<center>REVIEW</center>

# Regulatory RNAs and Cardiovascular Disease – With a Special Focus on Circulating MicroRNAs

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## Summary

MicroRNAs (miRNAs) are a class of short non-coding regulatory RNA molecules which play an important role in intracellular communication and cell signaling and which influence cellular processes such as proliferation, differentiation, and cellular death. Over the past two decades, the crucial role of microRNAs in controlling tissue homeostasis and disease in cardiovascular systems has become widely recognized. By controlling the expression levels of their targets, several miRNAs have been shown to modulate the function of endothelial cells (miR-221/222 and -126), vascular smooth muscle cells (miR-143/145) and macrophages (miR-33, -758, and -26), thereby regulating the development and progression of atherosclerosis. The stability of miRNAs within the blood suggests that circulating miRNAs may function as important biomarkers of disease development and progression. Numerous circulating miRNAs have been found to be dysregulated in a wide variety of different disease states, including diabetes, cancer, and cardiovascular disease.

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## Background

Despite the tremendous progress in diagnosis and treatment, cardiovascular disease (CVD) remains one of the leading causes of death in developed countries. In addition to the well-known environmental risk factors (smoking, lack of physical activity), genetic predispositions play an important role in CVD development.

Genome-wide association studies have revealed many changes within DNA sequence leading to increased risk of CVD development (Dubé and Hegele 2013). These changes/polymorphisms can either be associated with genetic markers such as variants within sortilin-1 (Kathiresan *et al.* 2008) and apolipoprotein A5 (Hubáček 2016), or they can be totally independent on traditional risk factors such as the markers on chromosome 9 (Roberts and Stewart 2012).

Nevertheless, a substantial proportion of CVD genetic risk remains unknown ("missing heritability") and it is supposed that epigenetic factors may be at least partially responsible for the "remaining" genetic risk of CVD (Muka *et al.* 2016).

Of the possible epigenetic mechanisms that lead to disease development, in addition to DNA methylation (almost exclusively cytosine methylation within the sequences with a high proportion of CG repeats) and histone modification (methylation, acetylation, phosphorylation, ubiquitination) more and more attention is now being focused on the analysis of regulatory RNA molecules (Udali *et al.* 2013, Kuneš *et al.* 2015, Kura *et al.* 2016).

Only 1-2 % of the human genome codes for mRNAs build a basis for protein synthesis. In contrast, there are many more non-coding RNAs (ncRNAs) in the genome, most of which have unknown functions. NcRNAs can be divided into infrastructural ncRNAs and

regulatory ncRNAs. Regulatory ncRNAs can be classified into Piwi-interacting RNAs (piRNAs), small interfering RNAs (siRNAs), microRNAs (miRNAs), and long non-coding RNAs (lncRNAs) (Thum 2015).

The majority of non-protein-coding transcripts belong to the group of lncRNAs. LncRNAs vary in size from 200 bp to >100 kb, are transcribed by RNA polymerase II, and are often spliced and polyadenylated. LncRNAs are usually localized in the nucleus, have a much lower rate of expression than miRNAs, and exhibit a lower ratio of similarity. They have been identified using a variety of means and the number of specific lncRNAs shown to influence genomic function is growing. These include lncRNAs with roles in imprinting, enhancer function, X chromosome inactivation, chromatin structure, and genomic rearrangements during the generation of antibody diversity. They appear to be critical for normal development and, in many cases, are deregulated in diseases such as cancer. LncRNAs include long intergenic ncRNAs, long intronic ncRNAs, telomeric ncRNAs transcribed from sub-telomeric promoters (telomeric repeat-containing RNA), pseudogene transcripts, circular RNAs, enhancer RNAs, and transcribed-ultraconserved regions (Caley et al. 2010, Kumarswamy and Thum 2013).

Probably the best known lncRNA is ANRIL (antisense non-coding RNA in the INK4 locus) identified at the 9p21.3 location. Variants within this lncRNA are associated with enhanced risk of coronary artery disease development, intracranial aneurysm, type 2 diabetes (T2DM), and some types of cancer (Pasmant et al. 2011).

PiRNAs are small ncRNAs of 24-31 nucleotides (nt) in size, and named for their ability to form complexes with Piwi proteins of the Argonaute family. The primary role of these small RNAs has been shown to be the suppression of transposon activity during the germline cycle. Transposon control also occurs in mammals during spermatogenesis through de novo DNA methylation (Iwasaki et al. 2015).

SiRNAs are linear perfectly base-paired dsRNAs, which are processed by Dicer into 20-24 nt siRNAs that direct silencing when loaded onto the RNA-induced silencing complex (RISC). They mediate post-transcriptional silencing similar to miRNA silencing. Compared with miRNAs, guide strand recognition is indistinguishable. In addition, siRNAs have also been found to direct sequence-specific transcriptional gene silencing by increasing epigenetic marks characteristic of heterochromatin (Kaikkonen et al. 2011).

MiRNAs are short non-coding single-stranded RNA molecules, ~22 nt in length, which act as post-transcriptional regulators. MiRNAs derive from transcripts that form distinctive hairpin structures. Processing of the hairpin into mature miRNA by Drosha and Dicer allows interaction with Argonaute (Ago) proteins to form RISC. Strand selection for RISC is dictated by the thermodynamic stabilities of the two duplex ends: the strand that has its 5′ terminus at the less stable base-paired end of the duplex is mostly favored (MacFarlane et al. 2010) (Table 1). The miRNAs then pair with mRNAs by binding to different target-gene regions: the 3′-untranslated region (3′UTR), 5′UTR, and promoter or coding sequences that repress or activate translation. The crucial binding location for translational regulation resides in the mature miRNA sequence, more accurately within the nucleotides 2-7 or 2-8 from the 5′ end of the miRNA, called the seed region (Obsteter et al. 2015). The mechanisms by which miRNAs reduce protein output are mostly triggered either by deadenylation of the target mRNA and inhibition of eukaryotic initiation factors, or by interference with translational elongation. In addition, the miRNA response has been reported to switch from inhibition of gene expression to enhancement, thereby inducing upregulation of target mRNAs during cell cycle arrest and repressing translation in proliferating cells.

MiRNAs are conserved in a wide range of organisms, from nematodes to humans (Kaudewitz et al. 2015). About 1,500-2,000 human miRNAs have been identified, and it is estimated that >60 % of all protein-coding genes are directly regulated by miRNAs. Furthermore, any given miRNA may bind to and regulate >1 target, sometimes as part of the same signaling pathway. Conversely, any given miRNA may harbor several distinct miRNA-binding sites within its 3′-UTR, adding multiple levels of regulation. As such, miRNAs are fine-tuners of gene expression patterns in response to pathophysiological stimuli. Most miRNAs are more ubiquitously expressed and are not cell-type specific. Thus, although many miRNAs are expressed at relatively low levels under basal conditions, they are strongly upregulated during pathological stress (Thum 2015).

Because we consider the area of this epigenetic research very important, we focused in this review on the role of miRNAs on cardiovascular disease, especially atherosclerosis.

**Table 1.** Basic components of miRNAs biogenesis. MiRNA transcripts are encoded both in intergenic regions and within introns of genes, and can be transcribed either through their own promoters or in concert with their host genes. In the canonical miRNA biogenesis pathway, transcript pri-miRNA is transcribed by the Polymerase II enzyme, after which it undergoes processing by the Drosha/DGCG8 complex to generate pre-miRNA. Following export to the cytoplasm, a pre-miRNA undergoes further cleavage by DICER to generate a mature miRNA duplex of 18-22 nt in length. Argonaute proteins then help mediate the separation of the miRNA strands and incorporation into the RISC. Within the RISC, miRNAs bind partially complementary target sites, primarily within mRNA 3´untranslated regions (3´UTRs), resulting in translational repression and/or mRNA destabilization.

| Component | Description | Function | Location |
|---|---|---|---|
| **Pri-miRNAs** | Long RNA precursors, several hundred or thousands of nucleotides long, has a ≈33-nt stem-loop configuration comprising a 5′ end cap structure and a polyadenylated 3′ tail sequence. | Primary transcripts – primary miRNA precursor. | *Nucleus* |
| **Drosha** | Ribonuclease type III. | Responsible for cropping pri-miR into pre-miR through a single-stranded RNA-double-stranded RNA junction-anchoring model. | |
| **DGCR8** | Double-stranded RNA-binding protein DiGeorge syndrome critical region gene 8. | Together with Drosha forms a protein complex which cleaves the pri-miRNA. DGCR8 recognizes the pri-miRNA at the ssRNA-dsRNA junction and directs Drosha to a specific cleavage site ~11 bp from the junction on the stem where Drosha cuts to liberate a ~60-70 bp miRNA hairpin pre-miRNA. | |
| **Pre-miRNAs** | ~70 nucleotide intermediate, long stem-loop structure. | Precursor miRNAs. | |
| **Exportin5** | Ran-GTP dependent nuclear transport receptor (Exp5)/RanGTP. | Hydrolysis of RanGTP to RanGDP releases the pre-miR in the cytoplasm. | *Cytoplasm* |
| **Dicer** | Ribonuclease type III. Multi-domain protein. | Pre-miR is processed into 18- to 22-nt miR duplex (miR:miR*) by Dicer, which is associated to another dsRBP. The duplex is probably unwound by a helicase activity, and 1 strand ("passenger" strand) is degraded, whereas the other strand ("guide" strand) accumulates as a mature miR. | |
| **TRBP** | Transactivating response RNA-binding protein. | TRBP associates with Ago2 and Dicer to play an integral role in the miRNA pathway. | |
| **PACT** | Protein Kinase R-activating protein | | |
| **Ago2** | Argonaute 2 protein with endonuclease activity. Ago2 is the only argonaute protein with "slicer" activity capable of catalyzing cleavage of mRNA. | Dicer, TRBP and Ago2 form a protein complex that recognizes and binds the pre-miRNA. Ago2 loads miRNA guide in RISC. | |
| **RISC** | RNA induced silencing complex. Core components: Dicer, Ago2, PACT and TRBP. | RISC serves as a guide, directing the silencing of target mRNA. Mature miRNAs are coupled with RISC and then regulate the target genes. | |

## MiRNAs in medicine

Numerous diseases, including cardiovascular disease and pathophysiology, are associated with the regulation of miRNA expression. MiRNAs have been identified as playing a key role in organ development, cellular differentiation, proliferation, and function.

## Intracellular miRNAs

Intracellular miRNAs are more abundant then extracellular miRNAs. In one study, over 250 small RNA libraries from 26 different organ systems and cell types of humans and rodents were sequenced to assess their expression patterns (Landgraf *et al.* 2007). The expression of some miRNAs is highly tissue-specific, e.g. miR-1 and -133 (cardiac and skeletal muscle), miR-122 (liver), and miR-124a (brain) (Lagos-Quintana *et al.* 2002). Among others, kidney-specific miRNAs (miR-192, -194, -204, and -215) have low expression in the liver, lung and heart (Landgraf *et al.* 2007). MiR-375 and -449 are lung tissue-specific (Williams *et al.* 2009).

Of the cardiac-specific miRNAs, miR-1 acts as a regulator of differentiation and proliferation during cardiogenesis and as a regulator of cardiomyocyte growth in the adult heart. MiR-133, transcribed from the same chromosomal loci as miR-1, enhances myoblast proliferation and is thus involved in cardiomyocyte proliferation (Chen *et al.* 2006). The miR-208 family, which includes miR-208a/b and -499 (exclusively expressed in the heart), are reported to play a pathological role in cardiac hypertrophy and, consequently, arrhythmia. MiR-499 has been shown to be downregulated under pathological conditions such as ischemia and cardiac remodeling (Rawal *et al.* 2014). MiR-21 is mechanistically involved in cardiac fibrosis development (Thum 2015). The neurologically enriched miR-212/132 family becomes activated during heart failure (HF) in human and animal models and has been shown to regulate cardiac hypertrophy in cardiomyocytes (Ucar *et al.* 2012).

Diabetes is a strong risk factor for the development of CVD. Significant differences have been found in the miRNA expression profiles of various tissues in T2DM patients and hyperglycemic animal models (Feng *et al.* 2016) in comparison to healthy subjects, e.g. the pancreas (miR-375, -184, and -33), adipose tissue (miR-93 and -223), and the liver (miR-143).

## Extracellular miRNAs

Extremely stable miRNAs also occur in extracellular fluids, e.g. serum, saliva, urine, milk, and tears. Concentrations of miRNAs in body fluids are low, which means they are likely to be more effectively detected.

MiRNAs can be released into the blood circulation by various mechanisms, including active secretion, apoptosis, and necrosis. These miRNAs circulate in different types of vesicles, such as apoptotic bodies, microvesicles, exosomes, and lipoproteins. It has been reported that a significant portion of circulating miRNAs in human plasma and serum is associated with Ago2 (Russo *et al.* 2014). Although miRNAs are mostly by-products of cellular activities and cell death, certain miRNA species may also function in cell-cell communication (Turchinovich *et al.* 2012).

Circulating miRNAs may be considered putative biomarkers of the cardiovascular system. A sensitive, specific, and non-invasive diagnostic blood test for CVD would be very desirable because it is difficult to obtain valid biopsies from patients. The ability to detect circulating miRNAs also provides an effective means for non-invasive detection and diagnosis in patients with CVD. Recent advancements in detection methods for circulating miRNAs present in the serum/plasma may become a powerful tool when performing minimally invasive diagnosis of different CVDs. Numerous studies have demonstrated that alterations in the spectrum of extracellular miRNAs are correlated with various cardiovascular conditions such as myocardial infarction (MI) (miR-1, -133a/b, and -499), hypertrophy (miR-29, -21), dilated cardiomyopathy (miR-423-5p), and arrhythmias (miR-29, -150) (Wang *et al.* 2010, Liu Z *et al.* 2012, Dawson *et al.* 2013, Roncarati *et al.* 2014).

In a patient cohort with ST-segment elevation MI, the early use of circulating miR-detection (miR-1, -133a, -133b, and -499-5p) in comparison with troponins as biomarkers was conclusively reported (D'Alessandra *et al.* 2010). Recently, Wang *et al.* (2016) found that circulating miR-19b-3p, -134-5p, and -186-5p are promising novel diagnostic biomarkers for the early phase of acute MI. Cardiac damage caused by MI, viral myocarditis, diastolic dysfunction, and acute heart failure has been reported as initiating the release of cardiomyocyte-specific miR-208b and -499 into circulation (Corsten *et al.* 2010). Other reports demonstrate decreased levels of miR-29 and -150 and

increased serum levels of miR-423-5p, -320a, -22, and -92b in subjects with systolic HF and atrial fibrillation (Goren *et al.* 2012, Goren *et al.* 2014). In another study, plasma concentrations of miR-126 were found to be reduced in patients with HF compared to healthy subjects (Fukushima *et al.* 2011).

Zhang *et al.* (2015) found that plasma miR-126 expression was significantly reduced in T2DM patients, which suggests that this miRNA may serve as a potential marker for the early prediction of diabetes. Coronary artery disease (CAD) is a complication of T2DM, and different circulating miR-126 expression patterns in the plasma of T2DM patients may serve as a biomarker of diabetic CAD (Al-Kafaji *et al.* 2016).

Diabetic nephropathy (DN) is one of the most serious complications of diabetes. Recently, a prospective case-control study showed that miR-21, -29a/b/c, and -192 reflect DN pathogenesis and may be of clinical significance in monitoring and preventing DN advancement (Chien *et al.* 2016). In Figure 1 are summarized the most prominent microRNAs involved in CVD pathogenesis.
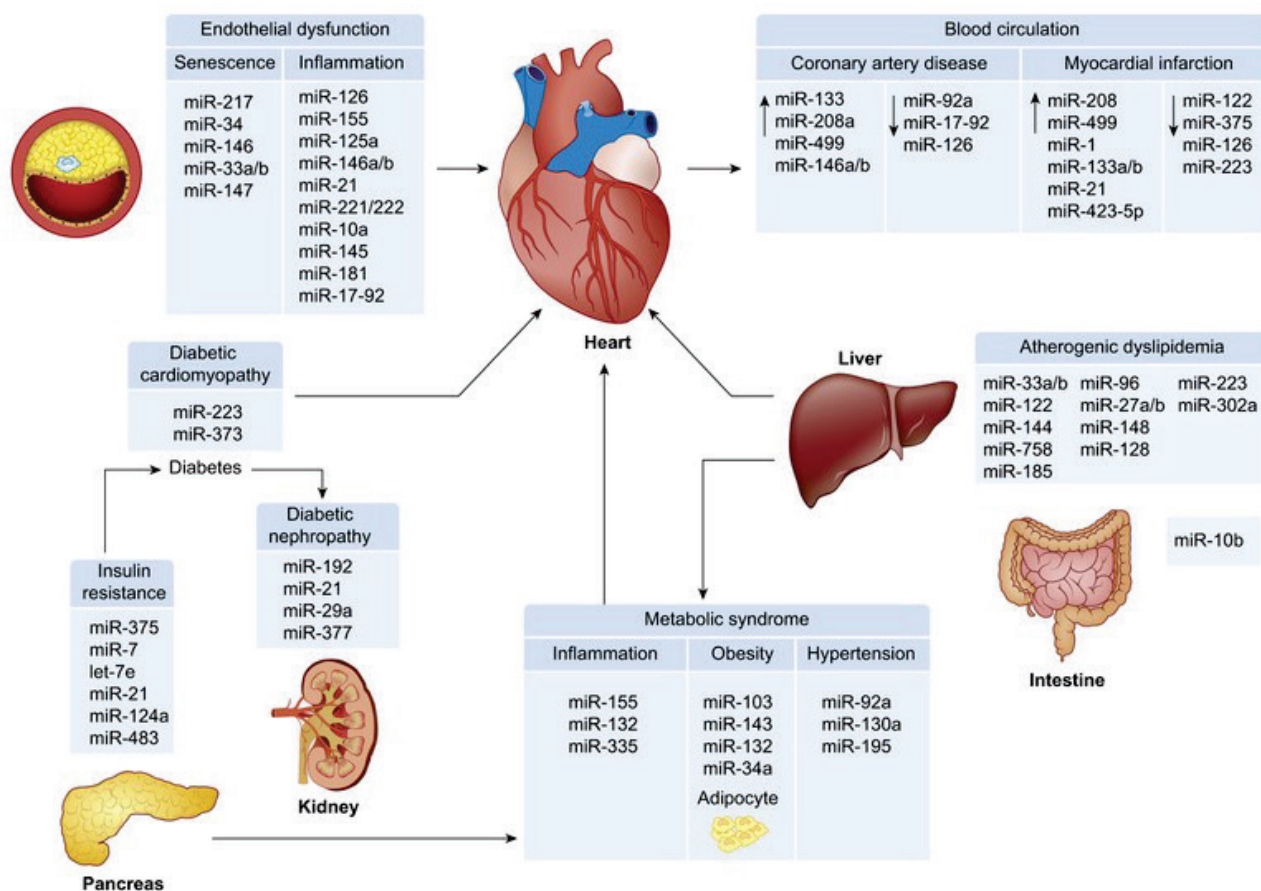


**Fig. 1.** Summarized features of the known roles of microRNAs in the development of CVD. Different up- and down-regulated microRNAs participate in the progression of endothelial dysfunction and regulations of metabolic processes.

## The role of miRNAs in the development of atherosclerosis

Atherosclerosis is the underlying condition in most CVD. Lipid and endothelial dysfunction, inflammation, immune response, and advanced ageing are major factors involved in the initiation and progression of atherosclerosis. Recent studies have shown the key roles of several miRNAs in endothelial dysfunction, cholesterol homeostasis, reverse cholesterol transport, plaque development, neoangiogenesis, and plaque rupture (Zampetaki *et al.* 2013, Menghini *et al.* 2014) (Tables 2, 3 and 4).

*Endothelial dysfunction*

Several miRNAs are involved in the modulation of endothelial dysfunction (Table 2) such as miR-10a, which inhibits a number of pro-inflammatory genes in

endothelial cells (ECs), including vascular cell adhesion molecule 1 (*VCAM-1*), E-selectin, and the nuclear factor (NF)-κB signaling pathway (Fang *et al.* 2010). This signaling pathway is also modulated by miR-181b, which directly targets the importing karyopherin subunit alpha-4 (*KPNA4*), a protein required for NF-κB nuclear translocation (Sun X *et al.* 2012). MiR-126, -31, and -17-3p also regulate vascular inflammation by controlling the expression of the adhesion molecules *VCAM-1*, intercellular adhesion molecule 1 (*ICAM-1*), and E-selectin (Suarez *et al.* 2010, Asgeirsdottir *et al.* 2012).

In addition to pro-inflammatory cytokines, shear stress also regulates endothelial cell activation through miRNAs. In this regard, atheroprotective laminar shear flow downregulates miR-92a and consequently increases the expression of well-known targets of this miRNA, such as Kruppel-like factors (*KLF2/4*) (Wu *et al.* 2011, Fang and Davies 2012). Some other miRNAs have important roles in endothelial ageing such as miR-146a, which delays EC senescence, and miR-217 and -34a, which promote EC senescence (Menghini *et al.* 2009, Ito *et al.* 2010, Vasa-Nicotera *et al.* 2011).

**Table 2.** MiRNAs associated with endothelial dysfunction.

| miRNA | Targets | Functions |
|---|---|---|
| **miR-10a** | MAP3K7; TAK1; βTRC, HOXA1 | Inhibits upstream canonical NF-κB signaling pathway |
| **miR-181b** | Importin α-3 | Reduces vascular inflammation *in vivo* |
| **miR-126** | SPRED-1; PIK3R; VCAM-1; CXCL12 | Inhibits leukocyte adherence to ECs. Regulates angiogenic signaling and vascular integrity |
| **miR-17-3p** | ICAM1 | Decreases leukocytes adhesion to activated ECs |
| **miR-92a** | KLF4/2 | Regulates or contributes to flow-mediated EC activation |
| **miR-217** | SIRT1 | EC senescence, suppresses cell proliferation |
| **miR-34** | | |

**Table 3.** MiRNAs involved in regulation of cellular cholesterol metabolism.

| miRNA | Targets | Function |
|---|---|---|
| **miR-33a/b** | ABCA1, ABCG1, NPC1, CPT1A, SIRT6, AMPK, HADHB, CROT, CYP7A1, ABCB11, ATP8B1, NSF, SRC3, PCK1, G6PC, IRS2, RIP140, NFYC, SREBP1 | Cholesterol efflux, HDL biogenesis, intracellular cholesterol transport, fatty acid oxidation, insulin signaling and glucose metabolism |
| **miR-122** | SREBP, MTTP, Klf6 | Fatty acid oxidation, cholesterol synthesis. Lipids accumulation in liver, fibrosis and tumor formation |
| **miR185/342** | SREBP | Fatty acid oxidation, cholesterol metabolism |
| **miR-370** | ABCA1; CPT1a, FASN, NR1H3, ACACA, SREBP | Cholesterol efflux, HDL biogenesis, fatty acid oxidation |
| **miR-27b** | PPARα, C/EBPα, ANGPTL3, NDST1 | Lipid metabolism, adipocyte differentiation |
| **miR-27a** | RXRα, ABCA1, FASN, SREBP1/2, PPARα/γ, ApoA1, ApoB100, ApoE | Lipid metabolism, cholesterol efflux |
| **miR-302a** **miR-758** **miR-144** **miR-106b** | ABCA1 | Cholesterol efflux |
| **miR-10b** | ABCA1; ABCG1 | |
| **mir-26** | ABCA1; ARL7 | Cholesterol efflux, HDL biogenesis |

*Cholesterol homeostasis*

Cholesterol is a key player in every stage of atherosclerosis development, and its membrane levels are mainly regulated at the transcriptional level by endoplasmic reticulum (ER)-bound sterol regulatory element-binding proteins (SREBPs) (Brown and Goldstein 1977). There are two *SREBP* genes, *SREBP1* and *SREBP2,* which encode three different transcripts. *SREBP1a* and *SREBP2* activate the transcription of cholesterol metabolism-related genes including 3-hydroxy-3-methylglutaryl coenzyme A reductase (*HMGCoAR*) and the low-density lipoprotein receptor (*LDL-R*), while *SREBP1c* enhances the expression of fatty acid metabolism-related genes such as fatty acid synthase (*FAS*) (Kim *et al.* 1995). Of the miRNAs that target the HDL/reverse cholesterol transport pathway, miR-33a and -33b are intronic miRNAs that are co-expressed with their host genes *SREBF1* and *SREBF2*, which code for transcription factors that regulate cholesterol and *FAS* (Feinberg and Moore 2016). On the other hand, miR-33a and -33b target genes involved in cholesterol reflux, ATP-binding cassettes (*ABCA1* and *ABCG1*), and fatty acid oxidation (*HADHB, CROT, CPT1a* and *AMPKa1*). Inhibition of miR-33 using modified antisense or locked nucleic acid oligonucleotides increases hepatic *ABCA1* expression and plasma HDL-C levels in both mice and monkeys by 40-50 % and enhanced cholesterol transport from macrophages to the plasma, liver, and feces by >80 % (Rayner *et al.* 2011).

MiR-122 is the most abundant miRNA in the liver, accounting for more than 80 % of the total miRNA content in this organ. Inhibition of miR-122 levels in the liver results in a significant reduction of plasma cholesterol levels in mice and non-human primates (Elmen *et al.* 2008). Micro-array and gene expression analyses in animals show that inhibition of miR-122 in the liver results in the downregulation of several genes involved in cholesterol synthesis, e.g. *HMGCS1, HMGCoAR,* and squalene epoxidase. Thus, miRNAs seem to play an important role in modulating cholesterol and fatty acid metabolism (Madrigal-Matute *et al.* 2013, Zampetaki *et al.* 2013, Menghini *et al.* 2014) (Table 3).

*Plaque development*

Lipid uptake and inflammatory responses in monocytes/macrophages are regulated by miRNAs such as miR-155 and -125a-5p (Huang *et al.* 2010, Chen *et al.* 2009). As a result, neointimal accumulation of foam cells and fatty streaks can be reduced, which is a main determinant of plaque development and instability. Vascular differentiation and apoptosis are regulated by transforming growth factor-β, which is a known target of miR-26a (Leeper *et al.* 2011). Metalloproteinase (MMP) expression, such as *MMP2/9*, controls vascular smooth muscle cell (VSMCs) proliferation. MiR-29b is upregulated in VSMCs treated with ox-LDLs and targets *MMP2*, thereby reducing VSMC migration (Chen *et al.* 2011). The vascular wall surrounding foam cells and extracellular lipid droplets (lipid core) is characterized by a switch from a contractile to a secretory phenotype in VSMCs. This phenotype switch may be modulated by miR-145, which favors a contractile phenotype, through the upregulation of *KLF4* and myocardin (Lovren *et al.* 2012). The fibroatheroma evolves into a more complicated plaque, often characterized by calcification that is regulated in VSMCs by miR-125b through targeting osteoblast transcription factor SP7 (Goettsch *et al.* 2011).

*Neoangiogenesis*

Normal vessels are nurtured through oxygen diffusion from the lumen of the adventitial *vasa vasorum*. But as the intimal wall thickens, the oxygen-effective diffusion distance becomes impaired and the *vasa vasorum* proliferates in the inner layers of the vessel wall. Neovessel formation is regulated by miR-222/221 and -155, which target endothelial nitric oxide synthase (Sun HX *et al.* 2012), and by miR-222, which silences the signal transducer and activator of transcription 5A (*STAT5A*) (Dentelli *et al.* 2010). Another interesting miRNA that regulates angiogenesis is the miRNA cluster miR-17-92 (Table 4). MiR-17-92 expression is regulated by vascular endothelial growth factor and targets thrombospondin-1, an antiangiogenic molecule (Suarez *et al.* 2008).

*Plaque rupture*

Neoangiogenesis and intraplaque hemorrhage drive blood components to the atherosclerotic lesion, promoting plaque vulnerability due to the enrichment of pro-inflammatory, pro-oxidant, and proteolytic factors. This pro-inflammatory scenario may be promoted by miR-146a, which drives peripheral blood mononuclear cells (PBMCs) towards a Th1 response (Guo *et al.* 2010). In this regard, miR-29 inhibits the expression of *Col3A1* and elastin (*ELN*), thus reducing vascular integrity. Interestingly, miR-29 expression has been shown to be upregulated in aortic aneuryms from fibulin-4 knockout and fibrillin transgenic mice (Li *et al.* 2009). MiR-365

can function as a pro-thrombotic factor by stimulating endothelial apoptosis, whereas miR-21 protects VSMCs from $H_2O_2$-induced apoptosis (Lin *et al.* 2009). MiR-221 and -222 have opposite effects in VSMCs and ECs, protecting from apoptosis or promoting cell death, respectively (Liu X *et al.* 2012).

**Table 4.** MiRNAs participated in vascular remodeling.

| miRNA | Targets | Function | |
|---|---|---|---|
| miR-155 | Bcl6, HBP1, AT1R; Ets-1; IL13 receptor α1 | Regulation of angiogenesis and inflammation | *Plaque development* |
| miR-125a-5p | LOX-1; CD68; SRB1, ORP9 | Lipid uptake, cytokine expression, oxLDL internalization | |
| miR-29b | DNTMP3b | Regulation of oxLDL mediated human aortic SMCs | |
| miR-143/145 | Klf4, myocardin, Elk-1 | Promote differentiation and repress proliferation of SMCs | |
| miR-125b | IRF4 | Inflammatory response of macrophages | |
| miR-26a | SMAD-1 | Protecting VSMCs from differentiation and apoptosis | |
| miR-222/221 | Ets-1 | Endothelial apoptosis regulation | *Neoangiogenesis* |
| miR-155 | AT1R; Ets-1 | Protective role in development of endothelial inflammation | |
| miR-17-92 | ABCA1; LDLR; SEMA6A | Cholesterol efflux; promoting of angiogenesis by regulation of ECs repulsion | |
| miR-27a/b | ABCA1; SEMA6A | | |
| miR-146a/b | TLR4; IRAK1; TRAF6 | Lipid accumulation, inflammatory response | *Plaque rupture* |
| miR-29a | LPL | Lipid uptake and inflammatory cytokine secretion | |
| miR-21 | FABP7, PPARα, PTEN, Bcl-2, PDCD4 | Depression of AP-1, inflammatory response, proliferation of SMCs, senescence | |
| miR-221/222 | c-kit, p27, p57 | Proliferation of SMCs, contractile gene transcription | |

## Circulating miRNAs as a biomarker of heart allograft rejection

Circulating organ/tissue-specific-enriched miRNAs may indicate direct organ/tissue injury or cell death, and circulating immune-associated miRNAs may serve as sensors of the immune state. Various cellular and immunological mechanisms regulate the process of organ rejection, and emerging evidence indicates that miRNAs play a vital role in these processes (Sarma *et al.* 2012).

Other studies of heart transplant models demonstrate the differential regulation of miRNAs compared to controls (Wei *et al.* 2012). MiRNAs have been shown to play a key role in T- and B-cell-mediated rejection and regulatory T-cell activity (Harris *et al.*

2010, Shan *et al.* 2011, Feng *et al.* 2013). Intracellular communication between antigen-presenting cells (APC) such as dendritic cells (DC), macrophages, and B- and T-cells is likely modulated by miRNAs. The differential expression of miR-155 and -221 has been detected as playing an important role in the differentiation of monocytes into immature DC and mature DC at various stages (Martinez-Nunez *et al.* 2009, Lu *et al.* 2011).

In a study by Sukma Dewi *et al.* (2013), significant changes were found in six circulating miRNAs (miR-326, -142-3p, -101, -144, -27, and -424) before, during, and after rejection. All six miRNAs are expressed by immune cells and three of them (miR-326, -142-3p, and -101) have crucial functions in the regulation and maintenance of self-tolerance. A study by Wang *et al.*

(2013) found dynamic change in three circulating cardio- or skeletal muscle-specific miRNAs (miR-33a/b, and -208a), which may reflect early myocardial injury after heart transplantation in humans. Necrosis and apoptosis of cardiomyocytes after cold ischemia may lead to the release of miRNAs into the blood. Recently, another study identified miRNAs (miR-10a, -31, -92a, and -155) that were differentially expressed for normal and rejecting heart allografts, and also showed that differential serological expression strongly correlated with tissular expression. These circulating miRNAs strongly discriminate patients with allograft rejection from patients without rejection (Duong van Huyen *et al.* 2014).

## Polymorphisms and miRNA

The miRNA regulome is defined as the compendium of regulatory elements that either regulate miRNA expression (transcriptional control elements and pre-miRNAs) or are regulated by miRNA activity (RNA target sites). Genetic variation in the miRNA regulome can perturb miRNA expression and/or function, potentially contributing to disease development and a wide variety of lipid-related phenotypes (Sethupathy 2013, Obsteter *et al.* 2015). Advancements in the miRNA field indicate the clear involvement of miRNAs and genetic variations within the miRNA pathway in the progression and prognosis of many diseases, including atherosclerotic cardiovascular diseases. Polymorphisms that may potentially affect miRNA-mediated regulation of cells can be present not only in the 3´UTR of a miRNA target gene, but also in the genes involved in miRNA biogenesis and in pri-, pre-, and mature-miRNA sequences. A polymorphism in processed miRNAs may affect expression of several genes and have serious consequences, whereas a polymorphism in the miRNA target site may be more target- and/or pathway-specific (Mishra and Bertino 2009). Additionally, polymorphisms within miRNA regulatory regions (variations which affect the ability of transcription factors to bind to DNA) have been shown to influence miRNA expression (Luo *et al.* 2011).

A single nucleotide polymorphism (SNP) rs2910164 in the precursor of miR-146a, a negative regulator of inflammation, has been found to be associated with risk of CAD and ischemic stroke (IS) (Ramkaran *et al.* 2014, Xiong *et al.* 2014, Huang *et al.* 2015). Bao *et al.* (2015) subsequently verified these relationships in a meta-analysis. Another study analyzed polymorphisms in pre-miRNA sequences (miR-146aC>G (rs2910164), miR-149T>C (rs2292832), miR-196a2T>C (rs11614913) and miR-499A>G (rs3746444)) and found the miR-146aC>G polymorphism and miR-146aG/-149T/-196a2C/-499G allele combination to be significantly associated with IS prevalence (Jeon *et al*. 2013).

**Table 5.** DNA polymorphisms within miRNAs and gene target sequences associated with different CVD.

| miRNA | SNPs within miRNA gene | Target gene | Biological function | Disease |
|---|---|---|---|---|
| miR-146a | rs2910164 | *IRAK1; TRAF6* | Negative regulator of inflammation | CAD; IS |
| miR-150 | rs7305605 | *MAPK9, IRAK2* | Inflammation and apoptosis pathway | IS |
| miR-200b | rs7549819 | *PRKAR2B* | Platelet biogenesis and activation | IS |
| miR-4513 | rs2168518 | *GOSR2* | Transport of angiontensin, insulin, leptin between Golgi compartments | CAD |
| niR-499 | rs3746444 | *SOX6* | Affect cardiomyocyte apoptosis *via* inhibiting mitochondrial apoptosis pathway | CAD |
| **miRNA** | **SNPs within 3´UTRs** | **Target gene** | **Biological function** | **Disease** |
| miR-224 | rs12190287 | *TCF21* | Regulation of coronary artery SMC and cardiac fibroblast | CAD |
| miR-155 | rs5186 | *AGTR1* | Vasoconstriction; cardiac hypertrophy and remodeling, heart failure, vascular thickening, and atherosclerosis | Hypertension |
| miR-616 | rs3735590 | *PON1* | Inhibits oxidation in LDL and HDL | IS |
| miR-149 | rs4846049 | *MTHFR* | Role in folate and homocysteine metabolism | CAD |

The CAD-associated polymorphism rs12190287, which resides in the 3'-UTR of *TCF21* (a regulator of the formation of coronary artery smooth muscle cells and cardiac fibroblasts), is predicted to alter the seed-binding sequence for miR-224 (Miller *et al.* 2014). MiR-155 differentially interacts with its polymorphism (rs5186) within the 3' UTR of the human *AGTR1* gene. MiR-155 downregulates the expression of the 1166A allele only, but not the 1166C allele, which is associated with hypertension. Rs3735590 C/T within the miR-616 target gene *PON1* has been found to be associated with reduced risk of CAD incidence, and carrying C alleles are an independent risk factor for CAD (Zhang *et al.* 2016). DNA polymorphisms within miRNA genes and gene target sequences associated with different CVDs are summarized in Table 5.

It has recently been determined that the expression of certain miRNAs is regulated by DNA methylation, which indicates that several layers of epigenetic mechanisms are involved in the regulation of gene expression (Saito and Jones 2006, Lehmann *et al.* 2008, Zhu and Fan 2011).

## MiRNA databases

Accurately identifying and cataloguing miRNA targets are crucial in understanding their function. To this end, numerous wet-lab methodologies have been developed, enabling the validation of predicted miRNA interactions or the high-throughput screening and identification of novel miRNA targets (Thomson *et al.* 2011). Currently available methodologies provide important research tools and are often used complementarily in investigative studies. The most prominent database is miRBase (http://www.mirbase.org/), which was set up to provide official nomenclatures for the miRNA research community. However, it contains limited information on miRNA functional annotations. A few other databases have been developed to focus more on miRNA function. For example, TargetScan (http://www.targetscan.org/vert_71) and PicTar (http://www.pictar.org) host miRNA targets predicted by different computational algorithms (Wang 2008).

DIANA-TarBase v7.0 (http://www.microrna.gr/tarbase) is the first database that aims to provide hundreds of thousands of high-quality manually curated experimentally validated miRNA:gene interactions, enhanced with detailed meta-data (Vlachos *et al.* 2014).

In the miRDB database, miRNA functional annotations are presented with a primary focus on mature miRNAs, which are the functional carriers of miRNA-mediated gene expression regulation. In addition, a wiki-editing interface has been established to allow anyone with internet access to make contributions on miRNA functional annotation (Wang 2008). All data stored in miRDB are freely accessible at http://mirdb.org.

MiRandola is the first comprehensive database of extracellular circulating miRNAs. The database is a useful reference tool for anyone investigating the role of extracellular miRNAs as non-invasive biomarkers and their physiological function and involvement in disease (Russo *et al.* 2012). The current version of miRandola focuses on human circulating miRNAs. MiRandola is available online at http://mirandola.iit.cnr.it/index.php.

## Factors interfering with analysis of circulating miRNAs

### Pre-profiling effects

Variability in the results of studies identifying miRNA biomarkers for the same disease may be attributed to pre-profiling sources, such as sample collection methods, patient lifestyle, and drug treatment. Analytical differences, such as the isolation methods of miRNAs, quantification platforms, and normalization methods, may also contribute to the variability of results (Zampetaki *et al.* 2012, MacLellan *et al.* 2014).

### Hemolysis

Some studies have previously described that the majority of miRNAs found in serum or plasma are also present in blood cells. Hemolysis caused during sample collection or processing may affect the miRNA profile of the sample. Due to their presence in blood cells, the most affected miRNAs are miR-451a, -16, -24, -15, and -223 (Blondal *et al.* 2012, Kirschner *et al.* 2013).

### Lifestyle

MiRNA expression in healthy subjects is altered as a result of fasting, dietary lipid intake, smoking, or regular exercise (Kroh *et al.* 2010).

### Obesity

Only a few studies have investigated circulating miRNA levels as potential biomarkers for obesity in humans. Circulating miRNAs are commonly deregulated in severe obesity. Specific circulating miRNAs are significantly regulated in pre-pubertal obesity, including

decreased miR-221 and -28-3p and increased concentrations of miR-486-5p, -486-3p, -142-3p, -130b, and -423-5p detected in plasma. The circulating concentrations of these miRNAs are significantly associated with body mass index and other measures of obesity (Prats-Puig *et al.* 2013). Elevated circulating miR-122, a liver-specific miRNA involved in cholesterol homeostasis, is positively associated with obesity and insulin resistance in young adults (Wang *et al.* 2015). A significant increase of miR-140-5p, -142-3p, and -222 and decreased levels of miR-532-5p, -125b, -130b, -221, -15a, -423-5p, and -520c-3p in morbidly obese patients have also been found. Weight loss-induced changes in this profile and in one study of *in silico* targets support this observation and suggest a potential mechanistic relevance (Ortega *et al.* 2013).

*Smoking*

Studies focusing on changes in the miRNA landscape in humans and animals exposed to cigarette smoke are fairly new and, therefore, *in vivo* studies are limited. Plasma levels of miR-223 and -126 affected by tobacco smoke exposure have been reported (Takahashi *et al.* 2013, Badrnya *et al.* 2014).

*Physical activity*

A few studies have shown that increased physical activity is associated with altered levels of circulating miRNAs. Altered expression of miRNAs, not only muscle-enriched miRNAs (miR-1 and -133a/b) but also liver-specific miRNAs (miR-143 and -145), has been shown to be dynamically regulated in response to both acute and chronic exercise interventions (Baggish *et al.* 2011). One study demonstrated that low maximal aerobic oxygen consumption is associated with high expression levels of three different miRNAs (miR-21, -210, and -222) (Bye *et al.* 2013). Most miRNAs altered due to exercise are involved in lipid metabolism.

*Comparing serum and plasma miRNA levels*

Although similar levels of miRNAs in serum and plasma have been previously reported (Blondal *et al.* 2013), inconsistencies between miRNA levels in both serum and plasma have also been found (Huang *et al.* 2013). It has been notably observed that thrombocyte-associated miRNAs in particular are found in much higher concentrations in plasma than in serum. Since thrombocyte-derived miRNAs such as miR-126, -223, and -197 seem to play a significant role in the prognosis

of future cardiovascular events (especially in the field of miRNA-based diagnostics of CAD), standardized methods are needed for future clinical miRNA quantification studies (Schulte and Zeller 2015).

*Biological aspects*

Various miRNAs are known to exert key roles in ageing, and other miRNAs are encoded on sex chromosomes (Boon *et al.* 2013). These facts suggest that a relevant portion of human miRNA profiles depends on the age and sex distribution of samples. In one study, the miRNAs most strongly affected by age were identified as miR-1284, -93-3p, -1262, -34a, and -145, while the miRNAs most influenced by sex were miR-219a, -548c, and -130a (Meder *et al.* 2014).

*Drug treatment*

Heparin, commonly used as an anticoagulant in cardiovascular diagnostics and interventions, inhibits RNA quantification by interfering with the DNA polymerases used in the quantitative PCR reaction. Moreover, heparin has also been described as strongly interfering with the detection of recombinant cel-miR-39 when spiked into samples for normalization purposes (Boeckel *et al.* 2012, Kim *et al.* 2012). Cel-miR-39 may be used for normalization to counteract the possible unfavorable influence of heparin treatment, which leads to distorted miRNA analysis results. More accurate results are produced when using a form of normalization that takes the mean of at least three circulating miRNAs stably expressed in plasma.

Quantification of miRNA levels altered in CAD may also be influenced by the intake of medications such as statins and ACE inhibitors (Fichtlscherer *et al.* 2010, Weber *et al.* 2011). Antiplatelet aspirin treatment has been shown to influence plasma miR-126 in subjects with T2DM (de Boer *et al.* 2013). MiR-221/222 expression was downregulated and a number of endothelial progenitor cells increased in one group of CAD patients after one year under statin therapy (Minami *et al.* 2009). In another pilot study, statins used for lipid-lowering therapy decreased inflammation-associated miRNAs (miR-106b, -21, -25, -451, and -92a) in patients with unstable angina (Zhang *et al.* 2015).

## Conclusions

The emergence of miRNAs as key regulators of cholesterol metabolism and potentially also vascular

function has attracted much interest over the years, not only from a physiological standpoint but also from a clinical perspective, as many miRNAs have been shown to be biomarkers of dyslipidemia and regulators of the mechanisms leading to atherosclerotic CVD. Ascertaining the relative contribution of each non-coding RNA in governing the initiation and development of the atherosclerotic process may shed further light on the influence of miRNAs on target-gene regulation and help to evaluate the therapeutic potential of manipulating miRNA expression in the treatment of cardiometabolic disorders. As miRNAs may represent therapeutic targets in atherosclerosis, it is therefore of utmost importance to study the mechanisms (e.g. delivery, target, and recognition machinery) that sort miRNA into exosomes. Clearly, the methods of collection and preparation used can significantly influence results and must be taken into account when evaluating miRNAs as circulating biomarkers of pathological processes.

## Conflict of Interest
There is no conflict of interest.

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