Emerging Role of MicroRNAs in Cardiovascular Diseases
Salvatore De Rosa, MD, PhD; Antonio Curcio, MD, PhD; Ciro Indolfi, MD

Despite the recent progress in the diagnosis and treatment of cardiovascular diseases, these are still a major source of morbidity and mortality worldwide. For this reason, a large research effort was directed to the identification of the underlying pathophysiological aspects of cardiovascular diseases. Nevertheless, many mechanisms still need to be more deeply investigated, limiting the development of efficient diagnostic and therapeutic strategies for a relevant number of patients. Recently, microRNAs (miRs) emerged as powerful regulators of biological processes, offering a further opportunity to better understand the biological mechanisms responsible for the development of cardiovascular diseases, including cellular function and cell-to-cell communication. At the same time, the recent demonstration that cell-derived circulating miRs can be measured in the blood opens up their use as powerful biomarkers. The present review summarizes the most relevant experimental evidences on the involvement of miRs in cardiovascular diseases, including vascular remodeling, coronary artery disease, heart failure and ischemic stroke, thus highlighting potential targets for novel therapeutic strategies. (Circ J. 2014; 78: 567–575)

Key Words: Aging; Cardiovascular diseases; Circulation; microRNAs

MicroRNAs and Their Biogenesis
MicroRNAs are short (17–25 nucleotides) non-coding RNAs, whose main function is to regulate gene expression by hindering the translation of specific mRNAs at the post transcriptional level. The nuclear RNA polymerase II is responsible for the transcription of the primary miRNA (pri-miRNA) from the genome, and the enzyme complex of Drosha–Dgcr8 facilitates the processing of pri-miRNA into an ~60–70 hairpin-structured precursor miRNA (pre-miRNA). The RanGTP-dependent nuclear export factor, exportin-5, facilitates their transport to the cytoplasm, where the RNAse III enzyme complex cleaves the pre-miRNA into the mature duplex miRNA (miRNA:miRNA*). One strand of this duplex RNA (the mature miRNA) is incorporated into the miRNA-induced
silencing complex (miRISC), while the other strand (passenger strand or microRNA*) is usually degraded. Depending on the degree of complementarity, a specific miRNA can either induce the degradation of its target mRNA or, most frequently, prevent its translation into proteins. 

More recently, non-canonical pathways for miRNA biogenesis have emerged. These include a Drosha-independent pathway, that exploits the splicing machinery or alternative RNA-mechanisms. Similarly, a Dicer-independent mechanism has been reported for the miR-451. Both the canonical and the non-canonical miRNA biogenesis pathways are depicted in Figure 1.

**Aging, Calcification and Vessel Degeneration**

It is known that age is closely associated with the development and progression of cardiovascular diseases. In particular, age-related alterations of the endothelium impair its physiological vasculoprotective action. A similar phenomenon has been reported to contribute to the development of heart failure. Very recently, it has been shown that the levels of selected miRs are modulated along with the increase in age. In fact, a significant regulation of the miR-29 family has been observed in old vs. young mice. Very interestingly, the same miRs are modulated in Zmpste24−/− mice, an animal disease model for the Hutchinson–Gilford progeria. Consistently, members of the miR-29 family showed an increased concentration in senescent cultured endothelial cells. The association becomes much more interesting as it is known that the miR-29 family is expressed in high amounts in the aorta of aged mice, where it induces a decrease in elastin and collagen, with a general reduction of extracellular matrix, which is responsible for a higher susceptibility to aneurysm formation. Interestingly, recent studies showed that the inhibition of the miR-29 family can be helpful in enhancing the structural stability of the vessel wall. In fact, the selective inhibition of miR-29 with a specific antagonist induced an increase in the expression of matrix proteins, which prevented aneurysm formation both in angiotensin II-induced aneurysm and in genetic models of aneurysm formation.

Despite being modulated in senescent cells and in aged mice, the miR-34 seems to affect age-related disorders in a further way. Its overexpression induces senescence in pro-angiogenic cultured endothelial progenitor cells, and promotes cell death in bone marrow-derived pro-angiogenic cells. Mir-34 is induced by p53, even though its levels can also be increased independently of p53. Important miR-34 targets include SIRT1, Bcl-2 and other cell cycle regulators.

The finding that the inflammation-related miR-146 is upregu-
In several ways, the heart and the vasculature are thoroughly regulated by miRs. In particular, the VSMC phenotype switch between a contractile and a synthetic status plays a pivotal role in vascular remodeling and neointimal hyperplasia. In particular, the VSMC phenotype switch between a contractile and a synthetic status plays a pivotal role in vascular remodeling and neointimal hyperplasia.53,54

Several other miRs have been related to aging, such as the miR-18 and -19, or the miR-22, that set additional regulating mechanisms for senescence, fibrosis and osteogenesis.52,55–58

A complete list of the most characterized age-related miRs is available in Table 1.

**Arterial Remodeling and Vascular Restenosis**

Pathological alterations in the regular regulation of the vessel wall is responsible for the progression of atherosclerosis, as well as neointimal hyperplasia.

Vascular remodeling is a highly integrated process that involves vascular smooth muscle cells (VSMCs), endothelial cells (ECs), monocytes/macrophages, platelets (PLTs), cells of the immune system and other non-vascular cells.52,53 However, VSMCs play a key role in these processes.54–57 In particular, the VSMC phenotype switch between a contractile and a synthetic status plays a pivotal role in vascular remodeling and neointimal formation, and is finely regulated by several pathways including the platelet-derived growth factor (PDGF), the transforming growth factor (TGF)-β/bone morphogenetic protein (BMP) pathways, the Ras-mitogen-activated protein kinase (MAPKs) proteins and cyclic AMP-protein kinase A (cAMP-PKA) signaling.54,55,56,58–62 All these processes are thoroughly regulated by miRs.45,56,59 In addition, miRs can mediate cell-to-cell communication between ECs and VSMCs in several ways.21,64,65 The most relevant miRs associated with arterial remodeling are reported in Table 2.

### Coronary Artery Disease and Acute Coronary Syndrome

Several miRs are expressed in biologically relevant levels in most cells and tissues of the cardiovascular system.63 In addition, miRs are seemingly released into the bloodstream, offering the opportunity to monitor the biological status of the cardiovascular system through the measurement of the expression pattern of specific miRs in the blood. In fact, selected miRs (miR-17, miR-92a, miR-126, miR-145 and miR-155) are down-regulated in the blood from patients with coronary artery disease (CAD), while others (miR-133, miR-208a) are upregulated.64 Furthermore, in a recent study, the extent of modulation of specific circulating miRs was associated with the plaque burden, evaluated using coronary computer tomography (CT) scans.65 Similarly, a specific circulating miRNA signature, including the miR-106b/25 cluster, miR-17/92a cluster, miR-21/590-5p family, miR-126* and the miR-451, represent a useful biomarker for the identification of vulnerable CAD patients.66 According, a cardiac release of specific miRs (miR-145, miR-126) was shown in patients with “vulnerable plaques”, identified by optical coherence tomography as those with high macrophage density or thin-cap fibroatheromas.67

However, the usefulness of circulating miRs goes beyond the identification of patients at a higher risk of developing an acute coronary syndrome (ACS). In fact, as both cardiomyocyte-specific (miR-208a) and muscle-enriched (miR-1, miR-133a/b, miR-499) miRs were described, several groups independently evaluated whether the measurement of their levels in the circulation could be useful to diagnose acute myocardial infarction.64,65,66–68 This approach appeared very promising from the initial steps; interestingly, it was shown that muscle-enriched miRs (miR-499, miR-133a) are released from the myocardium into the coronary circulation in ACS patients.66 In the same patients, the vascular-related miR-126 and miR-92a showed a negative concentration gradient across the coronary circulation, suggesting consumption either by degradation or tissue...
uptake during the passage through the myocardium.\textsuperscript{64} Among the most studied, both cardiac-specific (miR-208) and muscle cell-enriched miRs (miR-1, miR-133a/b, miR-499, miR-663b, miR-1291), as well as certain smooth muscle-enriched (miR-30k, miR-145) miRs, were evaluated as circulating biomarkers for acute myocardial infarction (AMI) and showed a good correlation to troponins.\textsuperscript{64,64,65,66} However, despite a number of alternative markers having been proposed, including circulating molecules and circulating cells,\textsuperscript{81-83} a valuable superiority to already established biomarkers of AMI should be demonstrated before miRs could be proposed as a real alternative to high-sensitivity cardiac Troponin T (hsTnT). In this regard, one interesting possibility could be an even earlier detectability of miRs compared to troponins. Interestingly, specific miRs were significantly elevated as early as 1 h after coronary artery ligation\textsuperscript{84} and even 15 min in a parallel murine model.\textsuperscript{85} In line with these results, very early myocardial release of muscle-enriched miRs was reported in humans within 30 min from symptoms onset, with consequent significant elevation of miRs at a time where hsTnT was still negative in some patients.\textsuperscript{84} In addition, miRs could provide a useful mean for diagnostic differentiation between the several clinical conditions that are potentially associated with an elevation of traditional cardiac biomarkers. In fact, as the introduction of hsTnT generated some confusion at the very beginning, making possible the detection of low Troponin T concentrations, which are often not diagnostic for an AMI but mirror a limited ongoing myocardial injury,\textsuperscript{86} the measurement of a selected pattern of miRs could be helpful to distinguish between dyspnea of a different origin.\textsuperscript{72,86,87} The most relevant circulating miRs associated with Acute Coronary Syndrome are reported in Figure 2.

Also, platelets play an important role in the evolution of CAD, as they can trigger the degeneration toward an acute coronary syndrome.\textsuperscript{88,89} As it has been reported, miR are present in large amounts in platelets, where they play a key regulatory role.\textsuperscript{90} Interestingly, increased levels of miR-340 and miR-624 were found in platelets from young patients with premature development of CAD, as compared to controls.\textsuperscript{91} In addition, specific miRs are involved in the regulation of several aspects of platelet function, such as activation or aggregation.\textsuperscript{92-94} The most relevant miRs associated with coronary artery disease are reported in Table 2.

\begin{table}[h!]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{miR} & \textbf{Molecular targets} & \textbf{Associated Disease} & \textbf{Net therapeutic effect} \\
\hline
1 & Cx43 & LVH, VT, MI & It's selective cardiac over-expression reduces LVH and prevents VT \\
2 & Irx5, Kcnq2 & Reduced HR, shortened PR interval, widened QRS interval & Combined loss of function of Irx5 and Irx4 causes ventricular repolarization abnormalities and a predisposition to arrhythmias \\
7 & Hyaluronic acid mediated EGF-R signaling & Its transcription is upregulated in aged cells & It restores the healing capabilities in chronic wounds in the elderly \\
9 & PDGF-β & LVH, fibrosis & It reduces paracrine angiogenic capacity of cardiomyocytes \\
15a/16 & VEGF, AKT3 & Critical limb ischemia & Relative inhibition through PACs improved post-ischemic blood flow, recovery and muscular arteriole density \\
20a & MKK3 & Reduced angiogenesis in ischemia settings & It represses the expression of MKK3 and VEGF-induced endothelial cell migration and angiogenesis \\
21 & PDCD4 & Restenosis & It's inhibition reversed vascular remodeling induced by balloon injury \\
23a & & HF & Combining its levels with NT-proBNP might add diagnostic value in HF diagnosis \\
24 & eNOS & Post-MI response & Local delivery increased angiogenesis and blood perfusion in the peri-infarct myocardium, reduced infarct size, induced fibroblast apoptosis and improved cardiac function \\
26 & KIR2.1 & AF & AF activates NFAT, enhancing its translocation into the nucleus, where it transcriptionally represses the expression of miR-26 genes \\
27 & SEMA6A & angiogenesis, adipogenesis, inflammation, lipid metabolism, oxidative stress, insulin resistance and type 2 diabetes & Increased levels distinguish HF in breathless patients \\
30b & & Lipid metabolism and atherosclerosis & \\
33a & KLF4, MKK4 & Angiogenesis and restenosis & It's inhibition increases endothelial proliferation and reduces NH after vascular injury \\
92a & cyclinD2 & LVH & It's upregulation by Trx1 inhibits cardiac hypertrophy \\
98/let-7 & Lipid metabolism and atherosclerosis & Inversely correlated with the collagen content contributing to myocardial fibrosis in AS \\
103 & Lipid metabolism and atherosclerosis & Increased levels distinguish HF in breathless patients \\
106 & TGF-β1 & Lipid metabolism, atherosclerosis. Downregulated in aortic stenosis & \\
122 & ET-1 & Takotsubo cardiomyopathy & It's decreased levels corroborate the microvascular spasm hypothesis in Takotsubo cardiomyopathy \\
125a-5p & Restenosis, MI, LVH & Upregulated in STEMI; controls phenotypic switch of VSMCs after injury; reduced in LVH & \\
\hline
\end{tabular}
\caption{Most Important MicroRNAs Involved in Cardiovascular Diseases}
\end{table}
### MicroRNAs in Cardiovascular Diseases

<table>
<thead>
<tr>
<th>miR</th>
<th>Molecular targets</th>
<th>Associated Disease</th>
<th>Net therapeutic effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>143/145</td>
<td>Acta1, Tnnc2, Tnnt2, Mylpf</td>
<td>Aortic aneurysms</td>
<td>Combining its levels with NT-proBNP might add diagnostic value in HF</td>
</tr>
<tr>
<td>199a-3p</td>
<td></td>
<td></td>
<td>It’s therapeutic inhibition improves cardiac function in HF</td>
</tr>
<tr>
<td>208</td>
<td>Myh6, Myh7, Myh7b</td>
<td>LVH, HF</td>
<td>Released from platelets, this miR promoted vascular endothelial cells apoptosis induced by advanced glycation end products</td>
</tr>
<tr>
<td>223</td>
<td>IGF1-R</td>
<td>Platelets play a significant role in atherosclerosis and stroke through active interaction with neutrophils, monocytes, and vascular endothelial cells.</td>
<td></td>
</tr>
<tr>
<td>324–5p</td>
<td></td>
<td></td>
<td>combining its levels with NT-proBNP may add diagnostic value in HF</td>
</tr>
<tr>
<td>328</td>
<td></td>
<td>AF</td>
<td>Increased levels distinguish HF in breathless patients</td>
</tr>
<tr>
<td>342–3p</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>350</td>
<td>MAPK 11/14 and MAPK9/8</td>
<td>Upregulated in hearts in response to late-stage AS</td>
<td>Ago-350 reversed cardiac hypertrophy by reducing cell size and apoptosis</td>
</tr>
<tr>
<td>370</td>
<td></td>
<td>Lipid metabolism and atherosclerosis</td>
<td>Provides protection against atherosclerosis by reducing lipid accumulation, and production of inflammatory cytokines in apoE−/− mice</td>
</tr>
<tr>
<td>467b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>590–3p</td>
<td></td>
<td>AF</td>
<td></td>
</tr>
<tr>
<td>758</td>
<td></td>
<td>Lipid metabolism and atherosclerosis</td>
<td></td>
</tr>
</tbody>
</table>

miR, microRNA; AgomiR, anti-miR-antisense oligonucleotide; Cx43, connexin43; LVH, left ventricular hypertrophy; VT, ventricular tachyarrhythmias; Irx4/5, Iroquois-class homeodomain protein IRX-4/5; Kdnd2, Potassium voltage-gated channel subfamily D member 2; Mi, myocardial infarction; HR, heart rate; EGFR, epidermal growth factor receptor; PDGFR-β, platelet-derived growth factor receptor-beta; AKT3, β-akt murine thymoma viral oncogene homolog 3; PACs, circulating proangiogenic cells; MIG, miRNA-induced gene; kinase 3; VEGF, vascular endothelial growth factor; PCDC4, programmed cell death protein 4; NT-proBNP, N-terminus fragment of brain natriuretic peptide precursor; HF, heart failure; eNOS, endothelial isoform of the nitric oxide synthase; KIR2.1, protein encoded by the KIR2.1 gene; AKT, atrial fibrillation; NFAT, nuclear factor of activated T-cells; SEMA6A, semaphorin6a; KLF4, Kruppel-like factor 4; NH, neointimal hyperplasia; Trx1, Thioredoxin 1; KCNJ2, Potassium inwardly rectifying channel subfamily J member 2; AgomiR, anti-miR-antisense oligonucleotide; Cx43, connexin43; LVH, left ventricular hypertrophy; VT, ventricular tachyarrhythmias; Irx4/5, Iroquois-class homeodomain protein IRX-4/5; Kdnd2, Potassium voltage-gated channel subfamily D member 2; Mi, myocardial infarction; HR, heart rate; EGFR, epidermal growth factor receptor; PDGFR-β, platelet-derived growth factor receptor-beta; AKT3, v-akt murine thymoma viral oncogene homolog 3; PACs, circulating proangiogenic cells; MIG, miRNA-induced gene; kinase 3; VEGF, vascular endothelial growth factor; PCDC4, programmed cell death protein 4; NT-proBNP, N-terminus fragment of brain natriuretic peptide precursor; HF, heart failure; eNOS, endothelial isoform of the nitric oxide synthase; KIR2.1, protein encoded by the KIR2.1 gene; AF, atrial fibrillation; NFAT, nuclear factor of activated T-cells; SEMA6A, semaphorin6a; KLF4, Kruppel-like factor 4; NH, neointimal hyperplasia; Trx1, Thioredoxin 1; TGFB1, transforming growth factor beta 1; AS, aortic stenosis; ET-1, endothelin-1; STEMI, ST-segment elevation MI; VSMCs, vascular smooth muscle cells; Acta, actin alpha2; Tnnc2, troponin C; Tnnti, troponin I; Mylp, myosin light chain phosphorylatable; Myh, myosin heavy polypeptide; IGF1-R, insulin-like growth factor receptor 2 and Collagen 1; MAPK, mitogen-activated protein kinase; apoE, apolipoprotein E.

### Congestive Heart Failure

As it became clear that miRs are responsible for the regulation of several biological functions, their association with heart failure (HF) was investigated by several groups. In this research area, the evaluation of miR levels provided the clue for the confirmation that fetal gene reprogramming underlies the development of HF. Also, interestingly, the study of the miR expression profile in the failing human heart revealed that modulation of specific miRs can identify selected alterations of specific regulatory pathways. In fact, it was observed that a decrease of miR-133b and miR-92 together with an increase of miR-100 and miR-195 were associated with changes in the gene expression profile in response to the modulation of the β-adrenergic system. Moreover, a recent report highlighted that left ventricular hypertrophy is associated with reduced miR-1 levels, and that forced expression of the antagonist-I reverted this phenotype in rodents. In contrast, it has been demonstrated that the silencing of miR-208a resulted in an improvement of cardiac function and increased survival in a HF model using Dahl salt-sensitive rats. Similarly, the myocardial delivery of miR-24 increased angiogenesis and blood perfusion in the peri-infarct myocardium, reduced the infarct size and improved cardiac function and improved survival and reperfusion after HF through the miR-206-mediated inhibition of TIMP-3. Further studies demonstrated that the levels of a specific subset of miRs (miR-7, -378, -214, and -181b) are associated with the typical alterations in the expression patterns of key molecules (epidermal growth factor receptor 2 and Collagen 1), which are commonly modulated in end-stage HF. In summary, knowledge about the regulatory role played by miRs in HF could disclose novel therapeutic avenues for heart failure. Furthermore, as specific changes in the plasma level of circulating miRs was independently reported by several groups, these changes offer the unique possibility of establishing novel HF biomarkers, potentially more reliable and sensitive than those currently available. In fact, the measurement of the circulating miR-423-5p or miR-134 could be of valuable help in distinguishing between dyspnea of different etiologies. The most relevant miRs associated with congestive heart failure are reported in Table 2. The most promising circulating miRs in relation to congestive heart failure are reported in Figure 2.

### Stroke

Recent studies evidenced an important regulatory role for miRs in the differentiation, plasticity and maintenance of the nervous system. Interestingly, a specific modulation of the miR expression pattern was observed both in the brain and in the circulating blood during cerebral ischemia. In particular, an increase in the levels of specific miRs was reported in the brain and in the bloodstream (miR-290, miR-292-5p and miR-497) or selectively in the blood (miR-210, miR-215, miR324-3p, miR-422b, miR-451 and miR-134) in rats after transient middle cerebral artery occlusion (MCAO). Similarly, a different subset of miRs was shown to be decreased in the ischemic brain. Of note, the miRs expression pattern showed a specific time-course after transient MCAO followed by reperfusion. The analysis of the potential targets of the miRs that were specifically modulated after brain ischemia revealed that they could potentially modulate several biological processes, including ionic homeostasis, neuro-protection or inflammation.
eases; an essential step toward the development of novel and more specific therapies. However, several challenges still need to be faced before those therapeutic hypotheses can be translated into effective and safe therapies. In particular, several concerns remain regarding the selectivity of such therapies, the regional delivery into the target tissues and the long-term safety. Nevertheless, with the fast advances in the understanding of microRNA biology, the development of specific cardiovascular miRs therapeutics can be foreseen in the near future.

The specific association between the levels of circulating miRs and several aspects of cardiovascular pathophysiology foresees the development of a new generation of biomarkers to better drive both the diagnostic process and the monitoring of cardiovascular diseases. However, a key issue should be addressed before miRs can be actually used as disease biomarkers, that is, the development of a reliable and easy detection method. Among the open problems, normalization remains the major challenge. In fact, because no housekeeping miR/small RNA is yet to be established to date, spiking of the plasma/serum samples with recombinant non-human miRs (e.g., miRs from the nematode, Caenorhabditis elegans) is the most used technique. Because real-time polymerase chain reaction (RT-PCR) results are subjected to large variations from day to day, even when recombinant miRs are used for standard curves, the establishment of an optimal normalization method remains mandatory before miRs can be exploited as disease biomarkers. Finally, as RNA isolation from plasma and subsequent quantification by real-time PCR is a demanding and time-consuming process, alternative detection methods are desirable, to warrant more expedited results.

Conclusions

In conclusion, the continuously growing body of evidence on miRs in the cardiovascular system opens up new possibilities to disentangle still unexplained aspects of cardiovascular diseases; an essential step toward the development of novel and more specific therapies. However, several challenges still need to be faced before those therapeutic hypotheses can be translated into effective and safe therapies. In particular, several concerns remain regarding the selectivity of such therapies, the regional delivery into the target tissues and the long-term safety. Nevertheless, with the fast advances in the understanding of microRNA biology, the development of specific cardiovascular miRs therapeutics can be foreseen in the near future.

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