Therapeutic Potential of Modulating MicroRNA in Peripheral Artery Disease

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Abstract

Peripheral artery disease (PAD) produces significant disability attributable to lower extremity ischemia. Limited treatment modalities exist to ameliorate clinical symptoms in patients with PAD. Growing evidence links microRNAs to key processes that govern disease expression in PAD including angiogenesis, endothelial function, inflammation, vascular regeneration, vascular smooth muscle cell function, restenosis, and mitochondrial function. MicroRNAs have been identified in circulation and may serve as novel biomarkers in PAD. This article reviews the potential contribution of microRNA to key pathways of disease development in PAD that may lead to microRNA-based diagnostic and therapeutic approaches.

Keywords

biomarker; peripheral arterial disease; epigenetics

There is a rising worldwide prevalence of disability attributable to peripheral artery disease (PAD).[1,2] Lower extremity ischemia in PAD causes suffering and functional impairment. [3] Cardiovascular complications of systemic atherosclerosis are markedly increased in PAD and persist despite available treatments.[4,5] In addition, the majority of patients with PAD remain undiagnosed and undertreated.[6] New methods for detecting PAD along with an improved understanding of the mechanisms driving vascular injury are critical to develop innovative strategies for prevention and management.

Emerging evidence identifies microRNAs (miRNAs) as novel regulators of vascular biology. [7] miRNAs are small, noncoding RNAs that interact with gene transcripts to repress expression.[8] Experimental work links miRNAs to key processes relevant to PAD including inflammation, angiogenesis, endothelial function, smooth muscle cell biology and restenosis (see Figure). Interestingly, miRNAs are present in circulating blood in humans and have potential as PAD disease biomarkers.[9] Endothelial-specific miRNAs may have specific

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relevance in atherosclerotic disease. Modulation of miRNA levels represents a novel treatment approach for limb ischemia. The current review focuses on miRNA in the mechanisms of disease development in PAD that may provide opportunities for miRNA-based therapies.

Determinants of Clinical Status in PAD

Atherosclerotic PAD involves the development of obstructive lesions in the arteries of the lower extremities. Patients with PAD have even higher cardiovascular event rates than patients with established coronary artery disease (CAD) that persist with aggressive risk factor control.[4] Epidemiologic evidence indicates that the relative impact of traditional risk factors differs between PAD and CAD.[10,11] In addition, prior studies report a stronger association of selected inflammatory markers with PAD as compared to CAD.[12,13] These findings substantiate the premise that the pathophysiology of PAD has distinctions from CAD. Thus, the miRNA signature and treatment approach may be different in PAD.

The determinants of clinical status and prognosis in PAD are complex. Classically, lower extremity symptoms have been attributed to fixed obstruction to flow. However, the severity of arterial obstruction is an incomplete predictor of clinical symptoms.[14–16] Vascular dysfunction may accelerate the clinical expression and progression of PAD.[17,18] Experimental studies suggest that endothelial-expressed miRNA have particular importance in vascular processes relevant to PAD.

MicroRNA in Vascular Function

miRNAs are small RNAs that regulate gene expression and direct vascular biology. Initially transcribed as long primary miRNAs, sequential processing by the enzymes Drosha and Dicer produces mature ≈22 nucleotide miRNAs. miRNA binding to the 3′ untranslated region of messenger RNA (mRNA) alters protein expression through translational repression or mRNA transcript degradation.[19] Individual miRNA may associate with functionally-related transcripts thereby governing complex processes in a coordinated fashion. There is considerable interest in miRNAs as therapeutic targets in vascular diseases as a single miRNA has the potential to influence entire gene networks.[8]

The broad role of miRNAs in vascular biology was established by studies examining genetic disruption of the processing enzyme, Dicer. Genetic disruption of Dicer impairs blood vessel formation leading to embryonic lethality in mice.[20] In cultured endothelial cells, silencing Dicer had marked effects on gene expression and functional properties. [21–23] In a mouse model, endothelial-targeted Dicer deletion reduced growth factor-mediated angiogenic responses confirming the importance of miRNAs to endothelial control of vascular growth. [24] Specific miRNAs demonstrate higher expression in endothelial cells.[25] Collectively, these findings indicate that miRNAs influence endothelial functions important to PAD.

MicroRNA in the Angiogenic Response to Limb Ischemia

An inadequate angiogenic response to lower extremity ischemia contributes to symptom manifestation in PAD patients. Growing evidence from animal models of limb ischemia

confirms the physiological role of miRNAs in angiogenesis. Conditional inactivation of Dicer in the endothelium impairs capillary growth and blood flow recovery after femoral artery disruption.[24] Dicer knockdown reduced expression of endothelial specific miRNAs and altered expression of proteins in the vascular endothelial growth factor (VEGF) signaling pathways including VEGF receptor 2 and Tie-1.[21] Further, vascular endothelial growth factor regulates expression levels of angiogenesis-related miRNAs in endothelial cells.[24]

More recent work has identified many specific miRNAs that modify the vascular growth response to limb ischemia (see Table 1). The endothelial expressed miRNA, miR-92a, is induced by ischemia and blocks blood vessel growth.[26] Inhibition of miR-92a enhanced neovascularization, reduced tissue necrosis and increased blood flow in a mouse model of limb ischemia.[26] Several proteins that facilitate angiogenesis including the integrin subunit alpha 5 and endothelial nitric oxide synthase are potential miR-92a targets. Similarly, miR-100 is suppressed by hindlimb ischemia and represses angiogenesis through mammalian target of rapamycin (mTOR).[27] Endothelial-selective overexpression of the anti-angiogenic miR-15a diminished leg perfusion recovery and capillary density in response to limb ischemia.[28]

Patients with diabetes have worse outcomes in PAD with a higher risk of limb loss reflective in part of limited angiogenic response.[29] A recent study provided evidence that microRNA are a novel mechanism for dysfunctional angiogenesis in diabetes.[30] MicroRNA analysis of endothelial cells exposed to high glucose demonstrated elevated miRNA-503. Overexpression of miRNA attenuated endothelial proliferation, migration and tube formation consistent with reduced angiogenic potential. In diabetic mice, delivery of a mir-503 inhibitory decoy restored the blood flow recovery response to hindlimb ischemia. The significance to clinical PAD was corroborated by the observation of higher miRNA-503 levels in the skeletal muscle of diabetic patients with critical limb ischemia, compared to controls. It should be noted that the sample size in the clinical portion of the study was small that limited the ability to control for potential confounders including diabetes and smoking. Taken together, these findings suggest that antagonism of miRNA-503 has therapeutic potential to improve limb ischemia in patients with diabetes.

Individual miRNAs may also facilitate angiogenesis. In limb ischemia models, injection of an antagomir that silenced the endothelial miR-126 reduced capillary growth though overall blood flow recovery was similar suggesting that compensatory mechanisms may exist to limit the effect of loss of a single miRNA.[31] The pro-angiogenic properties of miR-126 are mediated by targeting SPRED1 and PIK3R2, endogenous inhibitors of VEGF signaling. [32,33]

MicroRNA in the Regulation of Endothelial Function

In addition to participating in the control of angiogenesis, vascular miRNAs influence aspects of endothelial function critical to PAD. Recent investigations have characterized miRNAs that alter endothelial phenotype to affect atherogenic potential. Interestingly, global reduction of miRNAs through Dicer silencing increased eNOS expression and nitric oxide

bioavailability in cultured endothelial cells.[21] Thus, miRNAs serve as endogenous suppressors of eNOS levels suggesting that targeting specific miRNAs may enhance nitric oxide production. Subsequent work showed that miR-92a antagonism improved endothelial nitric oxide signaling, improved nitric oxide bioavailability, and increased flow-mediated dilation in cell culture and animal models.[26] [34] Further studies are needed to evaluate the relation of miRNA levels and nitric-oxide mediated endothelial function in patients with PAD.

Developing evidence links miRNAs to the endothelial response to flow. Local shear stress patterns modify endothelial biology in part through altered gene expression levels.[35] Laminar flow maintains endothelial health, whereas disturbed flow produces an adverse, pro-inflammatory endothelial state.[36] Multiple studies have demonstrated changes in miRNA expression with endothelial cell exposure to varying shear stress patterns.[37] Arterial regions exposed to pro-atherogenic flow display distinct miRNA expression profiles.[38,39]

Flow-sensitive miRNAs have also been studied in hindlimb ischemia reinforcing a potential connection to PAD. In endothelial cells, flow patterns induce differential expression of miR-92a with increased levels with oscillatory compared to pulsatile shear.[34] Complementary findings from porcine aorta showed higher miR-92a levels in regions exposed to flow turbulence.[39] Conversion from atheroprotective to atheroprone gene expression by oscillatory shear was mediated by a post-transcriptional reduction in kruppel-like factor (KLF) 2 and 4 levels through miR-92a.[34,39] miR-92a inhibition also prevented tumor necrosis factor alpha-mediated endothelial inflammatory activation by augmenting KLF4 levels.[39] A study in zebrafish embryos suggested flow-induced modulation of miR-126; however, miR-126 was not upregulated by laminar flow in cultured human endothelial cells.[40,41] miR-126 does appear to moderate endothelial inflammation by reducing vascular cell adhesion molecule-1 expression and decreasing leukocyte adherence. [42]

Additional miRNAs have been characterized that impact flow-dependent vascular inflammation. The amount of miR-10a is diminished in abnormal flow areas. In endothelial cells, mir-10a has anti-inflammatory properties by reducing expression of nuclear factor kappa B activators.[38] Conversely, oscillatory shear stress stimulates pro-inflammatory miR-663 and upregulates inflammatory gene expression and enhances leukocyte adhesion to endothelial cells.[43] Separate reports showed higher miR-21 levels with both oscillatory shear and laminar shear.[44,45] miR-21 exerts diverse endothelial effects by decreasing peroxisome proliferators-activated receptor-α expression and promoting adhesion molecule expression. However, beneficial effects have also been shown with miR-21 overexpression with reduction of PTEN, an inhibitor of eNOS activation, and increased nitric oxide activity. [45] Overexpression of miR-181b blunted endothelial inflammatory activation by suppressing importin-α3, a facilitator of NFκB activation.[46] Additional work will be required to determine whether the vascular inflammation present in PAD can be mitigated by microRNA-based therapies.[47]

MicroRNA and Vascular Reparative Function

Considerable enthusiasm exists for the prospects of employing cell-based interventions to facilitate vascular growth in PAD. The isolation of bone-marrow derived progenitor cells that promote neovascularization has prompted the development of investigational therapeutic strategies for advanced vascular disease. [48] Results from early clinical studies in patients with PAD have revealed modest benefits; however, multiple impediments persist that limit clinical efficacy. There is accumulating support for the role of miRNAs in optimizing stem cell function.[49] Investigations using Dicer reduction established that miRNAs are an essential component of stem cell maintenance and differentiation.[50,51] A recent study in cardiomyocytes detected a set of miRNAs that stimulated cell proliferation and cardiac regeneration following myocardial infarction.[52] These findings provide support for the concept that miRNA administration may bolster functional recovery produced by stem cells.

miRNAs have been detected that contribute to pluripotency, vascular differentiation, and adult progenitor cell function. [49,53,54] The transformation of embryonic stem cells to endothelial cells is accompanied by changes in miRNA levels relevant to angiogenesis including higher (miR-126, miR-210, let-7, mir-130a, miR-133, and miR-196) and lower (miR-20a, miR-20b, miR-221, and miR-222) expression.[55,56] In an animal model, genetic augmentation of embryonic stem cells to increase miRNAs critical to endothelial cell differentiation (miR-99b, miR-181a, miR-181b) improved blood flow recovery from hindlimb ischemia.[57]

Circulating pro-angiogenic endothelial progenitor cells have the capacity to foster vascular repair.[58] It has been proposed that in disease states, progenitor cell scarcity and dysfunction impair vascular regeneration.[59] In patients with coronary artery disease, endothelial progenitor cells showed differential expression of miRNAs related to angiogenesis, a phenotype that was reversed with statin therapy.[60] In patients with diabetes, endothelial progenitor cells displayed lower miR-126 expression that was associated with impaired angiogenic function.[61] A recent study demonstrated higher expression of miRNA-15a and 16 in endothelial progenitor cells from patients with critical limb ischemia.[62] These miRNAs reduced VEGF expression and alteration of endothelial progenitor cells to reduce the levels of miRNA-15a and 16 improved hindlimb blood flow recovery in a murine model. Together, the current evidence suggests that miRNA manipulation may be an approach to enhance cell-based therapies for PAD.

MicroRNA in the Development of Restenosis

In the past decade, the use of endovascular therapies to treat advance PAD has risen dramatically.[63] However, the efficacy of lower extremity revascularization is limited by the high rate of restenosis.[64] In vitro studies have identified miRNAs which regulate vascular SMC de-differentiation and processes relevant to myointimal hyperplasia.[65] Studies in animal models also demonstrate miRNA-dependent regulation of neointimal lesion formation.[66] Following carotid balloon injury in rats, expression profiling detects deviation in the vascular wall levels of multiple microRNAs.[67]

Endothelial damage with angioplasty depresses expression of miR-143 and 145, key supervisors of vascular smooth muscle cell phenotype.[67–69] Overexpression of miR-145 inhibits lesion formation and vascular smooth muscle proliferation.[70–72] Genetic models with miR-143/145 knockout mice have yielded complex information about neointimal growth. One study showed that young mice lacking miR-143/145 spontaneously develop femoral artery proliferative lesions.[69] Another report demonstrated limited neointimal growth to carotid artery injury in miR-143/145 depleted mice.[70] It may be that the proper regulation of miR-143/145 levels is required to stabilize downstream gene expression and prevent an aberrant injury response. Downstream targets of miR-143 and 145 that shift vascular smooth cell phenotype include KLF4, KLF5, platelet derived growth factor receptor, and angiotensin-converting enzyme.[68,69,71,73,74] Smooth muscle calcification is induced by bone morphogenetic protein-2 (BMP-2) downregulation of miR-30b and 30c, which increases Runx2 expression.[75]

Arterial injury also elevates levels of selected vascular smooth cell microRNAs. Angioplasty-induced vessel damage resulted in an increase in miR-21 levels in rats.[67] Depletion of miR-21 constrained the development of restenotic lesions along with lower vascular smooth muscle cell proliferation in both carotid and iliac artery models.[67,76] Fibroblast proliferation is also activated by miR-21 through programmed cell death 4/JNK pathway.[76] In vascular smooth muscle cells, the proliferative actions of miR-21 are mediated through PTEN and Bcl-2.[67,77] In patients with thromboangiitis obliterans, a non-atherosclerotic peripheral vascular disease, arterial miR-21 levels were increased and shown to modulate smooth muscle proliferation by targeting tropomyosin 1.[78] The number of samples in the clinical study was relatively small precluding any adjustment for potential confounders; thus a larger study will be necessary to replicate these findings. In a comparable fashion, angioplasty injury produces greater miR-221 and 222 expression.[79] Reduction of miR-221 and 222 interfered with vascular smooth muscle proliferation and neointimal hyperplasia through suppression of p27(Kip1) and p57 (Kip2). It is possible that derangements of miRNA expression translate to restenosis in PAD patients and could be manipulated by therapeutic inteventions.

MicroRNA and Mitochondrial Function

There is growing appreciation that peripheral artery disease produces skeletal muscle abnormalities characterized by mitochondrial dysfunction.[80,81] Abnormal muscle energetics combined with ischemia and endothelial dysfunction may amplify functional limitations in PAD.[82] Increasing experimental data associate miRNA with mitochondrial biology in muscle and the vasculature.[83] In skeletal muscle, limb ischemia induces differences in a set of miRNAs in mice.[84] Recent work shows detectable miRNA in the mitochondria that regulate energetics.[85,86]

Ischemia drives changes in miRNA expression relevant to mitochondrial function.[87] Multiple lines of evidence indicate that miR-210 is an integral regulator of the response to hypoxia.[88] miR-210 expression is stimulated in hypoxic conditions, in part, through HIF1α and suppresses mitochondrial respiration and reactive oxygen species generation. [89,90] MiR-210 expression, in the setting of hypoxia, augments the angiogenic response to

VEGF by suppressing Ephrin-A3 expression.[91] Additional studies show that ischemia generates a transition to greater mitochondrial fragmentation and apoptosis. Ischemic conditions decrease miR-499 that promotes DRP-1 expression and increased mitochondrial fission.[92] In patients with diabetes, aberrant mitochondrial dynamics relates to endothelial dysfunction.[93]

Restoration of mitochondrial function is a proposed pathway underlying the dramatic benefits of exercise therapy in PAD patients.[3,94]In animal models, specific miRNA have been isolated that couple physical activity with mitochondrial biogenesis. Exercise intervention reduced the expression of miR-494, a microRNA that is stimulated by limb ischemia.[84,95] Through an interaction with mitochondrial transcription factor A, miR-494 modulates mitochondrial content.[95] In a similar fashion, skeletal muscle levels of miR-696 decreased with exercise training and increased with inactivity. [96] The amount of miR-696 associated with proliferator-activated receptor-gamma coactivator-1alpha (PGC-1α) expression and with mitochondrial number.[96] Notably, PGC-1α, a controller of mitochondrial biogenesis, also determines blood flow recovery from limb ischemia and exercise-induced angiogenesis.[97,98] Further studies are warranted to establish whether miRNAs contribute to mitochondrial dysfunction in patients with PAD.

Circulating MicroRNA in PAD

Recent investigations demonstrate detectable levels of circulating miRNAs that serve as novel biomarkers in cardiovascular disease.[9,99,100] Prior studies show elevation of cardiac-specific miRNAs following acute myocardial infarction.[101–103] In prospective studies, miRNA expression profiles in plasma predicted cardiovascular outcomes in patients with atherosclerotic disease.[104,105] Levels of multiple miRNAs known to be expressed in cultured endothelial cells were lower in CAD patients.[106] Importantly, changes in circulating miRNA expression differ in animal models of myocardial infarction as compared to limb ischemia.[103] Potential advantages of miRNAs as biomarkers include: the ability to use amplification techniques to detect low level expression; long-term stability in blood; and, most interestingly, the possibility of evaluating expression networks across multiple miRNAs to examine coordinated responses to disease.[107]

In patients with PAD, individual miRNAs have been shown to be differentially expressed in circulation (see Table 2). Many of the miRNAs that have been studied in PAD were selected based on the animal studies discussed above that demonstrate relevance to limb ischemia. In diabetic patients, there was an association of lower circulating miR-126 with lower ankle-brachial index.[108] In patients with critical limb ischemia, levels of the anti-angiogenic miRs 15a and 16 were higher in serum and predicted the occurrence of amputation amongst diabetic individuals.[62] Similarly, plasma levels of miR-503 were higher in diabetic patients with critical limb ischemia.[30] In patients with thromboangiitis obliterans, circulating levels of miR-130, miR-27b, and miR-210 were increased.[109] Future studies are needed to perform a full profile of circulating miRNAs in a large cohort of PAD patients and controls.

The source and biological function of circulating miRNA remain a subject of active investigation. Developing evidence suggests cells actively release miRNA into microvesicles, which protects them from degradation by endogenous RNAses.[32,110] miRNAs specifically expressed in endothelial cells are enriched in plasma suggesting endothelial injury as a source.[106] In addition, miRNAs can be delivered to target cells. [111,112] Thus, there is the potential that the endothelium absorbs circulating miRNAs thereby regulating cell phenotype.[113,114] Transfer of microRNA from endothelial progenitor cell-derived circulating microvesicles improved perfusion recovery after hindlimb ischemia.[115] Platelets may also be an important determinant of circulating miRNA levels. [116] The diagnostic and prognostic implications of miRNA profiles require further characterization in patients with PAD.

MicroRNA therapies in PAD

Treatments based on miRNA targets are an area of active investigation.[8] In principle, both stimulation of beneficial miRNA pathways and inhibition of adverse miRNAs could be employed to confer clinical benefit in PAD.[117,118] Administration of mimics of pro-angiogenic miRNA or delivery with trophic expression vectors could promote neovascularization in PAD. The technology for opposing microRNA action systemically is further developed. Antimir development involves the creation of complementary oligonucleotides that reduce the levels of specific miRNAs. Multiple strategies permit adequate delivery and repression of miRNAs by antimirs including modification to increase binding capacity, avoid breakdown by nucleases, and enhance cell uptake, though novel tissue targeting modalities are sought. As described above, several strategies have been used successfully to promote vascular growth in animal models of hindlimb ischemia by antagonizing miRNAs known to be upregulated in ischemic tissue. Advantages of antimir therapies include the coordinated regulation of multiple gene targets by single miRNAs and the potential specificity of miRNA dysregulation to ischemic tissues. However, the fact that individual miRNAs have multiple targets also raises concern for off-target effects of antimirs. Additional approaches may be required to restrict the influence of antimirs to specific tissues or regions.

Conclusions

In summary, there is extensive support for intersections of miRNAs and vascular functions that determine clinical disease status in PAD. Individual miRNAs have been characterized that influence the lower extremity response to ischemia in animal models. Clinical studies in patients with PAD confirm alterations in vascular miRNAs in ischemic regions and in circulation. The translation of miRNA biology to the clinical arena for detection and management of clinical PAD holds significant promise.

Acknowledgments

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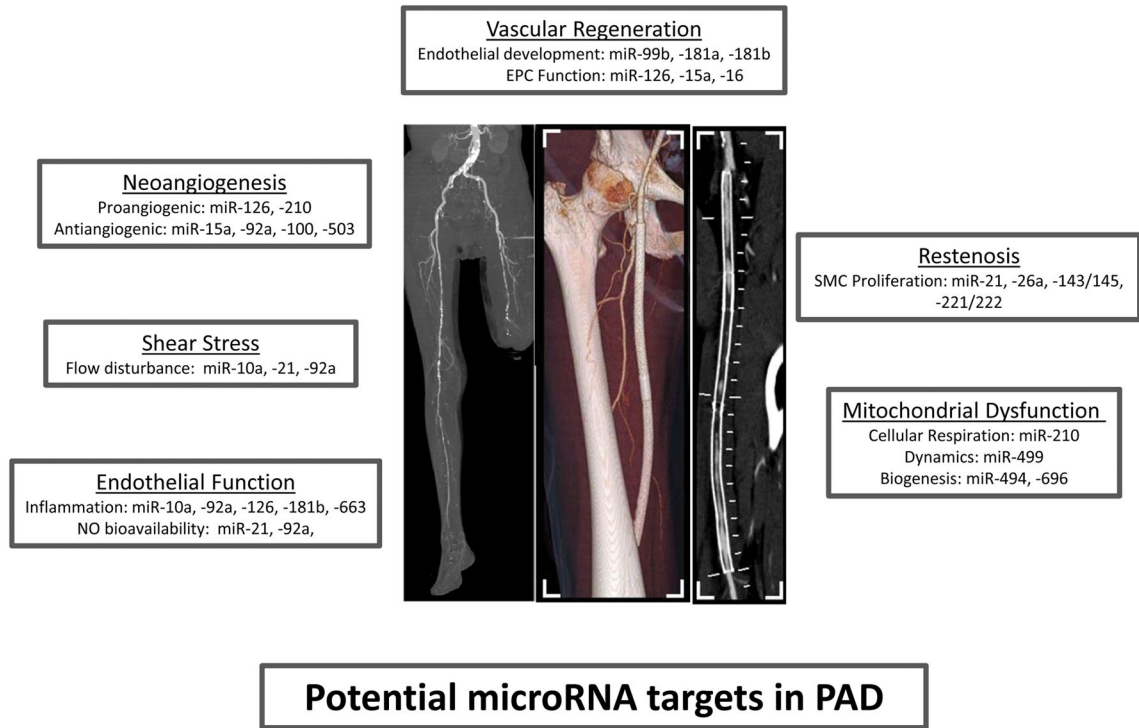
Figure 1.
Potential Contributions of MicroRNAs to PAD. MicroRNAs (miR) have been identified that determine key processes relevant to disease manifestation in PAD including neoangiogenesis, endothelial shear stress response, endothelial function, restenosis, vascular regenerative capacity, and mitochondrial function.

Table 1

MicroRNAs Relevant to Peripheral Artery Disease

MicroRNA	Putative Target	Putative Mechanism
miR-92a	eNOS, integrin-α5 KLF2/4	Anti-angiogenic, Inhibit NO Pro-inflammatory
miR-100	mTOR	Anti-angiogenic
miR-503	Cell cycle regulators	Anti-angiogenic
miR-126	SPRED1/PIK3R2/VEGF VCAM1	Pro-angiogenic, EPC function Anti-inflammatory
miR-10a	NFκB	Anti-inflammatory
miR-663	KLF4, ATF6	Pro-inflammatory
miR-21	PPARα PTEN PCD4/JNK PTEN/BC12	Pro-inflammatory Increase NO Restenosis SMC Proliferation
miR-181b	Importin-α3	Anti-inflammatory
miR-15a/16	VEGF	Impair EPC function
miR-143/145	KLF4/5, ACE, PDGFR	SMC Proliferation
miR-221/222	p27, p57	SMC Proliferation
miR-210	Ephrin A3/VEGF	Pro-angiogenic
miR-499	DRP-1	Mitochondrial dynamics
miR-494	MTF A	Mitochondrial biogenesis
miR-699	PGC1α	Mitochondrial biogenesis

Table 2

Circulating MicroRNAs as Biomarkers in Peripheral Artery Disease

MicroRNA	Clinical Findings
miR-503	Higher in patients with diabetes and critical limb ischemia
miR-126	Lower levels correlate with lower ankle-brachial index in patients with diabetes
miR-15a/16	Lower in patients with critical limb ischemia
miR-130, -27b,-210	Higher in patients with thromboangiitis obliterans