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MicroRNAs in peripheral artery disease: potential biomarkers and pathophysiological mechanisms

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***Abstract*:** Peripheral artery disease (PAD) is a disease of atherosclerosis in the lower extremities. PAD carries a massive burden worldwide, while diagnosis and treatment options are often lacking. One of the key points of research in recent years is the involvement of microRNAs (miRNAs), which are short 20-25 nucleotide single-stranded RNAs that can act as negative regulators of post-transcriptional gene expression. Many of these miRNAs have been discovered to be misregulated in PAD patients, suggesting a potential utility as biomarkers for PAD diagnosis. miRNAs have also been shown to play an important role in many different pathophysiological aspects involved in the initiation and progression of the disease including angiogenesis, hypoxia, inflammation, as well as other cellular functions like cell proliferation and migration. The research on miRNAs in PAD has the potential to lead to a whole new class of diagnostic tools and treatments.

Keywords: angiogenesis, biomarkers, critical limb ischemia, hypoxia, intermittent claudication, non-coding RNAs

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Introduction

Peripheral artery disease (PAD) refers to systematic atherosclerosis of arteries supplying the legs.[1] PAD is very common throughout the population, affecting at least 8.5 million people in the United States and 200 million people worldwide.[2] An individual with PAD faces an increase in cardiovascular burden along with limitations in mobility, with later stages of the disease resulting in critical limb ischemia (CLI) that may require amputation.[3] There are many different factors that play an important role in the pathophysiology associated with PAD, the first of which is altered hemodynamics. Atherosclerotic occlusions in PAD cause limited blood flow and oxygen delivery to the affected extremity. This aspect of PAD has been traditionally treated with revascularization surgery, and more recently, molecular mechanisms of angiogenesis have also been explored.[4–7]

Another well-studied mechanism believed to be linked to PAD manifestations is oxidative stress. Patients with PAD experience limb ischemia during exercise, and the temporary lack of oxygen causes a reduction in oxidative phosphorylation and an altered metabolism. Precursors of oxidative phosphorylation can accumulate, which can damage or conformationally change enzymes that can produce reactive oxygen species (ROS) upon the return of oxygen.[8] At rest, muscle is reperfused as oxygen is re-introduced; restoration of blood flow to ischemic tissue is accompanied by an increase in ROS production and oxidative stress, in a process known as ischemia/reperfusion (I/R) injury.[9] Thus, although re-oxygenation of ischemic tissue is critical, it can also be detrimental when oxygen is converted into ROS such as superoxide, a phenomenon that has been termed 'the oxygen paradox'.[10]

Increased oxidative stress in PAD is thought to result in an altered metabolomics profile and endothelial dysfunction, via reduction in NO bioavailability.[11,12] Likewise, increased oxidative

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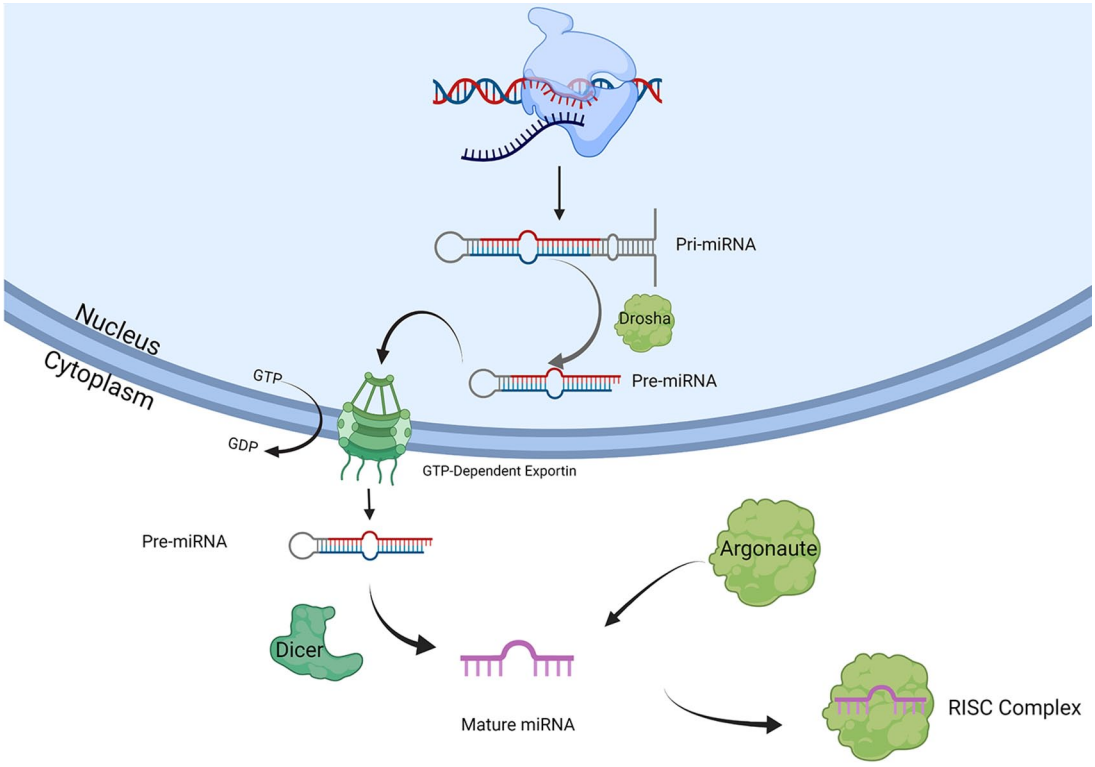

Figure 1. Summary of microRNA biogenesis. This figure summarizes the processing of miRNAs from transcription to a mature miRNA-RISC. First the pri-miRNA is transcribed and forms into a hairpin structure. RNAse III Drosha then cleaves the excess base pairs, forming a ~70 base pair stem-loop structure. Following cleavage, the GTP-dependent Exportin 5 transporter exports the pre-miRNA from the nucleus. Outside the nucleus, RNase III Dicer further cleaves the loop structure, separating the 2 strands of RNA, which leaves a 20-25 nucleotide single-stranded mature miRNA. Argonaute and other binding proteins are incorporated, forming the final RNA-inducing silencing complexes (RISC).

damage to ischemic muscles results in atrophy and degeneration of myofibers.[11,13–15] Chronic oxidative stress in the peripheral muscles may also induce fibrosis through the activation of transforming growth factor beta (TGF-β).[16–18] Finally, increased ROS production may result in mitochondrial dysfunction. Notably, decreased activity of electron transport chain complexes I, III, and IV has been demonstrated in the gastrocnemius muscle of PAD patients in association with elevated oxidative stress.[19] Each of these mechanisms contributes to the development and progression of PAD. Interestingly, in all of these mechanisms, miRNA's have been implicated to play an important role.[20,21]

MicroRNAs (miRNA) were discovered in 1993 and have had a substantial impact on the study of epigenetics ever since.[22] miRNAs consist of 20-25 nucleotides of single-stranded RNA that are highly conserved across many eukaryotic genomes. Their main function is to bind to and inhibit mRNAs, reducing their expression. In the first step of miRNA action, Pri-miRNA is transcribed and forms into a hairpin structure(Figure 1).[23] RNase III Drosha cleaves the excess base pairs so that it forms an approximately 70 base pair stem-loop structure.[23,24] After it has been cleaved, the pre-miRNA is exported from the nucleus by the guanosine triphosphate (GTP)-dependent Exportin 5 transporter.[25,26] Once outside the nucleus, the Pre-miRNA's 2 nucleotide overhang is recognized by RNase III Dicer, which cleaves off the loop structure and separates the 2 strands of RNA, leaving a 20-25 nucleotide single-stranded mature miRNA.[24,26–28] The last step of miRNA processing is the incorporation of Argonaute and other RNA binding proteins to form the RNA-induced silencing complexes (RISC)[29,30] (Figure 2). The RISC will typically

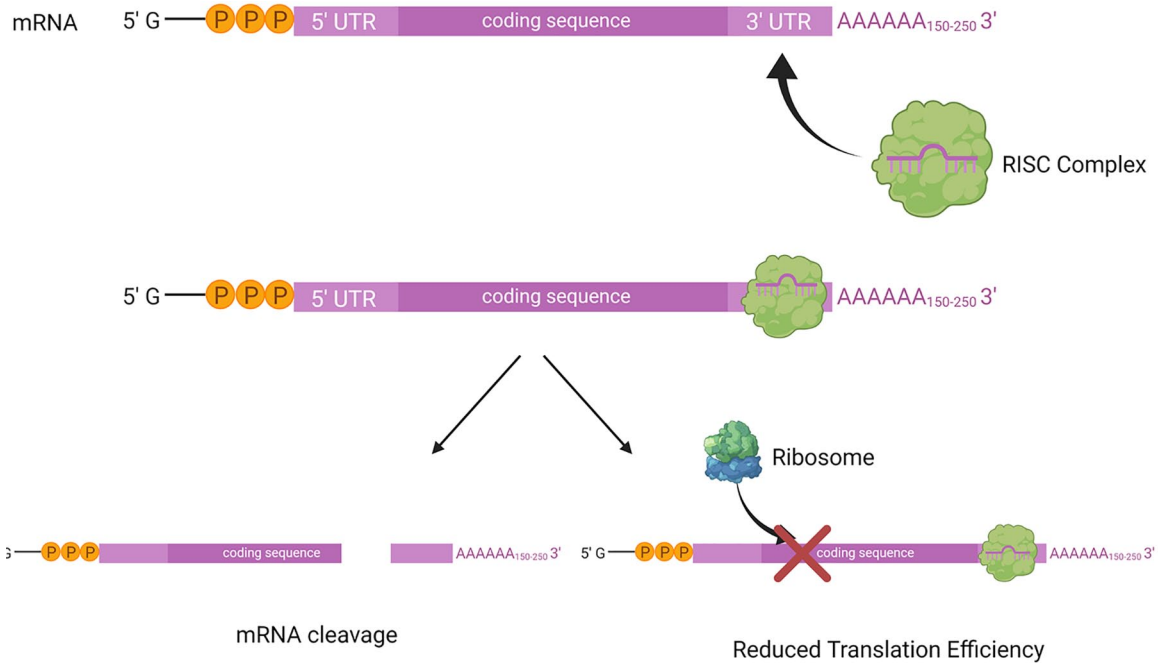

Figure 2. Summary of microRNA action. This figure summarizes the mechanisms of action of miRNA-RISC acting on mRNAs to downregulate their expression. The complex bind to the 5′ UTR of mRNAs and either cleaves them or reduces their translational efficiency or stability.
RISC, RNA-inducing silencing complexes.

attempt to bind to the complimentary sequences of the miRNA in the 3' UTR of mRNAs; however, there is preliminary evidence this can also happen at the 5' UTR.[31] This usually results in either the cleavage of the mRNA, destabilization of the mRNA, or reduced translational efficiency, all of which led to reduced expression of the mRNA's product[24,32] (Figure 2).

Because of their ability to affect a wide range of mRNA targets, miRNAs are highly regulated from transcription through maturation. MiRNAs are mostly transcribed by RNA polymerase II.[33] As such, they are subject to the same processes as other RNAs transcribed by RNA polymerase II such as polyadenylation of the 3′ end and 7-methyl-guanosine capping of the 5′ end.[33] There are also several transcription factors that have been shown to regulate the expression of miRNAs.[34] After translation, there are a number of co-factors that are associated with miRNA processing. Cleavage by Drosha requires the co-factor DiGeorge critical region 8, also known as Pasha.[35] Cleavage by Dicer also has co-factors involved that can be specific to the miRNA being cleaved. The regulation of these co-factors is another way that the expression of miRNAs can be regulated.

Diagnosis of PAD

Despite recent advances, PAD continues to be an underdiagnosed and undertreated condition.[4] Recent studies have explored the possibility of using miRNAs as biomarkers for PAD.[36–38] A biomarker can be defined as a molecule that can indicate the physiological state of a biological system, for example, by indicating the presence or severity of a specific disease state.[39] To be correctly considered a biomarker, a molecule must reliably and reproducibly predict clinical endpoints.[39] Using biomarkers could lead to the development of less-invasive tests that are more sensitive and selective. The search for miRNA biomarkers in PAD is influenced by the sampling location as well as the stratification or outcome measures (Table 1). In addition, comorbidities may also influence biomarker identification. For example, many of the studies in PAD also focused on diabetic patients specifically because they are at an especially high risk for the disease.[40]

Table 1. MicroRNAs in atherosclerosis and PAD diagnosis.

miRNA	Location	Condition	Reference
miR-3909	Blood plasma	PAD	Jain *et al.*[41]
miR-483-5p	Blood plasma	PAD	Jain *et al.*[41]
miR-29a-3p	Blood plasma	Repetitive Exhaustive *versus* Nonexhaustive Exercise	Håkansson *et al.*[42]
miR-193a-5p	Blood plasma	Repetitive Exhaustive *versus* Nonexhaustive Exercise	Håkansson *et al.*[42]
miR-210	Blood serum	Atherosclerosis obliterans	Li *et al.*[43]
		PAD	Signorelli *et al.*[36]
miR-130a	Blood serum	Stage I *versus* Stage II/III atherosclerosis obliterans	Li *et al.*[43]
		PAD	Signorelli *et al.*[36]
miR-27b	Blood serum	Stage I/II *versus* Stage III atherosclerosis obliterans	Li *et al.*[43]
		PAD	Signorelli *et al.*[36]
miR-1827	Blood plasma	Chronic limb-threatening ischemia	Syed *et al.*[44]
miR-320a	Blood plasma	In-stent restenosis	Yuan *et al.*[45]
miR-142-5p	Blood serum	In-stent restenosis	Pan *et al.*[46]
miR-142	Blood plasma	Postsurgery cardiovascular events	Barbalata *et al.*[47]
miR-323b-5p	Blood plasma	Critical limb ischemia in type 2 diabetes mellitus patients	Cheng *et al.*[48]
miR-4739	Blood plasma	Critical limb ischemia in type 2 diabetes mellitus patients	Li *et al.*[49]
miR-654-5p	Blood plasma	Atherosclerosis	Han *et al.*[50]
miR-409-3p	Blood plasma	Atherosclerosis	Han *et al.*[50]
miR-124-3p	Whole blood	PAD	Shi *et al.*[51]

PAD, peripheral artery disease.

In a recent study of patients with atherosclerosis, miR-654-5p and miR-409-3p were identified as potential biomarkers, and logistic regression models based on these 2 miRNAs distinguished atherosclerotic patients from controls.[50] Furthermore, in a study of arthrosclerosis obliterans, miR-21, miR-130a, miR-27b, let-7f, and miR-210 were all significantly increased, while miR-221 and miR-222 were significantly decreased in the intima. In serum samples, miR-21, miR-130a, miR-27b, and miR-210 expression were increased in arteriosclerosis obliterans relative to controls. Furthermore, miR-130a and miR-27b levels were significantly higher in stage III of the disease than in earlier stages.[43]

Other miRNAs that have been found to be misregulated in PAD are miR-27b, miR-130a, and miR-210. These miRNAs were shown to be highly expressed in patients with PAD compared with controls.[36] miR-124-3p was also identified as being significantly increased in PAD patients compared with controls.[51] Another group looked to identify miRNAs in the blood that were differentially expressed in athletes after exercise and compared with PAD patients. Interestingly,

miR-29-a-3p, and miR-495-3p were significantly different between athletes and PAD patients, only after exercise. This suggests that these miRNAs may be potential biomarkers of decreased muscle repair and recovery,[42] as decreased muscle repair and recovery after exercise can be signs of muscle dysfunction associated with PAD.

Other groups looked to diagnose specific subsets of PAD like critical limb ischemia. For example, miR-1827 expression was shown to be increased in patients with CLI compared with non-PAD controls.[44] In another study, miR-3909 and miR-483-5p were identified as potential biomarkers of CLI from the blood plasma.[41] Since critical limb ischemia disproportionally affects patients with type 2 diabetes mellitus, two biomarkers, miR-323b-5p and miR-4739, were also identified to predict critical limb ischemia in diabetic patients.[48,49]

Finally, another useful area for miRNAs is for monitoring incidents after surgical procedures. miR-320a[45] and miR-142-5p[46] have shown promise as biomarkers for in-stent restenosis, while miR-142 was identified to predict postsurgery cardiovascular events.[47] Despite the promising results of these studies, however, there still remains plenty of work to be done before the clinical application of miRNAs as biomarkers. It is important to note that there are significant limitations to many of the aforementioned studies, including small sample sizes. Future studies in large cohorts are warranted to confirm these preliminary findings.

Angiogenesis

One of the most studied areas when it relates to PAD and miRNAs is angiogenesis. Most of the studies on angiogenesis have taken place in the setting of murine hind limb ischemia. In hind limb ischemia, the femoral artery of the mouse is ligated or excised, which causes reduced blood flow to the hind limbs. This mimics the lower extremity ischemia observed in human PAD and results in a similar pathology, characterized by oxidative damage, myofiber degeneration, and mitochondrial dysfunction.[52] Because mice have the ability to generate new vessels rapidly, the hindlimb ischemia model allows for the study of angiogenesis and the factors that influence it.[53]

One miRNA that has been widely studied in hindlimb ischemia models is miR-210. miR-210 transcription is activated under low oxygen tension, which has earned its title as the 'master hypoxamir'.[54] Three studies by Zaccagnini *et al.*[55–57] investigated miR-210 in the context of acute hindlimb ischemia (HLI) (3 days) in mice, achieved by femoral artery removal. In this model, miR-210 overexpression was shown to improve angiogenesis and blood perfusion recovery and increase arteriolar and capillary density, and miR-210 blockade had the opposite effect. Other groups have confirmed these findings as well.[58,59] miR-210 has also been studied in circulating proangiogenic cell treatments. Mechanistically, miR-210 increased proangiogenic cells ability to migrate toward stromal cell-derived factor 1 (SDF-1), a cytokine shown to promote angiogenesis. miR-210 also improved the ability of the proangiogenic cells to adhere to endothelial cells, which is necessary for the sprouting of new vessels.[58]

Another common miRNA that has been studied in PAD is let-7. Let-7f has been associated with cigarette smoking, which is one of the major risk factors for PAD.[60] Exposure to cigarette smoke caused a significant decrease in let-7f levels in vascular cells. Increasing let-7f rescued the antiangiogenic effects of cigarette smoke exposure both *in vitro* and *in vivo*. Mechanistic studies suggest this occurs via let-7f-induced activation of TGF-β.[61] Let-7g has also been the subject of potential therapeutics. Under hypoxic conditions, let-7g levels are reduced, and recovery of let-7g has been shown to increase blood flow perfusion to the hind limb of mice with hind limb ischemia. Increasing let-7g also enhanced recruitment of endothelial progenitor cells, which play an important role in angiogenesis.[62]

Another miRNA that is involved in angiogenesis is miR-29a. Mice with type 1 diabetes have been shown to have higher expression levels of miR-29a along with impaired recovery after hind limb ischemia. Adding anti-miR-29a improved blood flow recovery of the diabetic mice, seemingly through increasing A Disintegrin and metalloproteinase domain-containing 12 (ADAM12) expression.[63] ADAM12 regulates angiogenesis by cleaving a tyrosine kinase receptor Tie2, which then activates proliferation pathways.[64,65] Similar effects were seen by the same group with miR-133a, which when blocked by an anti-miR, resulted in improved angiogenesis in diabetic mice. This occurred through the increased

expression of GTP Cyclohydrolase 1 (GCH1).[66] GCH1 catalyzes the biosynthesis of tetrahydrobiopterin, a critical co-factor of endothelial nitric oxide synthase.[66,67]

miR-124-3p has been shown to be increased in hind limb ischemic mice as well as hypoxia-exposed Human umbilical vein endothelial cells (HUVECs).[51] Bioinformatic analysis indicated that Signal transducer and activator of transcription 3 (STAT3) was a potential target of miR-124-3p, and this was confirmed by protein expression analysis. Accumulating evidence suggest that STAT3 is a critical regulator of angiogenesis, largely via the induction of genes such as vascular endothelial growth factor (VEGF).[51] Cells with higher amounts of miR- 124-3p showed lower levels of STAT3, which recovered when an anti-miR was introduced. Interestingly, cells expressing miR- 124-3p demonstrated a similar phenotype as cells treated with a siRNA against STAT3. *In vivo*, miR-124-3p levels also impacted perfusion of hind limb ischemic mice, with high levels of the miRNA associated with decreased perfusion recovery.[51]

miR-93 has been identified as another miRNA that is upregulated in the serum of patients with PAD.[68] It was also observed to increase throughout the progression of the disease as well. Cyclin-dependent kinase inhibitor 1 (CDKN1A), an important regulator of the cell cycle, was uncovered as a potential target of miR-93, and this was confirmed by its downregulation in EA.hy926 cells expressing high levels of miR-93. Increasing the expression of miR-93 in these cells also increased proliferation and migration. Finally, increasing miR-93 in a hind limb ischemia mouse model improved perfusion recovery.[68,69]

It is important to note that the physiological effects of modulating miRNAs in PAD conditions remains unclear. For example, while miR-155 targets eNOS, and potentially VEGF (which would suggest antiangiogenic effects), inhibition of miR-155 actually attenuates blood flow recovery after hind limb ischemia.[70] This highlights the importance of considering the multitude of effects a miRNA can elicit.

Surgical revascularization by endovascular stent insertion is a common vascular technique to treat PAD; however, one major problem is the occurrence of in-stent restenosis, the narrowing of the artery even after a stent has been placed. One of the miRNAs that may be involved in the process is miR-140-3p. Specifically, miR-140-3p was shown to be decreased in the arterial smooth muscle cells of restenotic arteries. Overexpression of miR-140-3p in arterial smooth muscle cells inhibits proliferation and increases apoptosis by targeting C-Myb, a transcription factor that promotes cell proliferation,[71] and BCL-2 B-cell lymphoma-2 (BCL-2), which plays a role in apoptosis.[72] Increased arterial smooth muscle cell proliferation is known to be involved in-stent restenosis, so miR-140-3p could potentially play a role in this process.[73] Similarly, miR-125a may also affect restenosis, as miR-125a expression is also decreased in the smooth muscle cells of restenotic artery walls. Likewise, miR-125a overexpression *in vitro* has also been shown to reduce smooth muscle cell proliferation.[74]

One of the issues that stands between miRNA research and potential treatments of PAD is related to the delivery of miRNAs. One possible solution is the use of nanoparticles. Tsumara *et al.* used poly lactic-co-glycolic acid nanoparticles to deliver miR-126 to mice post- hind limb ischemia surgery. Their results showed an increase in perfusion to the hind limb with the miR-126 nanoparticles compared with nanoparticles containing a scramble miRNA. They also observed an increase in capillary and arteriolar density in these mice.[75] These findings suggest that nanoparticle-delivered miRNAs may be a promising approach to therapy. However, as most of the data available are from rodent models of PAD, it is important to note that the homology, function, or targets of the specific miRNAs studied may not be conserved from murine species to humans. Thus, systemic evaluation of human tissue is critical for identifying miRNAs that may be relevant for human PAD.

Hypoxia and reactive oxygen species

Several miRNAs have been implicated in cellular ability to deal with ROS during hypoxic conditions. For example, miR-138 has been shown to significantly increase in endothelial cells subjected to hypoxia. Increased miR-138 expression was also shown in muscle tissue from both CLI patients and mice subjected to femoral artery resection.[76] Bioinformatic analysis indicated that miR-138 could potentially bind to the 3′UTR of S100A1, which is a Ca^{2+} sensor essential for nitric oxide synthase activation. Subsequent tests supported the claim that miR-138 acts through

the S100A1-3'UTR.[76] Other miRNAs identified related to antioxidant functions are miR-130a and miR-27b. Those miRNAs have both been shown to be increased in the blood of PAD patients, and bioinformatic analysis has linked both to oxidative stress pathways.[36]

Another miRNA that has been studied in relation to ROS is miR-210. Under hypoxic conditions, endothelial cells expressing increased miR-210 had higher cell viability. When blocked with an anti-miR-210, endothelial cells had a lower cell viability and an increase in ROS.[77] Knocking down miR-210 in mouse skeletal muscles increased apoptosis at 1 day and necrosis at 3 days. Transcriptome analysis of these muscles showed a deregulation of mitochondrial function and oxidative metabolism genes.[57] These data suggest miR-210 can be a potential therapeutic target of patients with PAD and warrant further clinical investigation.

Mitochondrial function

Several miRNAs are known to regulate mitochondrial function. For example, by targeting cytochrome c oxidase (COX) subunits, a key protein that comprises Complex IV of the electron transport chain, miR-338, miR-210, miR-130a, and miR-181c can reduce mitochondrial respiration.[78,79] Furthermore, miR-210 also targets mitochondrial iron sulfur cluster homologue (ISCU), which provides another mechanism for repressed mitochondrial respiration.[80]

Inflammation

Inflammation plays an important role in the development of PAD. One of the current focuses of inflammation in PAD is exosomes, which are excreted vesicles containing a number of different components, including miRNAs. In exosomes isolated from plasma, miR-21, 92a, 126, 143, 181b, and 221 were elevated in patients with severe PAD compared with mild PAD and control patients. Bioinformatic analysis showed that several of these miRNAs promote proinflammatory pathways. Furthermore, treatment of vascular smooth muscle cells with circulating exosomes from PAD patients resulted in enhanced migration.[81]

Exosomes secreted by macrophages were also studied in the context of smoking, which is a major risk factor for PAD. Nicotine was shown to induce macrophages to secrete exosomes. Injection of mice with the macrophage-secreted exosomes caused an increase in atherosclerotic plaque. Analysis of the exosomes identified miR-21-3p as the potential cause of the atherosclerotic buildup. When cocultured, macrophages secreting exosomes with miR-21-3p promoted vascular smooth muscle cell migration and proliferation. Computer models predict that miR-21-3p binds to and downregulates Phosphatase and tensin homolog (PTEN) mRNA, a phosphatase known to negatively regulate cell migration. PTEN inhibition resulted in the same effects as miR-21-3p in vascular smooth muscle cells.[82]

Another miRNA, miR-93, has been shown to modulate macrophage polarization. Macrophage cells transfected with miR-93 were shown to have increased arginase1 expression, which is indicative of M2-polarization. Re-introduction of miR-93 into mice deficient in miR-106, miR-93, and miR-25 restored angiogenesis, arteriogenesis, and perfusion recovery by promoting anti-inflammatory macrophages. RNA sequencing revealed that miR-93 is likely regulating interferon regulatory factor 1 (IRF-1) expression, which can regulate macrophage polarization through the immune-responsive gene-1 (IRG-1) pathway.[83]

The expression levels of the inflammation-associated miRNAs may offer diagnostic value in the context of PAD. They may also be useful in the risk stratification of PAD patients and in the prediction of clinical outcomes, since inflammation is associated with advancing degree stage as well as increased morbidity and mortality in PAD.[84] Furthermore, miRNA-based therapies may offer new therapeutic methods to treat the inflammatory component of PAD. However, in developing these treatments, caution must be taken due to the potential risk of impaired immunity or cancer that may accompany excessive inhibition of inflammation.

Other cell functions

Another miRNA-targeted mechanism relevant to PAD is endothelial cell apoptosis. In rats with arteriosclerosis obliterans in their lower limbs, an increase in miR-126 was shown. Through modeling, it was determined that the likely binding site of miR-126 was Phosphoinositide 3-kinase (PI3K), a kinase that is upstream of the Protein Kinase B (PKB/Akt) pathway. This pathway controls many

different cell functions such as metabolism, growth, proliferation, and survival.[85] miR-126-mediated suppression of PI3K was supported by western blots showing a decrease in p-Akt (active) in rats with atherosclerosis compared with control rats. When apoptosis was induced using oxidized LDL (Ox-LDL), which is known to act by signaling via the PI3K/AKT pathway, increasing miR-126 levels reduced the levels of apoptosis close to the control.[86] Similarly, another study observed similar effects in vascular smooth muscle cells using miR-15b. It was shown that miR-15b also acts through the PI3K-Akt pathway to regulate cell proliferation and apoptosis.[87]

Other studies have suggested a pro-atherosclerotic role for miR-210 as well. For example, in a mouse model of high-fat diet induced atherosclerosis, miR-210 was shown to induce aortic endothelial cell apoptosis by targeting 3-phosphoinositide-dependent protein kinase-1 (PDK1) and suppressing the mechanistic target of rapamycin (mTOR) signaling pathway.[88] Likewise, in the rat cardiomyoblast cell line H9c2, miR-210 overexpression increased hypoxia-induced cell injury and reduced cell viability, which was shown to be mediated by SMAD pathway suppression.[89] Finally, in a mouse model of liver ischemia-reperfusion injury, miR-210 promoted hepatocyte apoptosis, and miR-210 deficiency alleviated liver injury and hepatocyte apoptosis.[90] This was also shown to be mediated by miR-210 suppression of SMAD4.[90]

Recently, there has been growing interest in studying stem cell therapies using mesenchymal stem cells (MSCs). MSCs have been shown to have therapeutic potential through paracrine secreting mechanisms. When rabbits with hind limb ischemia were injected with MSC media, there was an increase in perfusion to the hind limb, which was accompanied by an increase in vascular density. miR-126 in the media was identified as a possible mechanism for the vascular regeneration, as levels of the microRNA were increased in gastrocnemius tissue samples of the rabbits, and *in situ* hybridization technique showed miR-126 being expressed in the vascular endothelium, smooth muscle cells of the arterioles, fibrous scars, and in the sarcoplasm of some muscle fibers.[91]

miRNAs may also play a role in the process of pathological fibrosis associated with PAD via modulation of the TGF-β pathway. For example, miR-29 overexpression in myoblasts inhibits fibrogenic differentiation and suppresses collagen synthesis.[92] Notably, miR-29 has been shown to be inhibited by TGF-β/SMAD signaling. Similarly, transfection of myoblasts with miR-146 results in reduced pro-fibrotic protein expression and collagen production by targeting SMAD4.[80]

Another area of miRNA research is the balance between the expression of a miRNA and its isomiR. An isomiR is a miRNA with a single base pair difference from an already established miRNA, which usually occurs as a result of differential processing.[93] For example, in primary human vascular cells, the isoform of miR-411, which has an extra adenosine on the 5' end, is expressed more highly than the original form. Interestingly, the expression of the isomiR of miR-411 was almost 5 times higher compared the original, canonical wild-type miRNA in veins from patients with limb ischemia. In contrast, lower limb veins of patients with coronary artery disease, with no limb ischemia, demonstrated a ratio that suggested only a 2-fold higher expression of the isoform, compared with wild-type miR-411. Functionally, it was shown that human umbilical arterial fibroblast cells expressing the miR-411 isoform had decreased wound healing ability relative to control miRNA-treatment as well as wild-type miR-411 treatment. These data suggest that it may be valuable to study specific miRNA isoforms when assessing miRNA effects.[94]

Finally, the interaction between other circulating genetic elements and miRNAs in atherosclerosis is important to consider. For example, miR-328-3p has been shown to be affected by a circulating RNA called Circ_0004104. In fact, knock-down of Circ_0004104 led to an increase in miR-328-3p levels, which further resulted in a reduction of ox-LDL.[95] miR-206 is acted on similarly by CircTM7SF3, another circulating RNA. As CircTM7SF3 levels are reduced, miR-206 levels increase, which results in a reduction of ox-LDL.[96] Table 2 summarizes the miRNAs involved in the PAD-related pathophysiological mechanisms discussed.

Conclusions

The study of miRNAs in health and disease is still a relatively new area, and there is still much that needs to be learned about the roles that they play. Using miRNAs as diagnostic and/or prognostic tools in PAD is a possibility for the near future, as

Table 2. MicroRNAs with pathophysiological relevance to PAD.

miRNA	Pathophysiological relevance	Target/mechanism	Reference
miR-210	Angiogenesis	SDF-1	Besnier *et al.*[58]
let-7	Angiogenesis	TGF-β	Dhahri *et al.*[61]
miR-29a	Angiogenesis	ADAM12	Chen *et al.*[63]
miR-124-3p	Angiogenesis	STAT3-VEGF	Shi *et al.*[51]
miR-93	Angiogenesis	CDKN1A	Shu *et al.*[68]
miR-140-3p	In-Stent Restenosis	C-Myb, BCL-2	Youle and Strasser[72]
miR-138	Oxidative stress	S100A1	Sen *et al.*[76]
miR-210	Mitochondrial function	COX, ISCU	Sun *et al.*[80]
miR-338	Mitochondrial function	COX	Latronico and Condorelli;[78] Li *et al.*[79]
miR-130a	Mitochondrial function	COX	Latronico and Condorelli;[78] Li *et al.*[79]
miR-181c	Mitochondrial function	COX	Latronico and Condorelli;[78] Li *et al.*[79]
miR-21-3p	Inflammation	PTEN	Zhu *et al.*[82]
miR-93	Inflammation	IRF-1-IRG-1	Ganta *et al.*[83]
miR-126	Apoptosis	PI3K-Akt	Li *et al.*[86]
miR-15b	Apoptosis	PI3K-Akt	Sun *et al.*[87]

COX, cytochrome c oxidase; ISCU, iron sulfur cluster homologue; PTEN, Phosphatase and tensin homolog.

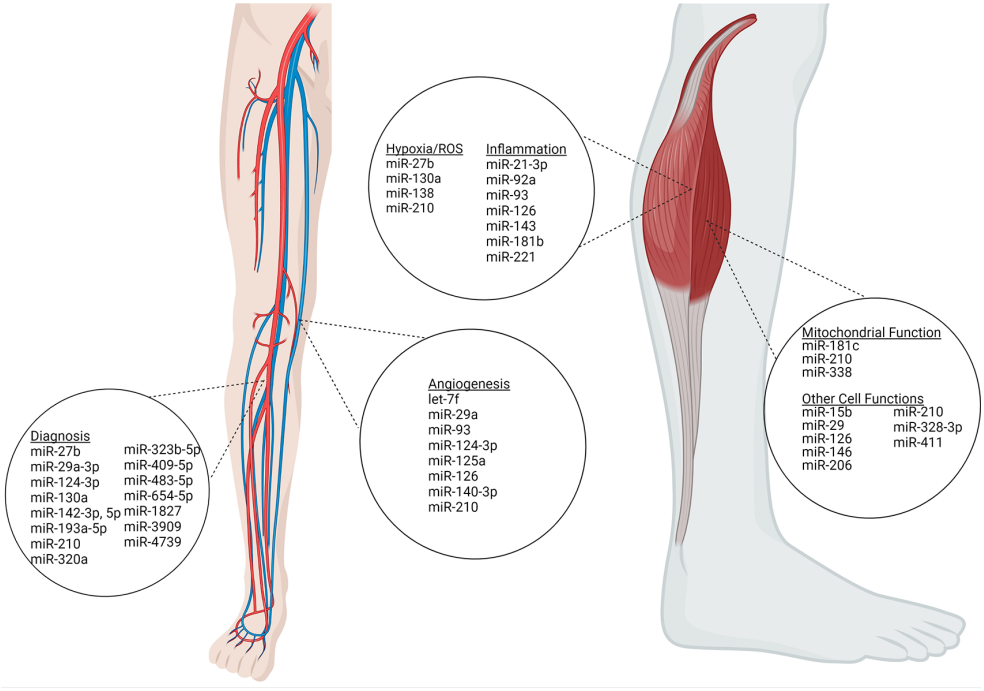

Figure 3. Summary of microRNAs in PAD. This figure summarizes the different miRNAs that have been suggested as biomarkers for PAD. In addition, other miRNAs presented have been found to play a role in different aspects of PAD pathophysiology including angiogenesis, hypoxia/reactive oxygen species, inflammation, mitochondrial function, or other cell functions.

many potential biomarkers have already been discovered and are continuing to be studied. Figure 3 summarizes the current literature on miRNA involvement in PAD (Figure 3). Future studies should validate these miRNA biomarkers in larger populations. Furthermore, as the influence of certain miRNAs in the progression of disease phenotypes and on specific pathological processes are becoming better understood miRNA-based therapies is becoming a promising possibility. However, significant gains must first be made in the general understanding of miRNA function and interactions, as well as in identifying particular miRNAs to target.

Consent for publication

All authors consent for publication

Author contribution(s)

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Ahmed Ismaeel: Conceptualization; Methodology; Writing – original draft.

Marissa Wechsler: Conceptualization; Methodology; Writing – original draft.

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