

## STATE-OF-THE-ART PAPERS

# microRNAs in Cardiovascular Diseases

## Current Knowledge and the Road Ahead

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Over the last few years, the field of microribonucleic acid (miRNA) in cardiovascular biology and disease has expanded at an incredible pace. miRNAs are themselves part of a larger family, that of non-coding RNAs, the importance of which for biological processes is starting to emerge. miRNAs are ~22-nucleotide-long RNA sequences that can legate messenger (m)RNAs at partially complementary binding sites, and hence regulate the rate of protein synthesis by altering the stability of the targeted mRNAs. In the cardiovascular system, miRNAs have been shown to be critical regulators of development and physiology. They control basic functions in virtually all cell types relevant to the cardiovascular system (such as endothelial cells, cardiac muscle, smooth muscle, inflammatory cells, and fibroblasts) and, thus, are directly involved in the pathophysiology of many cardiovascular diseases. As a result of their role in disease, they are being studied for exploitation in diagnostics, prognostics, and therapeutics. However, there are still significant obstacles that need to be overcome before they enter the clinical arena. We present here a review of the literature and outline the directions toward their use in the clinic.

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Although protein-coding and transcription-regulating sequences occupy <3% of our genome, it seems that at least 75% is transcribed (1). A certain amount of the genome has been known for some time to be dedicated also to encoding infrastructural ribonucleic acid (RNA)—such as transfer, ribosomal, and small nuclear and nucleolar RNAs (2)—but the fact that most of the genome did not seem to be functional was a mystery to be resolved. It is now understood not only that much of a cell's transcriptome is involved in the production of regulatory RNA species, but also that these RNAs rival proteins in importance for the control of biological processes (3). Therefore, a vast part of our genome is not dedicated to proteins but, rather, to the production of non-protein coding RNAs (ncRNAs) with regulatory functions. The first eukaryotic ncRNA was reported in the late 1980s (4), and since then their number has grown steadily, with reports demonstrating novel roles in many biological processes (5). A hint of the vital importance of ncRNA for higher organisms is conveyed by the hypothesis that eukaryotic complexity and phenotypic variation is engendered by the degree of intricacy of the regulatory

network—of which ncRNA are a chief component—rather than merely the size of the protein repertoire (3).

Among the different types of ncRNA, the microRNA (miRNA/miR) family has a central role in pathophysiological response to stress (6), regulating at least half of the transcriptome and constituting a layer of regulation that works in concert with more conventional protein-mediated mechanisms (7–9). miRNAs form an abundant ncRNA species, with nearly 2,000 human miRNA sequences catalogued hitherto (10), although it is not clear if all are bona fide. Many of these miRNAs have tissue-specific and developmental stage-specific patterns of expression (11).

microRNAs exert their function by regulating gene expression at the level of messenger RNA (mRNA) translation: It is solidly established that they regulate specific cellular processes through mRNA target recognition leading to the inhibition of protein synthesis. In particular, a specific miRNA may target multiple mRNAs (divergent miRNA pathway), a given mRNA may harbor binding sites for different miRNA (leading to combinatorial control by miRNAs), and sets of related miRNAs may affect a given pathway at different levels (convergent miRNA pathway) (Fig. 1). These characteristics create a 3-dimensional miRNA–mRNA interactome (the set of interactions occurring within a cell) that changes in relation to developmental stage, age, and pathophysiological state of the cell (12,13). A list of cardiovascular miRNAs and validated mRNA targets has been reported in a recent extensive review (14). It is possible, moreover, that they work also through other regulatory mechanisms, such as within the nucleus to regulate gene expression (15,16).

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**Abbreviations  
and Acronyms****CAM** = cell adhesion molecule**cTNT** = cardiac troponin T**DM** = diabetes mellitus**EC** = endothelial cell**HF** = heart failure**MI** = myocardial infarction**mRNA** = messenger ribonucleic acid**miRNA/miR** = microribonucleic acid**ncRNA** = non-protein coding ribonucleic acid**RNA** = ribonucleic acid**VSMC** = vascular smooth muscle cell

Once bound to the target, miRNAs can proceed to organelles called P-bodies, which fuse with endolysosomes. As a result, miRNAs can either enter a degradative pathway or, via the formation of multivesicular bodies, can be released into the extracellular space and circulation within small vesicles called exosomes (17). miRNAs can also be secreted from cells as vesicular bodies arising from the plasma membrane or can be simply extruded by cells through membrane shedding. Moreover, some cells, such as endothelial cells (ECs), can release miRNAs under the form of apoptotic bodies (17). Thus, miRNAs

can be found within the circulation either in a “free” form—that is, complexed with proteins, such as argonaute 2 or plasmatic proteins—or within membrane-bound bodies.

**miRNAs in Myocardial Development and Disease**

In 2002, 9 years after the description of the first miRNA in the nematode *Caenorhabditis elegans* by Lee et al. (18) and Wightman et al. (19), Calin et al. (20) published the first report describing a pathogenic role for an miRNA, specifically implicating deletion of the miR-15a/miR-16 cluster in the development of chronic leukemia. Since then, many other diseases have been linked with miRNA dysregulation, and today the importance of miRNA-mediated post-transcriptional regulation for the proper functioning of cardiovascular homeostasis, and the implications for heart disease pathogenesis, diagnosis, and prognosis are well established for the scientific community (21).

The role of miRNAs in myocardial development was first assessed in the fruit fly *Drosophila*, in which it was determined that miR-1—one of the most expressed miRNA in cardiac muscle—controlled the Notch 1 receptor (22). A causal link was established between cardiac hypertrophy and miRNAs by studying those preferentially expressed in the heart (23). In particular, miR-208a was found to be encoded by a gene residing within an intron of alpha-myosin heavy chain, to be regulated during cardiac hypertrophy, and to regulate this process itself by targeting a protein interacting with thyroid hormone receptor (24). At the same time, miR-1 and miR-133—which are encoded together in a bicistronic unit—were found to be inversely related to cardiac hypertrophy and to regulate cardiomyocyte size and function (25). miR-1 was found to modulate the insulin-like growth factor-1 pathway either directly, inhibiting insulin-like growth factor-1 and its receptor (26), or by down-

regulating secreted targets related to this pathway (27). The manipulation of miRNA levels with specific anti-sense molecules—called antagomiRs—was proven to work efficiently in the myocardium in vivo and to induce significant cardiac effects (25). A direct link between miR-21 and the miR-29 family with myocardial fibrosis during hypertrophy was also demonstrated (28,29), suggesting that miRNAs control different components of myocardial remodeling. Indeed, further studies demonstrated that miRNAs control fundamentally all critical aspects of cardiovascular biology, such as angiogenesis (30), metabolism (31–34), aging (35), and also the inflammatory component of myocardial remodeling: for instance, miR-155 was found to control macrophage activity, thereby regulating cardiac hypertrophy through an indirect, inflammation-dependent mechanism (36).

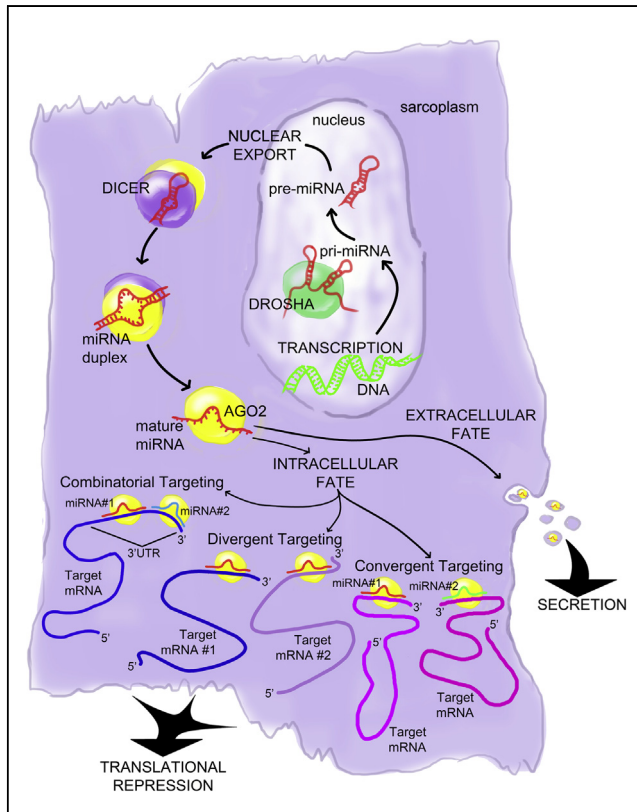
The involvement of miRNAs in human cardiomyopathies has also been suggested. For example, a rare mutation of miR-499—a muscle-specific miRNA—was found at its 3' end, outside the seed region thought to determine target recognition; this mutation was able to modify mRNA targeting and end-organ function, leading to heart failure (HF) in mice (37).

Very recently, exogenous administration of miRNAs, in particular miR-590-3p and miR-199a-3p, was found to enhance cardiomyocyte proliferation in newborn pups and in adult mice within the peri-infarct area (38). These results, if confirmed, imply that miRNAs could restore left ventricular mass and promote functional recovery after myocardial infarction (MI).

**miRNAs in Vascular Diseases**

As for the field of cardiology, that of vascular pathophysiology has seen an explosion of studies on miRNAs. miRNA were clearly demonstrated to play a fundamental role in controlling smooth muscle cell proliferation and maturation, vasculogenesis, neoangiogenesis, bone marrow cells, and endothelial function. We will briefly review some of the most critical discoveries in vascular biology.

In EC dysfunction, the differential expression of miR-10a was found to contribute to the regulation of the proinflammatory EC phenotype in atherosusceptible regions by inhibiting proinflammatory adhesion molecules, such as vascular cell adhesion molecule (CAM)-1, E-selectin, or the NF- $\kappa$ B pathway; similarly, miR-181, miR-126, miR-31, and miR-17-3p control vascular inflammation, acting on the expression of vascular CAM-1, intracellular CAM-1, and E-selectin (39–41). Cholesterol is a pivotal player not only in atherosclerosis development but also in metabolic syndrome and diabetes mellitus (DM). All the processes involved in the maintenance of cholesterol levels, such as de novo biosynthesis, internalization of exogenous cholesterol, and removal of its excessive cellular levels by high-density lipoproteins, are controlled at least partially by miRNAs. Two miRNAs have been implicated the most



**Figure 1** Schematic of miRNA Biogenesis and Function

Microribonucleic acids (miRNAs) derive from primary miRNAs (pri-miRNAs), of many hundreds or thousands of nucleotides in length, which are folded into characteristic hairpin structures. Over 50% of all miRNAs are polycistronic, that is, they are generated from a common transcript or a gene cluster, which is thought to efficiently allow cooperative miRNAs to be expressed simultaneously even when not part of the same miRNA family (i.e., when they do not have the same seed sequence). In the canonical miRNA biogenesis pathway, pri-miRNAs are cleaved in the nucleus by the ribonuclease drosha to form shorter, 70 to 100-nucleotides-long, preliminary miRNAs (pre-miRNAs), which are then transported out of the nucleus. In the cytoplasm, pre-miRNAs are recognized by another ribonuclease, dicer, and processed into short, double-stranded, immature miRNA. The strand with the 5' terminus that has less-stable base-pairing is stripped away from the duplex and transferred to argonaute 2 (AGO2). In addition to the canonical miRNA biogenesis pathway, others have been uncovered. miRNAs function as guides for a type of ribonucleoprotein called a miRNA-induced silencing complex (miRISC): the miRNA incorporated into each miRISC targets messenger RNAs (mRNAs) that harbor partially complementary binding site(s) to the targeting miRNA(s). Once bound to miRNA, the mRNA is negatively regulated through the action of the protein components of the miRISC. In mammals, miRNA-mediated negative regulation of protein synthesis probably occurs predominantly through mRNA destabilization rather than translation repression, but the mechanisms through which the miRISC acts remain to be fully elucidated. In any case, it is thought that the ultimate outcome of most mammalian miRNAs is <2-fold fine-tuning of their targets' expression. A primary determinant of miRNA-mRNA interaction is thought to be perfect Watson-Crick base-pairing of nucleotides 2 to 8 (called the seed region) at the 5' end of the miRNA, to nucleation sites typically present on the 3' untranslated region (UTR) of the targeted mRNA. miRNAs can also be secreted by cells through exosomes, apoptotic bodies, and other vesicular-like bodies. In this way, miRNA-mediated signals can be transferred to surrounding and/or distant cells/tissues. This phenomenon could be exploited for diagnostic/prognostic purposes by assessing the miRNA profile in biological fluids.

in lipid homeostasis: miR-122 and miR-33. miR-122 is mainly expressed in liver and was the first miRNA described as a regulator of lipid metabolism (42,43).

Antagonists of miR-122 resulted in a lowering of total cholesterol (both high- and low-density lipoproteins) and in an improvement of hepatic steatosis (42). Members of the miR-33 family, which includes miR-33a and miR-33b, have been found as intronic RNAs in sterol regulatory element-binding proteins and, being co-transcribed with their host genes, have been proposed as key regulators of cholesterol and fatty acid homeostasis (44-46).

In the intima, low-density lipoprotein uptake and inflammatory modulation in macrophages were also found controlled by miRNAs, such as miR-155 (47) and miR-125a-5p (48). Vascular smooth muscle cell (VSMC) proliferation in the neointima is responsible for the evolution from fatty streaks to fibrous atheroma, and an epigenetic mechanism mediated by miR-29b can regulate metalloproteinase expression involved in cell migration and VSMC proliferation, such as metalloprotein-2 and -9 genes (49). Moreover, the switch from the contractile to a secretory phenotype can be modulated by miR-145, which is highly expressed in VSMCs: VSMC-targeted miR-145 treatment markedly reduced plaque size in aortic sinuses, increasing the fibrous cap area, reducing the necrotic core area, and increasing plaque collagen content, and thus promoting a contractile VSMC phenotype. Therefore, VSMC-specific overexpression of miR-145 could be a promising novel therapeutic strategy for the stabilization of atherosclerotic plaque morphology and cellular composition, shifting the balance toward plaque stability and away from plaque rupture (50). Cholesterol-loaded macrophages are partly in charge of the cytokine synthesis that promotes neoangiogenesis: this process is mediated by miR-222/221 (51) and miR-155: miR-155 promotes atherosclerosis by repressing B-cell lymphoma 6 protein in macrophages (52). miR-342-5p fosters inflammatory macrophage activation through an AKT1- and miR-155-dependent pathway during atherosclerosis (53).

Expression of the miR-17~92 cluster is regulated by vascular endothelial growth factor, and, therefore, its role in regulating neoangiogenesis appears pivotal, although complex and not yet completely elucidated (54). Neoangiogenesis and intraplaque hemorrhage are the major steps in promoting plaque vulnerability, leading to plaque destabilization, rupture, and thrombus formation. Thus, the significant miRNA involvement in this setting needs to be clarified. Taken together, these findings emphasize the potential beneficial effects of up-regulation of atheroprotective miRNAs and down-regulation of proatherogenic ones, but we are still far from the direct translation of research into a concrete therapeutic strategy.

### VSMC Differentiation and Proliferation in Cardiovascular Diseases

The most studied miRNAs expressed by VSMCs belong to the miR-143/145 cluster, which has a pivotal role in VSMC differentiation and arterial pathogenesis (55-58).

The expression of miR-143/145 is decreased by acute and chronic vascular stresses (such as transverse aortic constriction and apolipoprotein E knockout-induced atherosclerosis); in human aortic aneurysms, the expression of miR-143/145 is significantly decreased compared with control tissue (58). Phenotypic modulation of VSMCs has been implicated in the pathogenesis of various proliferative vascular diseases, and miR-145 is able to control vascular neointimal lesion formation through its target Kruppel-like factor 5 and its downstream signaling molecule myocardin (59). Up-regulation of miR-145, but not of miR-143, was observed in pulmonary artery VSMCs and in lung tissue from patients with idiopathic and heritable pulmonary arterial hypertension, a pathology characterized by extensive remodeling of small pulmonary arteries and proliferation of pulmonary artery VSMCs (60). Other studies highlighted the regulatory role of miR-221 and miR-222 in VSMC proliferation and neointimal hyperplasia, showing that miR-221/222 down-regulation resulted in decreased VSMC proliferation *in vitro* and in rat carotid arteries (61). More recently, miR-663 was found down-regulated in human aortic VSMCs upon treatment with platelet-derived growth factor, and to be in charge of VSMC phenotypic switching by targeting JunB/myosin light chain 9 expression (62). Similarly, miR-133 plays a modulatory role in VSMC phenotypic switching in that it decreases when VSMCs are primed to proliferate *in vitro* and following vascular injury *in vivo*, whereas it increases when VSMCs are coaxed back to quiescence (63).

### Peripheral Ischemia and Vascular Consequences of DM

The conserved miR-17~92 cluster—which comprises miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92a—was found highly expressed in human ECs, and in particular overexpression of miR-92a blocked EC angiogenesis *in vivo* and *in vitro*, whereas administration of its antagomiR in mouse models of limb ischemia and myocardial infarction led to enhanced blood vessel growth and functional recovery of damaged tissues (64). Administration of bone-derived mononuclear cells in infarcted murine hearts reduced proapoptotic miR-34a expression in the infarct border zone, which is probably related to a paracrine effect because bone-derived mononuclear cell supernatants blocked H<sub>2</sub>O<sub>2</sub>-induced expression of miR-34a in cardiomyocytes *in vitro* (65); vice versa, reduced expression of miR-34a improved the survival of mononuclear cells *in vitro* and enhanced the therapeutic benefit of cell therapy in infarcted mice (66). In addition, the microangiopathy and damaging effects of DM were demonstrated in bone-marrow stem cells of diabetic patients, including microvascular rarefaction and shortage of progenitor cells, attributable to activation of a proapoptotic pathway regulated by miR-155 (67). DM also impaired circulating proangiogenic cells supporting post-ischemic neovascularization, through

miR-15a and miR-16 regulation (68). These miRNAs are also found in the serum upon chronic limb ischemia, and positively correlated with amputation after restenosis at 12 months post-revascularization in DM patients (68). Again in DM patients, miR-503 was found up-regulated in ECs and plasma (69).

### Circulating miRNAs: New Biomarkers of Cardiovascular Diseases?

miRNAs are remarkably stable in the extracellular milieu, and they are detectable in blood and other body fluids. In fact, circulating miRNAs are shielded from RNA-degrading enzymes either because of association in an extra-vesicular state with lipoproteins (high-density lipoprotein) (70,71) or protein (argonaute 2) complexes (72); or because they are found within vesicles, such as microparticles (73), exosomes (74,75), and apoptotic bodies (76). In particular, microparticles play a key role in a communication network for the local and systemic intercellular exchange of biological information, and harbor a specific concentrated set of cytokines, signaling proteins, mRNAs, and miRNA that can be effectively transferred to recipient cells (77). In fact, it has been suggested that miRNAs released from 1 cell type can be taken up by another in which they can then act: for instance miR-126 is contained in apoptotic bodies taken up by VSMCs, improving survival of the latter (76); similarly, miR-143 expression in ECs was found to be up-regulated by KLF2 and taken up by adjacent VSMCs, improving their functionality (78).

Interestingly, circulating miRNAs share many of the essential characteristics of a good biomarker: noninvasive measurability; a high degree of sensitivity and specificity, allowing early detection of pathological states; time-related changes during the course of disease; a long half-life within the sample; and rapid and cost-effective laboratory detection. miRNAs may fulfill most of these criteria. However, anticoagulation (79) and antiplatelet (80) drugs can affect quantification of miRNAs in blood samples, and must be taken into account when assessing circulating miRNAs (81).

Since their discovery in the circulation (82), the potential use of miRNA as serum biomarkers has been intensely studied (83,84). The value of currently available cardiovascular biomarkers in clinical practice—which are usually proteins or polypeptides—could be enhanced by the use of novel molecular and genetic biomarkers and improvements in risk prediction algorithms, with the ultimate goal of developing personalized medicine based on “omics” sciences. The use of these novel biomarkers for the prediction of cardiovascular-disease risk before clinical onset is appealing (84). Several groups have proposed circulating miRNAs as biomarkers for diagnosis and prognosis of cardiovascular pathologies ranging from HF (85), acute MI (86–89), and cardiomyopathies (90,91) to atherosclerosis (92) and DM (93). Despite their promise, miRNAs still have not entered the clinical scenario, mainly because of a lack of large cohort studies.

Another limitation to account for is related to the use of polymerase chain reaction technology: miRNA sequences are amplified using oligonucleotides specific for small nucleic acids. However, this amplification step may lead to artifacts if not properly titrated and conducted without rigorous controls. Recent advances are improving the precision of miRNA dosage: digital polymerase chain reaction is based on amplification of molecules diluted to the point that 1 single miRNA molecule is included per reaction (94,95); direct nucleic acid sequencing is another, very efficient, though still expensive, technique for accurately measuring RNAs (96). In large screening efforts, the cost of the study is obviously a limiting issue.

**Heart failure.** A unique miRNA signature for HF has not been identified yet (Table 1). miR-423-5p was found significantly increased in HF patients (97,98), but neither miR-423-5p nor miR-133a were associated with the level of brain-type natriuretic peptide, left ventricle function, or cardiac remodeling (99). In patients with acute HF, only miR-499 was significantly elevated (2-fold), with no significant changes in the other mRNAs studied in patients with diastolic dysfunction (100). More recently, miR-519e\*, miR-520d, miR-1231, miR-200b\*, miR-622, and miR-1228\* were found increased and correlating with brain-type natriuretic peptide levels in non-ischemic systolic HF (85).

**Cardiac hypertrophy.** Twelve circulating miRNAs were found significantly elevated in hypertrophic cardiomyopathy patients, but only 3 (miR-199a-5p, miR-27a, and miR-29a) correlated with hypertrophy; in particular, miR-29a was significantly associated with both hypertrophy and fibrosis evaluated with cardiac magnetic resonance, identifying it as a

potential biomarker for myocardial remodeling assessment in hypertrophic cardiomyopathy (91).

**Acute myocardial infarction and acute coronary syndrome.** In patients with acute MI, miR-1, miR-133a, miR-499, and miR-208a have been reported up-regulated in plasma as a result of cardiomyocyte necrosis and massive release into the bloodstream (Online Table 1) (86,87,101,102). The time-dependent release of acute MI-related miRNAs has been recently investigated: miR-1, miR-133a, and miR-208a increased continuously during the first 4 h after the induction of MI (103), before conventional biomarkers of acute MI could be detected. In a study comparing human and murine circulating miRNAs, human miR-1, miR-133a, and miR-133b peaked before cardiac troponin T (cTnT), whereas murine miR-499 appeared to be a more sensitive marker of acute MI in mice (89). miR-1 and miR-133a were increased in patients with acute MI, unstable angina, or Takotsubo cardiomyopathy (104). The circulating levels of miR-499 and miR-208b have been correlated with cTnT values in cardiopathic patients (100), and circulating miR-133a has been correlated with prognostic magnetic resonance markers, such as infarct size, microvascular obstruction, and myocardial salvage index, but was unable to independently predict clinical events (105). miR-133a was also substantially increased in patients with acute MI relative to those with Takotsubo cardiomyopathy: in fact, a unique signature comprising miR-1, miR-16, miR-26a, and miR-133a differentiated Takotsubo cardiomyopathy patients from healthy subjects and from acute MI patients (106). miR-208a has been proposed as a novel biomarker because it becomes detectable in plasma within 1 to 4 h from symptoms onset—when cTnT is still below the

**Table 1** Some Circulating miRNAs Found Associated With the Enlarged Heart

miRNA	Misexpression	Specimen	Study Population	Setting	Ref. #
<b>Heart failure</b>					
miR-423-5p, miR-320a, miR-22, miR-92b	Up-regulated in systolic HF patients	Serum	30 stable chronic HF patients + 30 controls	Hospital admission	(98)
miR-126	Negatively correlated with age and NYHA functional class in HF patients	Plasma	10 congestive HF patients + 17 controls	Hospital admission	(117)
miR423-5p	Up-regulated in HF patients	Plasma	50 patients with dyspnea (30 HF patients and 20 non-HF patients) + 39 controls	Hospital admission	(118)
miR-192, miR-194, and miR-34a	Up-regulated at early stage of post-ischemic HF	Serum	65 post-AMI patients and 65 controls	CCU	(119)
miR-200b*, miR-622, miR-1228*	Up-regulated in HF in patients with reduced ejection fraction	Whole blood and serum	53 nonischemic HF patients and 39 controls	Hospital admission	(85)
miR-210, miR-30a	Up-regulated in HF patients	Serum	22 HF patients and 18 controls + 9 pregnant women	Hospital admission	(120)
<b>Hypertrophic cardiomyopathy</b>					
miR-29	Up-regulated in HCM patients, biomarker of hypertrophy and fibrosis	Plasma	41 HCM patients + 41 controls	Hospital admission	(121)
<b>Dilated cardiomyopathy</b>					
miR-548c	Down-regulated in PBMCs from DCM patients	Whole blood	44 stable chronic HF + 48 controls	Hospital admission	(90)

AMI = acute myocardial infarction; CCU = coronary care unit; DCM = dilated cardiomyopathy; ED = emergency department; HCM = hypertrophic cardiomyopathy; HF = heart failure; miRNA/miR = microribonucleic acid; NYHA = New York Hear Association; PBMC = peripheral blood mononuclear cell.

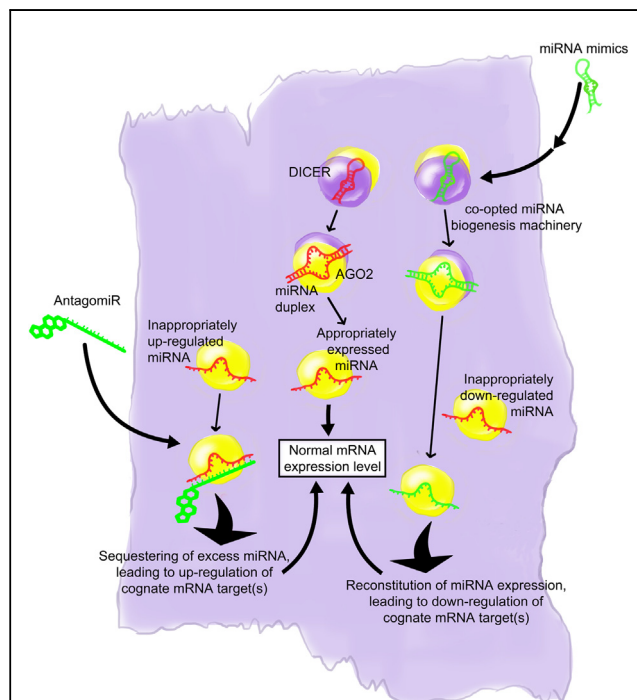
cut-off value—because it is cardiac-specific, and because it is absent in healthy subjects and patients without acute MI (86,107). However, the prognostic impact of circulating miRNAs (miR-1, miR-133a, miR-133b, miR-208a, miR-208b, and miR-499) in acute MI is still debated because a sizeable overlap exists between patients with unstable angina (108). Therefore, the current clinical use of miRNAs as biomarkers for these pathologies is still limited, with other biomarker analyses (such as high-sensitivity cTnT) being currently more easily conducted than the quantitative polymerase chain reaction-based assays needed to assess circulating miRNAs.

More recently, the prognostic value of circulating miRNAs has been evaluated. Despite the time-dependent changes in circulating miR-133a and miR-423, none were associated with indices of left ventricular function and remodeling or with brain natriuretic peptide (99). miR-208b was found to be a modest but significant predictor of 6-month mortality, but, along with miR-499 and high-sensitivity cTnT, was not a significant predictor of 6-year mortality in a cohort of 510 patients (109). To assess the association with incident MI and circulating miRNAs, a prospective study on a total of 19 miRNAs was conducted: miR-223 and miR-197 showed negative associations, while miR-126 showed a positive association with subsequent acute MI (88). miR-126, a pivotal regulator of endothelium and vascular integrity, is mostly abundant in platelets, but its role is still under debate (110).

**Vascular consequences of DM.** In a population-based study, miR-126 appeared inversely correlated with DM and its severity (93), and thus might have diagnostic and prognostic implications. Loss of miR-126 could be the result of altered secretion, degradation, or cellular uptake. Another study revealed that 7 miRNAs involved in insulin biosynthesis and secretion were significantly up-regulated in DM patients (111). More recently, miR-146a was found significantly elevated in newly diagnosed DM patients, but no prognostic value could be found (112).

### Therapeutic Perspective on miRNAs in Cardiovascular Diseases

The therapeutic use of miRNAs is currently being explored through 2 approaches: overexpression and inhibition (Fig. 2). The chemistry behind gene inhibition through antisense methodologies is significantly more advanced than miRNA overexpression through mimicry: whereas an increased miRNA number can be achieved through the use of viral vectors, oligonucleotide miRNA mimics are still under development. It is difficult, though, to foresee the application of viral vector-mediated gene expression. On the other hand, antisense oligonucleotides can be used to efficiently inhibit miRNAs. These antisense sequences need to be modified in order to improve their stability and tissue distribution. Since miRNAs can bind to targets with different sequences, and sequences outside the seed region



**Figure 2** Schematic of Hypothetical miRNA-Based Therapeutic Strategies

miRNA(s) found to have a role in pathogenesis and that are overexpressed during diseased states can be targeted for sequestration through the administration of anti-miRNAs, such as cholesterol-conjugated antisense oligonucleotides called antagomiRs, which compete with the cognate mRNA target(s). In this way, inappropriately down-regulated mRNAs return to normal levels of expression. In contrast, disease-down-regulated miRNAs may, in theory, be replenished through the administration of double-stranded oligonucleotides that contain the miRNA sequence. These are designed to co-opt the miRNA biogenesis machinery for maturation and incorporation of the artificial miRNA into the miRISC. Abbreviations as in Figure 1.

may be biologically active, anti-miRNAs may have a “promiscuous” effect. Thus, a rigorous approach is needed to assess their on-target effect on diseased tissues. Off-target effects are highly likely to occur when doses are increased. Reference (113) is a detailed extensive review on pharmacokinetics and pharmacodynamics of anti-miRNAs.

Anti-miRNAs are chemically modified to improve binding affinity, nuclease resistance, and cellular uptake. High-affinity interaction of antisense oligonucleotides with miRNAs is achieved through chemical modifications that enhance binding affinity and confer nuclease resistance and protein binding (phosphorothioate backbone linkages), and facilitate cellular uptake (2'-O-methyl-cholesterol-conjugation) (114). It has been demonstrated that they are efficiently taken up by cells of the cardiovascular system, including cardiomyocytes, ECs, and fibroblasts, and exert a potent biological effect within this context.

Anti-miRNAs can have different lengths: antagomiRs are antisense molecules of the whole miRNA sequence; they have phosphorothioate backbone modifications and are conjugated with (2'-O-methoxy)cholesterol (115). Locked

**Table 2** Effects of the Administration of Some miRNAs and Anti-miRNAs on the Cardiovascular System in Animal Models

miRNA	Type	Species	Therapeutic Effect	Ref. #
<b>Post-myocardial infarction remodeling</b>				
miR-15 family	Locked nucleic acid (LNA)-modified anti-miRNA	Mice and pigs	Reduced infarct size and cardiac remodeling and enhances cardiac function in response to MI	(122)
miR-24	2'-O-methyl-linked and 3'-cholesterol-linked antagomiR	Mice	Reduced infarct size, improved vascularity and cardiac performance after ischemic injury	(123)
miR-320	AntagomiR	Mice	Reduced infarct size via antithetical regulation of heat-shock protein-20	(124)
miR-29b	2'-O-methyl-linked and 3'-cholesterol-linked mimics	Mice	May reduce increase in fibrosis after MI	(29)
miR-590 and miR-199a	Transfection with human miR-590-3p and miR-199a-3p	Mice	Reduced infarct size and improved cardiac function, consistent with the effect of these miRNAs in actively stimulating cardiomyocyte proliferation	(38)
<b>Cardiac remodeling, cardiac hypertrophy, and fibrosis</b>				
miR-208a	LNA-modified anti-miRNA	Dahl rats	Reduction in cardiac remodeling with improvement in cardiac function	(125)
miR-212/132 family	miR-212/132 knockout mice with TAC	Mice	Protection from pathological cardiac hypertrophy induced by pressure overload via TAC	(126)
miR-133	antagomiR-133	Mice	Induction of a sustained cardiac hypertrophy	(25)
miR-21	2'-O-methyl and 3'-cholesterol antagomiR	Mice	Reduced cardiac fibrosis, leading to enhance cardiac function in response to pressure overload	(28)
miR-21	LNA-modified anti-miRNA oligonucleotide	Mice	Failed to block the ventricular remodeling response to stress	(127)
<b>Atrial fibrillation</b>				
miR-328	2'-O-methyl and 3'-cholesterol antagomiR	Mice and dogs	Rescue from the atrial fibrillation phenotype and the associated atrial remodeling properties	(128)
<b>Atherosclerosis</b>				
miR-145	Adenoviral overexpression and 2'-O-methyl antagomiR	Rat	Regulated vascular SMC phenotype and controlled vascular neointimal lesion formation	(59)
miR-145	Lentiviral overexpression	Mice	Promoted a contractile phenotype in vascular SMC and reduced plaque size	(50)
miR-21	2'-O-methyl antagomiR	Rat	Decreased vascular SMC proliferation and increased apoptosis	(129)
miR-33	LNA-antisense oligonucleotides	Mice	Elevated HDL cholesterol	(44)
miR-33	anti-miRNA	Non-human primates	Raised HDL cholesterol and lowered VLDL cholesterol	(130)
miR-122	2'-O-methoxyethyl phosphorothioate antisense oligonucleotide	Mice	Decreased plasma HDL and LDL cholesterol levels and improved liver steatosis	(42)
miR-122	LNA-anti-miRNA	Non-human primates	Decreased total plasma cholesterol without any evidence for LNA-associated toxicity	(43)
miR-126	miR-126 enriched in apoptotic bodies	Mice	Stabilized and decreased size of atherosclerotic lesions	(76)
<b>Aneurysm</b>				
miR-29b	LNA-anti-miR-29b	Mice	Increased collagen expression, reducing abdominal aortic aneurysm progression	(131)
miR-29b	LNA-anti-miR-29b	Mice	Induced extracellular matrix expression and inhibited angiotensin II-induced dilation of aortic aneurysm	(132)
<b>Angiogenesis</b>				
miR-92a	2'-O-methyl-linked and 3'-cholesterol-linked antagomiR	Mice	Enhanced blood vessel growth and functional recovery of ischemic tissue	(64)
miR-23/27	LNA-anti-miRNA	Mice	Repressed angiogenesis in a laser-induced choroidal neovascularization model	(133)
miR-24	2prime;-O-methyl-linked and 3'-cholesterol-linked anti-miRNA	Mice	Prevention of endothelial apoptosis and enhancement of vascularity after MI	(123)
miR-503	adenovirus-mediated transfer of miR-503 decoy	Mice	Corrected diabetes mellitus-induced impairment of post-ischemic angiogenesis and blood flow recovery	(69)
<b>Pulmonary hypertension</b>				
miR-145	LNA-anti-miRNA	Mice	Prevented the development of pulmonary artery hypertension	(60)
<b>Stroke</b>				
miR-320a	LNA-anti-miR-320a	Rats	Reduced cerebral edema and infarct volume in cerebral ischemia	(134)
<b>Acute myocarditis</b>				
miR-155	LNA-anti-miRNA	Mice	Attenuated cardiac infiltration, decreased T lymphocyte activation, and reduced myocardial damage	(135)

HDL = high-density lipoprotein; LDL = low-density lipoprotein; LNA = alpha-linolenic acid; MI = myocardial infarction; SMC = smooth muscular cell; TAC = transverse aortic constriction; other abbreviations as in Table 1.

nucleic acids are 8- or 15-base-long antisense molecules with 2' sugar modifications (2'-fluoro and 2'-O-methoxyethyl groups), which confer a very high affinity for the target sequence. These molecules are for all intents and purposes drugs: their efficacy needs to be titrated against their toxicity, which can lead to off-target effects, including interference with gene expression in non-diseased tissues. In addition, the use of locked nucleic acids has been suggested to induce interference with the complement cascade and activation of innate immunity, and hepatotoxicity has been described in some reports (113).

The antisense approach has been used for therapeutic purposes with a high degree of success in the context of experimental myocardial hypertrophy and HF, atherosclerosis, arterial restenosis, and dyslipidemia (Table 2). However, the use of anti-miRNA in the cardiovascular arena is still in the pre-clinical phase. To date, only 1 antagomiR, anti-miR-122, has made it through to a phase-II clinical trial for the treatment of hepatitis C virus infection (116). Large-animal studies and phase I/II trials on humans are still lacking for cardiovascular medicine. Obstacles still need to be overcome before miRNAs can become a therapeutic option in the near future, such as the development of effective animal models, more precise regulation of miRNA mimics, and antagonists that take into account different individual cardiovascular risk factors and age-related issues. Despite these obstacles, the road ahead looks incredibly appealing: besides the knowledge that will be generated by the understanding of the basic mechanisms of cardiovascular pathogenesis, the possibility to develop new diagnostic and therapeutic tools in this sector of medicine is at hand.

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**Key Words:** cardiovascular disease ■ heart disease ■ microRNA.

 **APPENDIX**

**For a supplemental table and an expanded references section, please see the online version of this article.**