Specific microRNAs and heart failure: time for the next step toward application?

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A number of microRNAs are involved in the pathophysiological events associated with heart disease. In this review, we discuss miR-21, miR-1, miR-23a, miR-142-5p, miR-126, miR-29, miR-195, and miR-499 because they are most often mentioned as important specific indicators of myocardial hypertrophy and fibrosis leading to heart failure. The clinical use of microRNAs as biomarkers and for therapeutic interventions in cardiovascular diseases appears highly promising. However, there remain many unresolved details regarding their specific actions in distinct pathological phenomena. The introduction of microRNAs into routine practice, as part of the cardiovascular examination panel, will require additional clinically relevant and reliable data. Thus, there remains a need for additional research in this area, as well as the optimization and standardization of laboratory procedures which could significantly shorten the determination time, and make microRNA analysis simpler and more affordable. In this review, we aim to summarize the current knowledge about selected microRNAs related to heart failure, including their potential use in diagnosis, prognosis, and treatment, and options for their laboratory determination.

Key words: microRNA, heart failure, miREIA, two-tailed-qPCR, miRNA therapeutics

Received: April 7, 2022; Revised: June 3, 2022; Accepted: June 9, 2022; Available online: June 20, 2022
https://doi.org/10.5507/bp.2022.028

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INTRODUCTION

Cardiovascular diseases, especially heart failure (HF), are a leading cause of death worldwide, especially in developed countries. Therefore, it is important to understand the molecular pathophysiology, and to identify new biomarkers that are effective for early and correct diagnosis and disease prevention. Circulating microRNAs (miRNAs) appear to be promising candidates[1-3]. These small non-coding RNA biomolecules significantly affect physiological and pathophysiological cellular processes[4]. In the cardiovascular system, they control the functions of various cells, such as cardiomyocytes, endothelial cells, smooth muscle cells, and fibroblasts[5]. They play essential roles in important processes, including angiogenesis, cardiac cell contractility, control of lipid metabolism, plaque formation, heart rhythm physiology, and cell growth[2]. Since many miRNAs are tissue-specific, they can be advantageously used as sensitive biomarkers of occurring disease[6,7]. The miRNA concentrations in a cell can significantly affect the cell metabolism, cycle, or function. Knowledge of these mechanisms enables them to be controlled, such that some miRNAs could be useful as therapeutic agents[8]. At this time, small miRNA biomolecules are most commonly assessed by quantitative polymerase chain reaction with reverse transcription (RT-qPCR), microarray-based detection technology, next-generation sequencing (NGS), and microRNA enzyme immunoassay (e.g. miREIA) quantification[9].

HEART FAILURE AND ITS CAUSES

According to the European Society of Cardiology in 2016, HF is defined as a clinical syndrome with classic symptoms including dyspnoea, ankle swelling, and fatigue. Cardiac structural and/or functional abnormalities may lead to concomitant symptoms – such as pulmonary crackles, peripheral oedema, and increased pressure in the jugular veins – which result in increased intracardiac pressure or decreased cardiac output during stress or at rest[1]. HF is primarily characterized from left ventricular ejection fraction (LVEF) measurement. Current recommendations classify the disease as HF with reduced ejection fraction (HFrEF) (LVEF<40%), HF with preserved ejection fraction (HFpEF) (LVEF≥50%), and mid-range ejection fraction (HFmrEF) heart failure (LVEF 40–49%). In the adult population, HF prevalence is around 1–2%, and increases with age. About 64.3 million people are living with HF worldwide[10,11]. Due to the improved treatment of acute conditions – especially after acute myocardial infarction (AMI) – many patients survive but develop a chronic disease state due to myocardium damage. The prognosis of patients with chronic HF is not favorable, with five-year mortality of approximately 50% – worse than in many cancers[12]. Prognosis is even poorer among patients in NYHA functional class III, and especially NYHA IV, with one-year mortality of 20–50%. Chronic HF treatment represents a significant economic burden, accounting for 1–2% of healthcare costs[13].

HF occurs as a result of ventricular myocardial dysfunction – most commonly primary or secondary impairment of left ventricular systolic and/or diastolic function. The most common cause of chronic systolic HF (in up to 70% of patients) is coronary heart disease, in which coronary artery atherosclerosis plays an important role. Valve defects are the cause in about 10% of patients. Specific heart muscle diseases (inflammatory, metabolic, endocrine, and toxic) are uncommon. HF can occur due to various pathophysiological mechanisms[14], which cause cardiomyopathy (CMP) in about 10% of patients, mainly dilated (DCM) (ref.[15]) and hypertrophic (HCM) (ref.[16]). Cardiomyopathies are a major cause of HF (ref.[17]), and can have a variety of symptoms, although many patients may experience sudden death without prior manifestation.

DCM is a frequent cause of heart failure in young individuals, but can occur at any age. The exact prevalence of DCM is unknown, but rates of approximately 1:250 have been reported[18,19]. The pathophysiology of HF in DCM involves 30–50% genetic causes. It can also be caused, for example, by direct damage to the myocardium due to myocarditis or toxic substances (ethanol, drugs, amphetamines, anabolics, chemotherapeutics, etc.). Other possible causes include endocrine and metabolic abnormalities or autoimmune disorders[17,18]. The disease is characterized by dilatation of the heart compartments, and the presence of thrombi in the atria and ventricles. Areas of interstitial fibrosis can be found in the tissue. It is primarily a disorder of cardiomyocyte function, i.e. a disruption of myocardial contractility[20].

Some sources describe HCM as the most common cause of sudden death in athletes and young persons, although it can also occur at any age[21,22]. HCM is a very common genetic heart disease with a prevalence of about 1:500 (ref.[23]). In 60% of patients, the disease shows genetic inheritance of the autosomal dominant type with frequent familial occurrence[17]. The disease is characterized by myocardium hypertrophy (wall thickening ≥ 15 mm) without left ventricular dilatation, and without other myocardial diseases that would cause hypertrophy. HCM is commonly observed with left ventricular outflow tract obstruction associated with abnormal mitral valve anterior tip movement. Obstruction occurs and is involved in HF development[21,24].

Additionally, HF is associated with changes in the size and shape of the left ventricle. The left ventricle dilates and acquires a spherical shape, and its walls thin out. Left ventricular remodeling entails not only changes in the size and shape but also changes in the biological properties of cardiomyocytes, and changes in the ratio of cardiomyocytes and neomyocyte mass. These changes result from the response to stimulation of cardiomyocyte membrane receptors by neurohumoral activation mediators[25].

In clinical practice, various biomarkers are assessed to confirm HF, which can help with early diagnosis and prognosis, or can be an indicator of the response to a selected therapy. In most cases, these requirements are met by assessment of B-type natriuretic peptide (BNP) and its N-terminal propeptide (NT-proBNP), which have become part of the chronic HF diagnostic algorithm. Other classical laboratory parameters are also determined – including creatine kinase, troponin, C-reactive protein, glucose, liver enzymes, iron metabolism parameters, urea, uric acid, electrolytes, hormone metabolites, infectious serology parameters, and autoimmune parameters – which may have meaningful value in chronic HF diagnostics. The limits of the clinical use of biomarkers for HF diagnosis and prediction are the basis for the creation of panels of biochemical markers that can reflect the pathophysiological processes associated with HF. MicroRNAs could also be part of these panels of biochemical parameters, to serve as predictive and diagnostic markers of cardiovascular disease, as has been discussed in many studies[26].

MicroRNAs (miRNAs) AND THEIR DISCOVERY

The first discovered miRNA, *lin-4*, was reported in 1993, in a study of the nematode *Caenorhabditis elegans*[27]. Since then, *lin-4* has been identified as important in the cascade of factors required for direct postembryonic proliferation and differentiation of hypodermal stem cells into specialized skin cells[28-30]. Importantly, another discovered miRNA, *let-7*, was also detected in a number of other organisms, including humans[30-32]. Subsequently, many scientists focused on investigating other small non-coding RNA molecules from both the plant and animal kingdoms, especially those that are important in the human body. In 2001, these small RNA molecules, comprising 19-24 nucleotides, were named microRNAs, abbreviated as miRNAs or miRs (ref.[27,33]).

MiRNA NOMENCLATURE AND miRBase DATABASE

The miRNA nomenclature is determined according to a precise naming system (with a few exceptions, such as the early originated group called "*let*"). The name consists of a three-letter abbreviation, where the lowercase "mir" indicates precursor hairpins, and the abbreviation "miR" with an uppercase "R" indicates mature miRNA sequences. This name is followed by a hyphen and a number that indicates the order in which the miRNA was discovered, e.g. mir-318 was discovered and named before mir-319. This abbreviation is preceded by an abbreviated three- or four-letter prefix that indicates the species of the organism to which the miRNA is specific, e.g. "hsa-miR-101" (in *Homo sapiens*) or "mmu-miR-101" (in *Mus musculus*). These two miRNAs are orthological. Paralogous sequences, in which the mature miRNAs differ in sequence by one or two nucleotides, have a letter suffix at the end of the name, e.g. "mmu-miR-10a" and "mmu-miR-10b". Such miRNAs differ very little in structure but may have different functions, tissue expression, or targets. To specify the location of the active strand from which the miRNAs come, the suffix "-3p" or "-5p" is included after the discovery number. For example, "miR-17-5p" and "miR-17-3p" are two different mature miRNA

sequences that were excised from opposite arms of the same hairpin precursor[34-36].

The publicly available online repository miRBase (http://mirbase.org/) can be used to search for existing miRNA sequences and their annotations[37]. The miRBase database is designed to provide an integrated interface with comprehensive data on miRNA sequences and their intended targets[35]. The miRBase Sequence database includes hairpin portions of the miRNA transcripts (referred to in the database as "mir"). There is also information regarding the sequences of the mature miRNAs ("miR"), genomic coordinates, data obtained by NGS, comments, and references. For each miRNA sequence, the database includes records of associated publications, e.g. 520 publications are available for hsa-mir-1-2. All sequence data and annotations are available for download. The last updated miRBase database (v 22) contains 38,589 entries of hairpin miRNA precursors from 271 species, which is about a one-third increase in entries compared to the previous version. Additionally, a total of 48,860 different mature miRNA sequences are derived from these hairpin precursors. To date, 1917 hairpin precursors and 2654 mature miRNA sequences have been identified for the human genome[37].

BIOGENESIS AND miRNA FUNCTION

MiRNAs are small non-coding RNA molecules that do not serve as a template for protein synthesis but that have regulatory capabilities. They act as gene regulators in physiological processes, such as proliferation, differentiation, apoptosis, etc., and their dysregulation is associated with various pathological disorders[38]. Friedman et al. states that miRNAs can modulate up to 60% of genes encoding proteins in the human genome at the translational level[39]. By binding to the complementary 3'-region of its target mRNA molecule, miRNA prevents its translation and thus affects gene expression at the post-transcriptional level. In heart development, miRNAs are important for the growth of normal functional cardiac tissue, and many of them are characteristic of the various phases[40,41]. In contrast, in HF pathophysiology, various miRNAs are involved in the process of cardiac remodeling, hypoxia, or cardiomyocyte apoptosis. The involvement of miRNAs as regulatory biomolecules in the development of myocardial hypertrophy and fibrosis suggests that they play essential roles in the initiation of HF (ref.[42,43]).

MiRNA biogenesis is a multi-step process. The classical pathway of miRNA formation is referred to as the canonical pathway (Fig. 1), in which a primary transcript is formed from nuclear DNA, which can be several kilobases long and carries hairpin-like structures, so-called pri-miRNA. This process involves the enzyme RNA polymerase II (ref.[44]). Pri-miRNA is further processed by the Drosha (RNase III endonuclease) and DGCR8 microprocessor complex (DiGeorge syndrome critical region 8, called Pasha) into precursor miRNAs (pre-miRNAs) of approximately 70-90 nucleotides in length[45]. Precursor miRNAs with a typical hairpin shape are exported from the nucleus to the cytoplasm using the exportin 5/RanGTP (GTP-binding nuclear protein Ran) protein system. Pre-miRNAs are cleaved in the cytoplasm by the RNase III endoribonuclease Dicer, which is complexed with trans-activation response RNA-binding protein (TRPB). After cleavage of the hairpin loop, double-stranded miRNA molecules are formed. These short molecules are packaged in a so-called miRNA-induced suppressive complex (miRISC), which includes the Argonaut-2 protein (Ago2). Double-stranded miRNAs are unwound by the Ago 2 protein, and one strand (passenger) is degraded in the cytoplasm while the other (guide) remains in the complex. This miRISC is responsible for the post-transcriptional regulation of gene expression. The mRNA includes a so-called miRNA response element (MRE) on the 3' untranslated region, which miRISC recognizes and may bind based on the complementarity of the bases (A-U and C-G) (ref.[4]). A single miRNA can have many targets on an mRNA molecule, and a number of different miRNAs can be involved in regulating a single mRNA. The mechanism of microRNA in the regulation of gene expression is based on translation repression, and the degradation or deadenylation of the target mRNA molecule[38].

CIRCULATING miRNA

In 2008, it was discovered that miRNAs also occur extracellularly and circulate in the bloodstream. Extracellular miRNAs are usually secreted into the bloodstream bound to RNA binding proteins, such as nucleophosphine-1, argon protein (Ago-2), low-density lipoproteins (LDL), or high-density lipoproteins (HDL). They can also be packaged into exosomes, microvesicles (active transport across the cell membrane), or apoptotic vesicles (passive transport during cell death), and transported into the blood. Thanks to these "protective carriers", extracellular miRNAs are highly stable and resistant to degradation by circulating ribonucleases[46,2,47]. Many miRNAs have been detected in a variety of body fluids, such as plasma, serum, urine, tears, saliva, breast milk, and amniotic, pleural, cerebrospinal, or seminal fluid[48]. Since circulating miRNAs are characterized by a high degree of stability, they can be involved in so-called intercellular communication. In this process, circulating miRNAs that have been expelled, e.g. from an apoptotic cell, are taken up by another cell, where they participate in the regulation of gene expression[49]. For example, in the hypertrophic myocardium, due to cardiac pressure or angiotensin II, fibroblasts secrete miR-21-5p exosomes. These extracellular vesicles capture the surrounding healthy cardiomyocytes, and the released miRNA triggers signaling pathways that lead to cell hypertrophy and fibrosis. Bang et al. pharmacologically inhibited this miRNA in a mouse model, and found that myocardial hypertrophy was partially reversed. For their potential therapeutic use, it is advantageous that the regulatory capabilities of miRNAs can be directly altered[50].

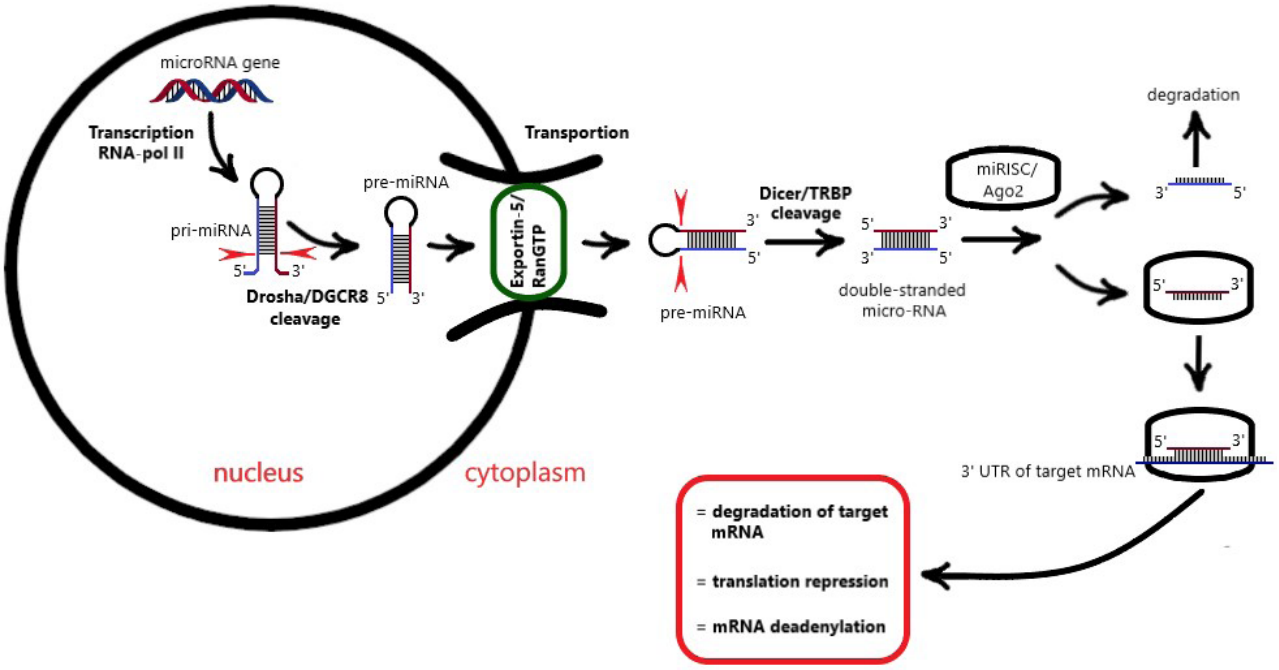

Fig. 1. The classical pathway (canonical pathway) of miRNA biogenesis involving DNA transcription, enzymatic processing, splicing, cytoplasmic export, maturation, and finally binding to a target region in the mRNA.
RNA pol II, RNA polymerase II; DGCR8, DiGeorge syndrome critical region 8; RanGTP, GTP-binding nuclear protein Ran; TRBP, trans-activation response RNA-binding protein; miRISC, miRNA-induced suppressive complex; Ago2, Argonaut-2 protein.

SPECIFIC miRNAs FOR HEART FAILURE

Circulating miRNAs are potential biomarkers of cardiovascular disease, including HF (see Table 1). During HF development, it is almost impossible to detect the initial changes at the molecular level using imaging techniques. Protein biomarkers are indicators of more serious damage. Therefore, miRNAs can play a key role in early diagnosis and prognosis. A more accurate understanding of pathophysiological processes at the RNA level may lead to better targeting of appropriate treatment in selected patients[51,43].

Cardiac hypertrophy often leads to HF, and is associated with dysregulated miR-21-5p expression in the heart. It has been reported that miR-21-5p plays important roles in the proliferation and apoptosis of vascular smooth muscle cells, and in the growth and death of cardiac cells, and also affects the function of cardiac fibroblasts[52]. MiR-21 levels selectively increase in the cells of a failing heart, thereby increasing ERK-MAP kinase (extracellular-signal-regulated kinase–mitogen-activated protein kinase) activity through inhibition of the Sprouty homolog 1 (Spry1) protein. This mechanism regulates fibroblast survival and growth factor secretion, apparently regulating the extent of interstitial fibrosis and cardiac hypertrophy[53]. Cardiac fibroblasts secrete miRNA-21-enriched exosomes, which mediate cardiomyocyte hypertrophy through a paracrine signaling mechanism[50]. In a mouse model, in vivo blockade of miR-21 by a specific antagomir led to decreased cardiac ERK-MAP kinase activity, thereby inhibiting interstitial fibrosis and suppressing cardiac dysfunction[53], suggesting that miRNAs may also be targeted for the treatment of heart disease. In other studies, elevated levels of miR-21, along with miR-1 and miR-499-5p, have been associated with AMI. Oerlemans et al. investigated these three miRNAs, along with others, in 332 patients with suspected acute coronary syndrome, and found that the combination of miR-1, miR-21, and miR-499 may have higher diagnostic value than high-sensitive troponin T (hs-TnT) (ref.[54]).

Specific miRNAs can be used to distinguish between DCM and HCM. Li et al. reported that miR-21 is upregulated in DCM but not in HCM. ROC analysis revealed that miR-21 determination was highly sensitive and specific (AUC=0.944) for distinguishing patients with DCM from control samples. Among patients with DCM, myocardial fibrosis severity, as determined by magnetic resonance imaging, was associated with high miR-21 expression, and the presence of myocardial fibrosis may be an important factor related to treatment failure in patients with end-stage DCM. In patients with HCM, only miR-1-3p was correlated with the left ventricular end-diastolic diameter (LVEDD) and LVEF. MiR-1-3p levels were significantly lower in patients with HCM compared to the DCM and control groups. In patients with HCM, decreased miR-1-3p levels were associated with higher LVEDD and lower LVEF, reflecting severe cardiac dysfunction. Furthermore, miR-23a was significantly upregulated in both DCM and HCM patients (AUC ranging from 0.925-0.933) (ref.[55]). Due to the smaller numbers of probands in the individual groups, these important findings should be confirmed in a larger group of patients.

Muscle-specific miRNAs include miR-1, which regulates cardiomyocyte growth and differentiation. This miRNA is released into the blood only when cells are damaged, and is nearly non-existent in the blood under physiological conditions. Concentrations of miR-1 or miR-499 correlate well with cardiac troponin levels, and thus are abundant in the blood when coronary arteries are affected[6]. MiR-1 has been evaluated as a prognostic marker of AMI. Circulating miR-1 is used as an independent predictive indicator of LV remodeling after STEMI (ST-elevation myocardial infarction). Combined determination of miR-1 and NT-proBNP shows predictive value comparable to magnetic resonance imaging[56].

Specific miR-499-5p has been identified as a promising biomarker of acute NSTEMI (non-ST-elevation myocardial infarction) (ref.[57]). Zang et al. reported the detection of elevated miR-499 levels in 142 patients with AMI, as early as 1 h after the onset of chest pain, with a further increase over the next 9 h, exhibiting a sensitivity of 80% and specificity of 80.28% (AUC=0.86) (ref.[58]). Another study demonstrated that elevated miR-499 levels were associated with atrial fibrillation (AFib), which is a leading cause of HF, stroke, sudden death, and cardiovascular morbidity worldwide. MiR-499 negatively regulates the CACNA1C (calcium voltage-gated channel subunit alpha1 C) gene, which encodes the L-type voltage-gated calcium channel Cav1.2, and thereby contributes to the arrhythmia in AFib. MiR-499 levels are reported to be significantly increased in patients with paroxysmal AFib (n=64) compared to the control group (AUC=0.83; 95% CI: 0.74-0.90). This miRNA could serve as a therapeutic target in AFib therapy[59]. Increased miR-499 expression has also been reported in patients with ischemic CMP and patients with HF due to hypertension[60].

The miR-29 family (miR-29a/b/c) has been widely studied in connection with many cardiovascular diseases. These miRNAs exert physiological regulatory effects in cardiomyocytes, aortic and myocardial tissue, vascular endothelial cells, and cardiac metabolism. They also play various regulatory functions in the pathophysiological mechanisms of cardiomyopathies, atrial fibrillation, myocardial fibrosis, atherosclerosis, and HF. MiR-29a/b is a prognostic biomarker in HF development. Expression of the miR-29 family tends to be reduced in the affected cardiac tissue. In animal models, congestive HF significantly increases collagen and fibrin I/III expressions in fibroblasts, while miR-29b levels are decreased. This was demonstrated using a lentivirus that reduced miR-29b expression and thereby increased collagen expression in fibroblasts. On the other hand, high levels of miR-29b reduced collagen expression[61]. Thus, miR-29 family members are considered anti-fibrotic markers. They act directly on extracellular matrix synthesis, regulating the mRNA of elastin and fibrillin 1 proteins. MiR-29 is a suitable therapeutic target for improving cardiac function[61,62]. Tran et al. examined 44 different extracellular circulating miRNAs and their association with cardiac remodeling and HF. They found that miR-29c-3p is involved in the signaling pathways of the p53 protein and cardiac transforming growth factor β (TGF-β). TGF-β reduces miR-29c-3p expression, thereby upregulating collagen expression, and influencing the development of cardiac fibrosis and cell death. MiR-29c-3p has been identified as one of three important biomarkers involved in the development of cardiac remodeling and HF in patients with acute coronary syndrome (n=296) (ref.[63]).

Another miRNA associated with cardiac remodeling and myocardial hypertrophy is miR-23a-3p, which is prohypertrophic, and its expression is regulated by nuclear factor-activated T cells (NFATc3). MiR-23a targets and suppresses the translation of antihypertrophic factor, muscle-specific ring finger protein 1 (MuRF 1). This miRNA is upregulated during pressure-induced cardiac hypertrophy, and its high levels initiate further hypertrophic reactions. These findings facilitate the detection of cardiac hypertrophy at the molecular level, as well as the development of new therapeutics for HF treatment[64]. Long et al. reported that miR-23a promotes cardiomyocyte apoptosis by inhibiting mitochondrial superoxide dismutase (MnSOD) expression. Cardiac cell apoptosis is associated with AMI and subsequent HF, and miR-23a may be a suitable therapeutic target for treatment of these conditions[65].

MiR-195 is also associated with apoptosis. This miRNA targets the mRNA of the Sirt1 protein, which inhibits reactive oxygen species (ROS) production and protects cardiomyocytes from palmitate-induced apoptosis. Regulation by miR-195 reduces Sirt1 protein production, thereby mediating palmitate-induced cardiomyocyte apoptosis. MiR-195 may be a key factor in lipotoxic CMP, as well as an important new therapeutic target[66]. This miRNA is also an important factor in the development of cardiac hypertrophy and HF. Overexpression of miR-195 stimulates myocardial hypertrophy and is a key regulator of the whole process. He et al. demonstrated that circulating miR-195-3p could be a promising biomarker of HF (AUC=0.831) (ref.[67,68]).

MiR-126 is an important regulatory factor in the process of atherosclerosis. Apoptotic bodies arising from endothelial cells during atherosclerosis are taken up by surrounding surviving cells, which then transmit signals to other cells in the vicinity to activate production of the chemokine CXCL12 (C-X-C motif chemokine 12). This activation occurs through miR-126, which is part of apoptotic bodies. It acts by blocking the negative regulator RGS16 (regulator of G-protein signaling 16), thereby releasing the CXCR4 (C-X-C chemokine receptor type 4) receptor, leading to increased expression of the anti-apoptotic factor CXCL12 and mobilization of progenitor cells[69]. MiR-126 is also an important regulatory factor for endothelial cell homeostasis, and an interesting indicator of endothelial dysfunction, and appears to be a promising prognostic biomarker of vascular damage. Jiao et al. recently reported the functions of miR-126-3p and miR-142-5p as potential biomarkers for DCM diagnosis in pediatric patients (n=46, age: 9 months-13 years). ROC analysis revealed that both miRNAs showed excellent diagnostic relevance for discriminating between healthy and

sick individuals (miR-126-3p: AUC=0.950, miR-142-5p: AUC=0.983). The miR-142-5p assay showed a sensitivity of 93.3% and a specificity of 93.8% for disease compared to healthy individuals. MiR-126-3p also appears to be a suitable biomarker for detection of HF in DCM among children. Significantly higher miR-126-3p levels were found among children with DCM with HF compared to in the control group of children with DCM without HF (P<0.05; AUC=0.782). Mir-126-3p was negatively correlated with LVEF (ref.[15]). In another study, Qiang et al. demonstrated that miR-126-3p was associated with the diagnosis of ischemic and non-ischemic CMP, and the prevention and treatment of HF (ref.[70]).

THE THERAPEUTIC POTENTIAL OF miRNAs

Since miRNAs modulate protein expression and regulate biological processes, they are becoming promising therapeutic agents for many diseases, including cardiovascular conditions. There are currently two approaches for purposefully targeted miRNA activity. One is the suppression of miRNA expression, which is upregulated in many HF-related disease states. The other is to mimic (replace) the function of a miRNA that is reduced in the disease state. Its supplementation can be ensured by the application of so-called "miRNA mimics", which are double-stranded synthetically prepared small RNA molecules that mimic miRNAs in the body. The synthetic double-stranded RNA molecule is composed of one strand that is identical to the native miRNA, and another strand that is complementary. The double-stranded arrangement is important for RISC to be able to recognize a synthetic molecule in an organism. Inhibition of upregulated miRNAs can be accomplished using synthetic oligonucleotides called "antagomiR", which complementarily bind to a specific miRNA, thereby blocking its binding to the target region of the mRNA. Another way to prevent specific miRNAs from binding to the complementary region in the target mRNA is to use so-called "miRNA sponges". These transcripts have multiple complementary regions for miRNAs, and are thus useful for reducing the amount of free active miRNAs in a cell due to the binding of specific miRNAs.

However, the application of miRNAs as therapeutics is not easy. They must be chemically modified to be able to pass through the cell membrane. Synthetic miRNAs can be modified through binding to a cholesterol molecule, or can be delivered to a target site using locked nucleic acid (LNA) or modified bicyclic oligonucleotides – which ensure greater miRNA stability, lower toxicity to surrounding tissues, and nuclease resistance. Polymer nanoparticles or liposomes into which miRNA is incorporated can also be used[2,5]. Although a number of studies show that miRNAs are interesting therapeutic agents for heart disease treatment, many obstacles must be overcome before miRNA therapy can become routine practice. For example, the ability of a single miRNA to target multiple different mRNAs can result in a number of side effects. Other research challenges include the ability to specifically target the effect of a miRNA on a particular cell type, and ensuring the drug's stability and duration of action in an acute attack or chronic disease[71].

Clinical trials are currently testing potential miRNA therapeutics that could play key roles in HF treatment. One is the miR-92a inhibitor, MRG-110, which is now in Phase I clinical trials. MiR-92a is an important anti-angiogenic miRNA, the inhibition of which has increased angiogenesis, for example, after myocardial infarction. If the Phase I clinical trials are successful, Phase II trials will continue in patients with ischemic cardiomyopathy and HF and/or peripheral arterial disease. Similarly, the

Table 1. MicroRNA dysregulations associated with pathological-anatomical changes leading to cardiovascular disease (CVD), including HF.

microRNA	Up- or Downregulated in CVD	Pathological-anatomical changes	Diagnosis	Ref.
miR-21-5p	↑	fibrosis, hypertrophy	HF	50, 52, 53
miR-21, miR-1, miR-499-5p	↑	atherosclerosis, cell death	AMI (STEMI, NSTEMI)	6, 54, 56, 57, 58
miR-21	↑	fibrosis	DCM	55
miR-1-3p	↓	↑LVEDD, ↓LVEF	HCM	55
miR-23a-3p	↑	hypertension, hypertrophy, cell death	HCM, HF, AMI	64, 65
miR-499	↑	arrhythmia; endothelial dysfunction, hypertension	AFib, CMP, HF	59, 60
miR-29b, miR-29c-3p	↓	fibrosis	ACS, HF	61, 62, 63
miR-195, miR-195-3p	↑	cell death, hypertrophy	CMP, HF	66, 67, 68
miR-126; miR-126-3p, miR-142-5p	↑	atherosclerosis, endothelial dysfunction	vascular damage; DCM, HF	15, 69, 70

↑, upregulated; ↓, downregulated.
ACS, acute coronary syndrome; AFib, atrial fibrilation; AMI, acute myocardial infarction; CMP, cardiomyopathy; CVD, cardiovascular disease; DCM, dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy; HF, heart failure; LVEDD, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction; NSTEMI, non-ST-elevation myocardial infarction; STEMI, ST-elevation myocardial infarction.

Remlarsen miR-29-mimic (MRG 201) is being tested. The effects of Remlarsen on extracellular matrix remodeling and myocardial fibrosis will be investigated in Phase II clinical trials in patients with HF (ref.[72]).

METHODS FOR miRNA DETERMINATION

Circulating miRNAs are ideal biomarkers because their levels are stable at different pH values; they can resist boiling temperatures, and repeated freezing and thawing; and they can be stored for a long time. They can also be easily and non-invasively isolated from fluids. They are a current reflection of events occurring in the body, and are an important means of intercellular communication[73].

However, the determination of miRNA concentration is not easy due to their small molecule size and small base differences, usually low concentrations in the assay materials, and different approaches to isolation and quantification[74]. The three most common methods for quantifying miRNAs are techniques based on PCR, microarrays, and sequencing technologies. Another suitable technology is the detection based on the principle of immunoassays, and we can also mention methods based on isothermal amplification, northern blotting, electrochemical methods, and nanotechnological procedures[75-77]. All methods have both advantages and disadvantages. For routine practice of miRNA determination, it will be most important to have a method that is fast, inexpensive, accurate, and sufficiently sensitive.

NGS is the most sensitive method for finding new miRNAs. NGS analysis enables the detection of one-base differences in miRNA sequences, and the simultaneous assessment of several heterogeneous samples. However, due to the need for a considerable number of readings, this method is very time-consuming and sample-intensive. Multiplex analysis based on microarrays using a hybridization process – usually between a probe bound to a solid surface and a miRNA of interest – allows the measurement of several hundred miRNAs in a single sample. Nevertheless, microarrays are most often used for relative quantification, as miRNA quantification tends to be skewed due to unreliable background corrections. The sensitivity of this assay is lower than that of NGS. Both methods are better suited for searching for new miRNAs within samples from patients in various disease states than for routine analysis of specific miRNAs. These methods are demanding in terms of instrumentation and evaluation equipment, and are expensive.

On the other hand, methods based on RT-qPCR are widely available and well-automated. Since miRNA molecules are very short, special PCR primers are used in the RT-qPCR techniques from various companies, which limits the occurrence of non-specific reactions (e.g. non-specific binding, penetration of primer dimers, etc.). The miRNA length can be extended during the reverse transcription step. It is possible to use a specific stem-loop primer that binds to the 3'-end of the miRNA molecule. Another variation is the binding of a 3'-polyA tail to the 3'-end of miRNA, followed by the attachment of a polyT primer to this 3'-end[75].

One means of modifying RT prior to qPCR itself is a ligation reaction – for example, using SplintR ligase – which joins complementary DNA oligonucleotides that are linked to miRNA by hybridization. Efficient ligation requires overlap of only 4-6 bp between the DNA probe and the miRNA. This enables greater flexibility in designing miRNA-specific ligation probes[78]. However, a short overlap between the DNA probe and the targeted miRNA can lead to increased non-specific sensitivity, and thus to the quantification of entire miRNA families in a single measurement. Another type of RT-qPCR modification is the application of so-called two-tailed qPCR, which uses specific two-tailed reverse primers with a unique hemiprobic structure. These primers have both free ends, 5´ and 3´, which detect most of the miRNA sequence, and thereby generate a cDNA library using only the specifically targeted miRNA. This cDNA template enters the qPCR reaction, where the forward primer is targeted to the RT primer, and the reverse primer specifically targets the desired miRNA sequence. Due to the high specificity of both steps, the assay has one of the highest resolutions on the market, and the amplification step ensures high sensitivity of the assay[76]. Kappel et al. reported the initial determination of miRNAs based on immunochemical reaction. The principle of the assay is the detection of specific biotinylated DNA/RNA heterohybrids bound to immobilized streptavidin-modified microparticles, using an acridinium ester-labeled monoclonal antibody. This was the first quantitative assay performed using chemiluminescent miRNA detection[79]. Immunochemical reaction is also involved in the miREIA method using the ELISA format (Enzyme-Linked ImmunoSorbent Assay). A monoclonal antibody against specific DNA/RNA heterohybrids, labeled with the enzyme horseradish peroxidase (HRP), is immobilized on the surface of the microtiter plate and the complexes are visualized using a chromogenic substrate. The miRNA concentration in the sample is read from the calibration curve generated by the synthetic miRNA in absolute values[80]. With the use of a thermocycler for miRNA hybridization with DNA probes, the miREIA method can be carried out in a classical immunochemical laboratory. It does not require any amplification steps, and results can be achieved in less than three hours, including evaluation. Nevertheless, two-tailed qPCR is certainly a more sensitive method, and is more user-friendly for biomolecular laboratories.

LIMITATIONS OF THE USE OF miRNAs AS HF BIOMARKERS

MiRNAs regulate many important processes of cardiovascular disease. Specific miRNAs could be included in the diagnostic panel of biomarkers for heart disease, mainly due to the predominant expression of a miRNA in cardiac tissue, and/or because a miRNA is required for cardiac development or function, or for repair of specific

heart damage. However, the roles of many miRNAs overlap in different cardiac pathologies. This may be partly explained by the fact that many studies have been performed with a low number of enrolled patients. Other important factors include the use of different assay methods, conditions for miRNA isolation, normalization parameters, and collection times, and the analysis of miRNAs in different types of biological materials, which may include anticoagulants (serum, plasma, and whole blood). Reducing the variability between studies would help establish a recommended set of normalizing endogenous control miRNAs for specific pathological conditions. To more accurately identify miRNAs associated with cardiovascular disease, it may be crucial to evaluate them at precise time intervals from the onset of symptoms, as well as to determine their relationship to a detailed personal history[51,60].

CONCLUSION

MiRNA biomarkers can provide key information for early diagnosis, prognosis, and (thanks to a more accurate understanding of pathophysiological processes at the RNA level) the determination of appropriate treatment for selected patients. There are still many challenges to overcome for the development of safe and effective miRNA-based therapeutics. At this time, many therapeutic approaches are only in the experimental phases. However, ongoing clinical trials may provide valuable information regarding the therapeutic use of miRNAs. Many methods are currently available for assessing miRNAs. As clinical trials are increasingly focused on the potential use of miRNAs as biomarkers of acute disease states, there is a need to continue methodological development to facilitate their routine analysis in clinical laboratories. In particular, emphasis should be placed on high sensitivity and specificity, automation, and shorter analysis times. Another major challenge is the standardization of the whole process – from miRNA sampling, isolation, and determination, to the evaluation of the obtained results. Due to the many potential benefits of using miRNAs for the diagnosis and subsequent treatment of cardiovascular diseases, there is an urgent need for extensive studies that can help address their "shortcomings" and establish them in routine clinical use.

ABBREVIATIONS

AFib, Atrial fibrillation; Ago2, Argonaut-2 protein; AMI, Acute myocardial infarction; BNP, Brain natriuretic peptide; CACNA1C, Calcium voltage-gated channel subunit alpha1 C; CMP, Cardiomyopathy; CVD, Cardiovascular disease; CXCL12, C-X-C motif chemokine 12; CXCR4, C-X-C chemokine receptor type 4; DCM, Dilated cardiomyopathy; DGCR8, DiGeorge syndrome critical region 8; ELISA, Enzyme-linked immunosorbent assay; ERK-MAP kinase, Extracellular-signal-regulated kinase–mitogen-activated protein kinase; HCM, Hypertrophic cardiomyopathy; HF, Heart failure; HFmrEF, Heart failure with mid-range ejection fraction; HFpEF, Heart failure with preserved ejection fraction; HFrEF, Heart failure with reduced ejection fraction; HRP, Horseradish peroxidase; hsTNT, High-sensitive troponin T; LDL or HDL, Low-density or high-density lipoproteins; LVEDD, Left ventricular end-diastolic diameter; LVEF, Left ventricular ejection fraction; MI, Myocardial infarction; miREIA, microRNA enzyme immunoassay; miRISC, miRNA-induced silencing complex; MnSOD, Mitochondrial superoxide dismutase; MRE, miRNA response element; mRNA, Messenger RNA; MuRF 1, Muscle-specific ring finger protein 1; NFATc3, Nuclear factor-activated T cells; NGS, Next-generation sequencing; NSTEMI, Non-ST-elevation myocardial infarction; NT-proBNP, N-terminal prohormone of brain natriuretic peptide; NYHA, New York Heart Association; RanGTP, GTP-binding nuclear protein Ran; RGS16, Regulator of G-protein signaling 16; ROS, Oxygen radicals; RT-qPCR, Quantitative polymerase chain reaction with reverse transcription; STEMI, ST-elevation myocardial infarction; TGF-β, Transforming growth factor β; TRBC, Transactivation response RNA-binding protein.

Search strategy and selection criteria

Our research strategy focused on selected specific microRNAs associated with heart failure. A search of valuable literature was performed in the PubMed, Web of Science, and SCOPUS databases using the technical terms "microRNA", "heart failure", "cardiomyopathy", "miRNA biogenesis", "miRNA nomenclature", "circulating miRNA", "miRNA determination" and "miRNA therapeutics". All scientific articles were current as of December 2021.

Acknowledgements: This study was supported by the Ministry of Health Czech Republic–conceptual development of research organization (05-RVO-FNOs/2018) and by grant LF OU SGS13/LF/2019-2020. The authors are indebted to Dr. Martina Hložánková and Dr. Eva Bače, BioVendor – Laboratory Medicine Corp., Brno, Czech Republic for cooperation in proofreading and critical evaluation of analytical-biochemical parts of the manuscript. We would also like to thank Dr. Jana Vavrošová for consultations and English proofreading of the manuscript.

Author contributions: RS: literature search, data collection, manuscript writing/editing; LE: literature search, data collection; LE, VP, DS: correction and critical evaluation of the clinical portion of the manuscript; ZS, DS: revision and critical evaluation of the analytical-biochemical portions of the manuscript, incited a review article, final approval.

Conflict of interest statement: The authors state that there are no conflicts of interest regarding the publication of this article.

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