleotides) and long non-coding RNAs (>200 nucleotides).

**MICRORNAS**

The microRNAs (miRNAs) belong to the short non-coding RNAs and were first described in the roundworm *Caenorhabditis elegans* by Lee et al.\(^{12}\) and Wightman et al.,\(^{13}\) in 1993. They showed that the gene lin-4 was not coding for proteins but instead produced a short RNA of 22 nucleotides long, which was later found to be capable of binding to lin-14 and thereby reducing the production of the LIN-14 protein. In 2001, several more miRNAs were discovered in *C. elegans* and could also be identified in other invertebrates, vertebrates and humans.\(^{14-16}\) These milestone papers paved the way for several years of continuously increasing miRNA research (Figure 1). So far, 2588 mature miRNAs have been discovered in humans (www.mirbase.org).

In the nucleus, primary miRNAs (pri-miRNAs) are transcribed from DNA. These long stemloop structures with single stranded RNA extensions on both ends are then cleaved by processing proteins DGR8 and Drosha, resulting in precursors hairpin miRNAs (pre-miRNAs). The pre-miRNAs leave the nucleus and are further processed by Dicer in the cytoplasm of the cell. This results in the loss of the hairpin structure and a mature miRNA is formed. These mature miRNAs can then be cleaved by helicase, creating 2 separate

![Figure 1. Number of PubMed publications containing the search term “microRNA” from the year 2001 to 2016](image-url)
miRNA strands. The active miRNA strand binds to an Ago protein which can be loaded into a RNA-induced silencing complex (RISC). This complex is capable of binding to the complementary 3’-untranslated region of the mRNA strand, and with perfect complementarity the mRNA transcript will degrade. With incomplete binding the mRNA will not degrade but repressed translationally. Figure 1 in Chapter 2 graphically presents the miRNA biogenesis and function. Thus, by targeting the complementary mRNA, miRNAs are able to regulate gene expression at the post-transcriptional level.

The current understanding is that miRNAs function intracellularly, and hence in tissue. Dysregulation of miRNAs have been linked to specific diseases and underlying pathways. Of all mature miRNAs, a small minority was found to be organ specific, such as miR-122 (which is only expressed in the liver) or miR-9 (expression limited to the brain). MiRNAs with high specificity for the heart are miR-208, miR-133, miR-1 and miR-499, which are all associated with cardiac development and disease. For example, deletion of the mature miR-1-2 sequence in mouse embryonic stem cells resulted in ventricular septum abnormalities, rapid heart dilatation, rhythm disturbances and ventricular dysfunction. MiR-208a was shown to be required for normal cardiac conduction and contraction, while upregulated levels were found to be related to cardiac hypertrophy, fibrosis and a disturbed electrical conduction in mice. As such, methods were developed to alter miRNA expression in order to inhibit or stimulate their gene expression-modifying function. Several pre-clinical studies were published to date and have shown promising results in the cardiovascular field, although no clinical trials have entered the arena yet.

Circulating microRNAs

Although miRNAs have a gene silencing function within cells, in 2008, researchers discovered extracellular miRNAs in blood and later also in other body fluids such as urine, saliva and cerebrospinal fluid. In this thesis, we specifically focus on miRNAs in blood plasma. These miRNAs are protected from early degradation by RNases in the circulation since they are either bound to proteins or encapsulated by exosomes, apoptotic bodies or other microparticles. Even extreme conditions such as boiling and long-term storage do not affect circulating miRNA levels. As it appeared that miRNAs can be transported through the circulation, the hypothesis that miRNAs may exert paracrine functions led to a plethora of studies investigating this possibility. Indeed, several studies showed that miRNAs are able to change gene expression and thereby protein synthesis in target cells in controlled, experimental settings. Besides their function as cell-to-cell communicators, the stability and relative straightforward measurability prompted the investigation of circulating miRNAs as biomarkers of disease, also in heart failure.

Biomarkers can reflect biological processes in health and disease, and are therefore helpful tools in the management of disease. In heart failure, the biomarkers N-terminal
pro b-type natriuretic peptide (NT-proBNP) and b-type natriuretic peptide (BNP) can help establishing the diagnosis of heart failure and are currently the gold standard.\(^1\) Additionally, several other, novel biomarkers such as soluble ST2, growth differentiation factor 15 (GDF-15) and galectin-3 have been described to have a role in heart failure and mainly reported as prognosticators of survival.\(^2\) However, biomarkers may also be used to evaluate therapy and can provide insight into underlying disease mechanisms. Circulating miRNAs could add value to the already available heart failure biomarkers and have been detected in blood and plasma of patients with cardiovascular disease. Distinct circulating miRNA patterns were reported for patients with myocardial infarction in comparison to control subjects, and also in cardiac arrhythmias and hypertension.\(^3\) In heart failure, most studies focused on differential miRNA expression in chronic heart failure patients compared to healthy subjects,\(^31\) while only few research groups directed their attention to acute heart failure. In these relatively small studies, circulating miRNAs capable of differentiating patients presenting at the emergency room with dyspnea owing to heart failure and breathlessness caused by other causes were described.\(^34,35\) Some groups tried to use circulating miRNAs to distinguish HFrEF from HFrEF, and identified specific differential miRNA patterns, although very few overlap was found between these studies.\(^35\) Because of the lack of robust circulating miRNA studies in acute heart failure, we focused on the investigation of a circulating miRNA profile in acute heart failure patients. In addition, the association between circulating miRNAs and outcome in acute heart failure has not been described before.

Although the proper identification of the most differentially expressed circulating miRNAs in heart failure is the first step, understanding the findings is a crucial next stage in the study of circulating miRNAs. These miRNAs may represent several clinical factors and disease processes in heart failure, however, information on this topic is limited. Increasing our knowledge concerning the potential meaning of these miRNAs in the circulation of heart failure patients is therefore one of the primary aims of this thesis.

**AIMS AND OUTLINE OF THE THESIS**

The main aims of this thesis are as follows:

1) Discover differentially expressed circulating miRNAs in acute heart failure patients
2) Provide additional information regarding clinical factors and disease processes these circulating miRNAs may reflect
3) Identify a suitable animal model for future translational research
In Chapter 2 we discuss the current knowledge of miRNAs in heart failure, specifically focusing on circulating miRNAs and their release and uptake from the circulation, their role in paracrine cell signalling and biomarker potential. Further, we describe miRNAs with important functions in cardiac hypertrophy and fibrosis and discuss the possibilities of inhibiting or overexpressing these miRNAs in the context of future drug targets.

Although some studies identified a pattern of differentially expressed circulating miRNAs in heart failure, most studies were conducted in a small number of chronic heart failure patients and few validated their findings in independent patient cohorts. Therefore, we aimed in Chapter 3 to identify a miRNA signature in several different heart failure cohorts representing different stages of acute and chronic heart failure.

While the identification of these differentially expressed miRNAs in plasma is the first step in the study of heart failure-related circulating miRNAs, not much is known regarding their origin, role and function in the circulation of heart failure patients. In the next chapters, we try to provide more background on our previous findings described in Chapter 3, in which we show that the most differentially expressed circulating miRNAs in heart failure are downregulated. We explore several different hypotheses in Chapter 4, 5 and 6, as presented in Figure 2. In Chapter 4 we investigate the previously identified miRNAs in relation to other, well-known biomarkers. We also sought to find associations between these circulating miRNAs, their potential targets and relevant cardiac-related pathways. In Chapter 5 we focus on another import aspect of acute heart failure, namely dilution and fluid overload. We explored whether volume status was associated to circulating miRNA levels by relating changes in haemoconcentration to changes in miRNA levels in patients with acute heart failure. We also investigated the hypothesis that these heart failure-related miRNAs may be associated to vascular processes. In general, the current concept is that miRNAs in the circulation mainly derive from blood cells. Further,
in heart failure it has been shown that the most abundantly expressed miRNAs in the circulation of do not originate from the heart, but from blood and endothelial cells. Therefore, we aimed in Chapter 6 to measure the heart failure-related set of miRNAs in another cohort of heart failure patients and investigated the potential relation with vascular dysfunction as reflected in heart failure patients with atherosclerotic disease. Further, we assessed the associations between circulating miRNA levels and biomarkers reflecting atherosclerosis-related processes including angiogenesis, inflammation and endothelial dysfunction.

In order to gain insight into the potential function of these circulating miRNAs, proper animal models suitable for further mechanistic follow-up studies are needed. However, very few circulating miRNAs identified in human heart failure have been measured in animal models of heart failure. In Chapter 7, we report about 3 mechanistically different rodent heart failure models in which we investigate our set of previously described heart failure-related circulating miRNAs.

Finally, in Chapter 8 we discuss the main findings and conclusions and place our results in perspective. Moreover, we describe the future directions and new developments in non-coding RNA research.
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