CHAPTER 2

The role of microRNA-126 in vascular homeostasis

Introduction

The vascular network comprises a large network of arteries, veins and capillaries that facilitates the circulation of blood to maintain homeostasis. The endothelium constitutes a thin layer of endothelial cells (ECs) that form a semi-permeable barrier between the circulating blood and the other structural compartments of the vascular wall. ECs are key mediators of vascular homeostasis and, therefore, the maintenance of a healthy endothelium is critical. Pathological conditions such as tissue ischemia, inflammation, hyperglycemia and hypercholesterolemia lead to EC activation, endothelial dysfunction and ultimately to EC apoptosis, which can accelerate the risk of premature atherosclerosis. Maintenance of endothelial integrity is of central importance for cardiovascular health and in large part determined by the balance between EC injury and repair [1]. One of the key repair mechanisms that aid in the replacement of damaged ECs is angiogenesis, the process by which new blood vessels bud from pre-existing capillary ECs. Angiogenesis is a tightly regulated process, that requires the coordination of numerous signaling pathways in which ECs act as both active participants and regulators [2]. The formation of novel capillaries may not be restricted to the sprouting capability of ECs as circulating, bone marrow-derived cells are also thought to be contributing to neovascularization and re-endothelialization [3]. This process that involves endothelial progenitor cells (EPCs) is called neo-vasculogenesis. The relative contribution and the exact phenotype of the cells involved in this process is a topic of active investigation. It has been demonstrated that EPCs can support the formation of independent functional vascular structures after migration towards a hypoxic region in the skin [4]. Others claim that the induced neovascularization mostly depends on stabilization of novel vascular structures by recruited supporting perivascular mural cells [5-6]. Although their role is elusive, it is clear that bone marrow-derived circulation cells contribute positively to maintain vascular integrity.

Insight into the cellular and molecular mechanisms that can control vascular homeostasis is of high relevance in pursuit of understanding and treatment of a broad range of diseases that involve deficient or aberrant neovascularization such as cardiovascular disease and cancer. It is becoming increasingly apparent that microRNAs (miRNAs) are key regulators of vascular homeostasis [19].

These short non-coding RNAs were initially discovered in 1993 [7]. However, the impact of miRNAs on cellular biology has only recently started to unfold. The genomic sequences encoding miRNAs are generally harboured within intronic regions of genes and have been found to be well conserved between species [8]. After synthesis and processing miRNAs are incorporated in the RNA-induced silencing complex (RISC), where the miRNA can guide the RISC to the 3'UTR of the designated target sequence. [9]. The seed sequence, defined by 2-8 nucleotides located at the 5' region of the miRNA, is critical for target recognition and

silencing of the mRNA [10-11]. Translation of the mRNA is repressed after association of a miRNA with its target sequence. The exact mechanism by which translational arrest is induced involves both degradation of the mRNA and the inhibition of the initiation and elongation steps of translation [12-14].

MiRNAs are expressed in a tissue- and cell-specific manner during development suggesting a role for miRNAs in specifying and maintaining tissue identity [15]. Also, there is growing recognition that one single miRNA can have multiple targets, and therefore impacts multiple pathways. These features also predict regulatory roles for miRNAs in the control of vascular homeostasis and recent studies identified a number of miRNAs with pro-angiogenic [16-19] as well as anti-angiogenic functions [20, 21].

Of particular interest with respect to a controlling role in neovascularisation is miRNA-126, a miRNA that was found to be highly enriched in the endothelium [22-23]. Initially it was thought that miRNA-126 was exclusively expressed in ECs, however miRNA-126 is also present in several cancer cell types [24-31], airway epithelium [32-34], circulating cells [35-39], and platelets [40-42]. Significant progress has been made in identifying its mRNA targets and function both in endothelial cells and other cell types that express this miRNA (listed in Table 1). Clearly, like many others, miRNA-126 appears to fulfil different functions in different stages of cell life and can work on several targets within the same cell.

Interestingly, miRNAs are also detected outside the cellular compartment as they can readily be detected in microvesicles in human plasma, such as apoptotic bodies [43] and exosomes[44] or 'free-floating' non-vesicle argonaute-2 (Ago2) complexes [45]. Unlike other miRNAs, miRNA-126 is not restricted to one of these groups and is present in each of these configurations [45].

More recently, miRNA levels have been quantitated in human plasma using miRNA-arrays and quantitative real-time PCR, and it is been reported that

Box 1.

Depending on the target prediction website and algorithm that is used to identify targets for (human) miRNA-126 the total of hits can vary between 17 in TargetScan (http://www.targetscan.org) and 937 in MicroCosm Targets (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5). TargetScan was developed in 2001 to identify the targets of vertebrate miRNAs, the algorithm combines thermodynamics based modelling of RNA:RNA duplex with comparative sequence analysis to predict miRNA targets across multiple genomes [93]. MicroCosm Targets uses the Miranda algorithm, which follows about the same rules as the TargetScan algorithm. However, the genome of two other organisms, the zebrafish (***Danio rerio***) and the fugu (***Fugu rubripes***) was scanned for potential targets. In addition to the analysis of 3'UTRs, all protein coding regions for high scoring miRNA target sites are calculated, leading to far more possible target sites [94]. Despite numerous lists of potential targets per miRNA, only a small number of target sites on target genes have been experimentally verified.

lowered levels of miRNA-126 correlate with age, coronary artery disease (CAD) or subjects diagnosed with type II diabetes mellitus (DM2) [46-48]. These studies indicate a potential link between a reduction of this vascular miRNA and endothelial dysfunction. While the abundant expression of miRNA-126 by ECs suggests that these cells are the main source of circulating miRNA-126 in the circulation. However, other sources such as platelets [40-42] and bone marrow-derived circulating cells [38] express significant levels of miRNA-126 and can therefore also contribute to the circulating pool of miRNA-126.

MiRNA-126 in the endothelium

The locus encoding miRNA-126 resides within intron 7 of the EC-restricted epidermal growth factor like-domain 7 (EGFL7) [49]. EGFL7, that can be found on chromosome 9 or 2 in human and mice respectively [50], is a secreted protein of 41 kDa [51], that is up regulated after arterial injury *in vivo*. This augmentation of EGFL7 recruits ECs, angioblasts and supportive cells to sites of injury for vascular repair [52]. Upstream of the EGFL7/miRNA-126 locus are two E26 transformation-specific sequence (Ets) binding sites that are evolutionarily conserved. It has been established that the binding of Ets-1 or Ets-2 to an Ets binding site is required for the trans-activation of the EGFL7/miRNA-126 gene in ECs [53]. EGFL7 is restricted to the endothelium in adult mice and humans [50], and its expression can contribute to the presence of miRNA-126 in these cells, however, the presence of miRNA-126 in other cells, which do not express EGFL7 is counter-intuitive. A first explanation could be that the EGFL7 gene is also expressed shortly during early embryogenic development [51, 54], which might be the reason for the expression of miRNA-126 in other cells of the hematopoietic lineage. It is likely that the primary transcript of EGFL7 is post-transcriptionally silenced, independent of the nuclear and cytoplasmatic processing of miRNA-126. Furthermore, it has been described that the mRNA of EGFL7 harbours a binding site for miRNA-126 [55], indicating that miRNA-126 itself can block the translation of EGFL7. However, a positive or negative association for miRNA-126 with its host gene was absent in tumour samples taken from a cohort of 110 colon cancer patients [56]. These data indicate that miRNA-126 expression may react to different stimuli then those that lead to the expression of EGFL7.

Interestingly, the targeted deletion of miRNA-126, either via genetic deletion in animals [49, 57, 58] or following administration of antagomirs [59], perturbed vascular development [49, 57, 58], attenuated recovery after myocardial infarction [58], and impaired angiogenic capacity after ischemic hind limb injury [59]. Furthermore, mutant mice and morphant zebrafish demonstrated drastic vascular abnormalities, such as heart valve elongation defects [60], oedema, haemorrhaging and embryonic death [49, 57, 58]. As such, the diminished angiogenic capacity and vascular defects observed as a result of decreased

Table 1. Overview of validated targets and pathways of miRNA-126

Cell type / tissue	Target protein	Process	Ref.
Endothelium	Spred-1, PI3KR2, PAK1	vascular development angiogenesis	[57-59, 90]
Endothelium	VCAM-1	inflammation leukocyte adhesion EC heterogeneity	[23, 71]
Endothelium	RGS16	atherosclerosis Sca-1+ incorporation	[43]
Endothelium	CXCL12	ischemia mobilization of Sca-1+	[66]
CD4+ T cells	Dnmt1	DNA methylation	[39]
Hematopoietic stem cells	HOXA9, c-Myb, PTPN9	hematopoietic development erythropoiesis	[35, 36, 38]
Mast cells	Spred-1	mast cell differentiation cytokine production	[37]
Epithelium (lung)	?	allergen exposure Th2 response eosinophil recruitment	[32, 33]
Epithelium (lung)	TOM1	immune response modulation of TLRs	[34]
Epithelium (mammary)	PGR, β-casein	mammary gland development lactation	[91]
Breast cancer	IRS1, VEGF-A, PI3KR2	tumour development	[30, 31]
Colon cancer	p85β subunit	tumour development	[28]
Lung cancer	Crk, VEGF-A SLC7A5, EGFL7	tumour invasion tumour angiogenesis tumour cell proliferation	[24, 26, 27, 55]
Gastric cancer	SOX2, Crk	tumour cell proliferation tumour invasion	[25, 29]
Pancreatic cancer	Adam9	tumour invasion	[92]

miRNA-126 in ECs strongly suggests that miRNA-126 has an essential role in regulating EC responsiveness to angiogenic stimuli. Support for this notion can be derived from the fact that miRNA-126 regulates the angiogenic signalling pathways, downstream of fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) by binding to the 3'UTRs of sprouty-related EVH1 domain containing 1 (SPRED-1) and phosphoinositide-3-kinase regulatory subunit 2 (PI3KR2) [57, 58]. By effectively blocking the expression of these aforementioned proteins by miRNA-126, the v-raf-1 murine leukaemia viral oncogene homolog 1 (RAF1) and phosphoinositide-3-kinase (PI3K) are able to trigger ECs to elicit a vascular response to injury [57, 58]. Therefore, it is likely that ECs require miRNA-126 to maintain the integrity of the vasculature during vascular development as well as in adult life. Recently, a role for miRNA-126 has been confirmed in zebrafish, where embryos were treated with the myosin ATPase inhibitor 2,3-butanedione 2-monoxime or the anaesthetic tricaine methanesulphonate to arrest the heart and block circulation [61], the expression of miRNA-126 and Kruppel-like factor 2a (KLF2a) were down regulated, leading to enhanced translation of SPRED-1. The resultant repression of VEGF-stimulated angiogenesis led to major (vascular) developmental defects [62].

Next to the role of miRNA-126 in regulating angiogenesis, a regulatory role has also been established for the development of atherosclerosis. In response to proapoptotic stimuli, ECs lining atherosclerotic plaques can generate apoptotic bodies [63-64]. Both the release and abundance of these apoptotic bodies have been found to be associated with endothelial dysfunction, suggesting that they may serve as a diagnostic marker of atherosclerotic vascular disease [63]. The incorporation of apoptotic bodies secreted by ECs by an acceptor cell can dramatically change its miRNA content, impacting cellular function. It has been established that miRNA-126 is the most abundant miRNA in these EC-derived apoptotic bodies [43]. The *in vitro* uptake of EC-derived apoptotic bodies by human umbilical vein ECs (HUVEC) resulted in a marked increase in intracellular expression and secretion of chemokine ligand 12 (CXCL12) [43]. However, the 3'UTR of CXCL12 is a direct target of miRNA-126, thus the increase in protein expression can not be the result of elevated miRNA-126 levels in the recipient cell. Regulator of G-protein signalling 16 (RGS16), a negative regulator of the CXCL12 receptor chemokine (C-X-C motif) receptor 4 (CXCR4), was identified to be a target of miRNA-126, and to be involved in the regulation of CXCL12 expression [65]. This was validated by intravenously injecting miRNA-126-containing EC-derived apoptotic bodies into ApoE-/- mice. After placing these mice on a high fat diet for a period of six weeks, mice that were administered miRNA-126-containing apoptotic bodies displayed a higher luminal incorporation of CXCR4-dependent Sca-1+ stem cells into the aortic root plaque than mice injected with non-EC derived apoptotic bodies. Prolonged treatment with EC-derived apoptotic bodies elevated CXCL12 levels

andreduced atherosclerotic plaque size in the aortic root [43]. These data implicate that the delivery of miRNA-126 by microparticles, such as apoptotic bodies, might play a key role in diet-induced atherosclerosis.

In addition, miRNA-126 can also directly influence CXCL12 expression by binding to 3'UTR of its mRNA. It has been established that attenuation of miRNA-126 with antagomirs increases the expression of CXCL12 in HUVEC. *In vivo* administration of antagomir-126 led to elevated protein levels in the circulation and ischemic tissue after inducing ischemic injury. The increase in CXCL12 expression triggered the mobilization of Sca-1+/Lin- stem cells into the circulation [66]. These findings suggest that miRNA-126 potentially plays an important role in regulating vasculogenesis after ischemic injury by targeting CXCL12.

It is well established that systemic inflammation leads to EC activation. Since miRNA-126 is a central regulator of EC function and homeostasis, it is likely that miRNA-126 might influence the EC response to inflammatory stimuli. A primary response to systemic inflammation is the augmentation of vascular cell adhesion molecule 1 (VCAM-1) expression, leading to the clustering of VCAM-1 on the endothelial surface. The formation of these clusters results in the transmission of numerous intracellular signals that facilitate adhesion, migration and diapedesis of leukocytes through the EC permeability barrier into the adjacent tissue [67-70]. A potential binding site for miRNA-126 was localized in the 3'UTR of the mRNA of VCAM-1, which suggests a role for miRNA-126 by controlling the expression of VCAM-1 upon inflammation. Indeed, over-expression of miRNA-126 in combination with the induction of an inflammatory response with tumour necrosis factor alpha (TNFα) in HUVEC suppressed the protein levels of VCAM-1 and the ability of these cells to adhere leukocytes [23]. Moreover, it has recently been shown that the expression of miRNA-126 in microvascular compartments is a governing factor in acute inflammation in the kidney. Upon induction of anti-glomerular basement membrane glomerulonephritis as well as TNFα, lipopolysaccharide or anti-myeloperoxidase-induced glomerulonephritis, VCAM-1 mRNA expression was highly increased in both arterioles and glomeruli, while the protein was only expressed to a limited extent in the glomerular compartment. Extensive RNA analysis in the glomerular and arteriolar vascular segments suggested that these two vascular compartments display different levels of miRNA-126. High miRNA-126 levels were found in the glomerular compartment and coincided with low VCAM-1 protein expression, while in the arterioles low miRNA-126 levels associated with increased VCAM-1 levels [71]. These elevated levels for miRNA-126 in glomerular ECs coincided with increased expression of Ets-1, an established transcriptional regulator of miRNA-126 [53]. The interaction between miRNA-126 and Ets-1 adds an extra level of complexity to the regulation of vascular inflammation. Vascular inflammation induces Ets-1

expression, thereby activating the transcription of pro-inflammatory proteins, including VCAM-1 [72]. In contrast, Ets-1 also induces miRNA-126, subsequently inhibiting the translation of VCAM-1 [23]. Through its influence on the expression and clustering of VCAM-1, miRNA-126 may contribute to the heterogenic response of ECs to inflammatory stimuli. The multi-regulating capacity of miRNA-126 in various stages of vascular development, neovascularisation and inflammation underlines the importance of miRNA-126 in ECs (summarized in Figure 1).

MiRNA-126 in progenitor cells

The function of miRNA-126 extends beyond its expression in endothelial cells. Several cancer types display elevated levels of miRNA-126 [24-31], and cells coming from the hematopoietic compartment also show expression of miRNA-126 [35-42]. For instance, elevated miRNA-126 expression has been detected in human CD34+ hematopoietic stem cells and progenitor cells following erythroid induction [36, 73], and mobilization with G-CSF [74]. Interestingly, miRNA-126 expression was found to be decreased during megakaryocytopoiesis [75]. Since differential expression of a miRNA highly impacts the expression of target genes, one can speculate that alterations in miRNA-126 expression in

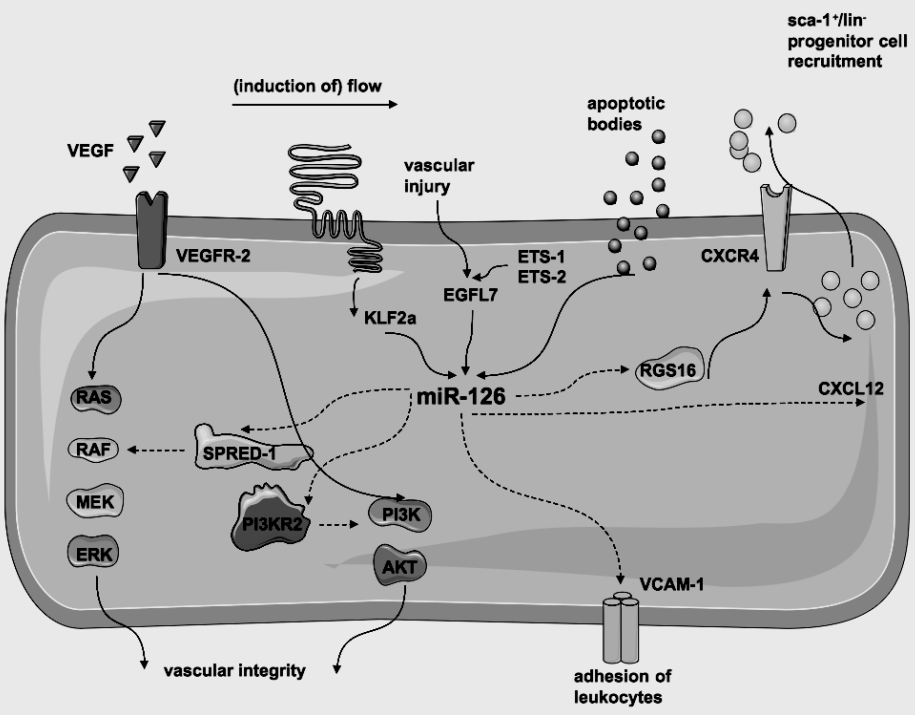

Figure 1. The multi-regulating capacity of miRNA-126 in endothelial cells

hematopoietic progenitor cells could profoundly impact cellular function.

Since the evolutionary conserved homeobox (HOX) genes play an important role during development and hematopoiesis[76-77], Shen and co-workers hypothesized that miRNA-126 could impact hematopoiesis by regulating HOX mRNA transcript stability [38]. However, only two HOX genes, namely HOXA3 (miRanda, http://www.microrna.org/) and HOXA9 (PicTar, http://pictar.mdc-berlin.de/), contain predicted binding sites for miRNA-126. *In vitro* experiments revealed that the abrogation of endogenous miRNA-126 in murine bone marrow cells increased the expression and activity of HOXA9 protein. Furthermore, it was demonstrated that the expression profile of miRNA-126 parallels HOXA9 mRNA expression in normal murine bone marrow. These findings suggest a potential role for miRNA-126 in controlling hematopoietic development by regulating the levels of HOXA9 protein [38].

In addition to murine bone marrow cells, human embryonic stem cells (hESCs) have been used to study the function of miRNA-126 in hematopoietic differentiation. For this, miRNA-126 was over-expressed in hESCs upon embryoid body formation, yielding a reduced number of erythroid colonies. Co-expression of tyrosine-protein phosphatase non-receptor type 9 (PTPN9), which contains a predicted binding site for miRNA-126, led to a partial recovery of erythropoiesis [36]. The inability to fully restore erythropoiesis suggests that another target of miRNA-126 might be found in the erythroid pathway. The role of miRNA-126 in erythropoiesis is also suggested by the notion that PTPN9 is hyper-activated in the erythroid progenitors in patients with polychytemia vera, a disease that results in erythrocyte overproduction. This hyper-activation is combined with the lack of miRNA-126 expression in the erythroid progenitors of these patients [78-79].

Circulating miRNA-126

Blood plasma samples harvested from subjects with cardiovascular disease (CVD) risk factors have extensively been studied for the presence of biomarkers. Interestingly, miRNAs could serve as novel biomarkers as they can be detected in the circulation, making it possible to readily assess the miRNA-profiles of healthy and diseased subjects. Importantly, miRNAs are surprisingly stable despite the high endogenous RNase activity in the circulation [80]. Circulating miRNAs are present in both serum as well as plasma and can be measured using quantitative real-time PCR [81]. To date, miRNAs have been detected in the circulation in two forms, notably as being carried by cell-released vesicles [82] or in association with Ago2 complex, the catalytic component of the RISC [45]. While most miRNAs exist in the circulation in only one of both forms, miRNA-126 has been found to be both vesicle-bound as well as in a complex with Ago2 [45].

In several different patient cohorts the circulating miRNA content was compared to healthy controls. In a limited study with 12 heart failure patients and

healthy controls, no differences were found in miRNA-126 levels [83]. In contrast, the presence of cardiovascular risk factors such as age, CAD and DM2 correlated with decreased expression of miRNA-126 as compared to healthy controls [46-48]. The loss of miRNA-126 could explain the observed impairment of angiogenic signalling in the periphery of patients diagnosed with CAD and DM2. It is likely that fine-tuning of miRNA-126 expression in CVD is essential to elicit the appropriate response in the case of acute endothelial activation. MiRNA-126 is abundantly expressed in ECs, and is required for the stimulation of neovascularization, while curtailing its expression in the face of chronic endothelial activation and injury, to avoid EC death.

This thesis sheds a light on cellular sources of miRNA-126 in the circulation, including ECs and circulating hematopoietic stem cells [35-39]. Recently, platelets have also been found to express miRNA-126 [41]. Although platelets have no nucleus and therefore do not possess the machinery to transcribe or generate mature miRNAs, miRNAs (including miRNA-126) are both abundant and functional in platelets [41]. The source of these mature miRNA-126 molecules is likely to be the megakaryocyte, since megakaryocytes have been found to express significant levels of miRNA-126 [75], which indicates that their miRNA content is transferred from the megakaryocyte to the budding platelets. Furthermore, it is possible that platelets actively endocytose vesicle-bound or Ago2-associated miRNAs from the periphery, thereby adding to their miRNA-126 content.

Upon endothelial injury platelets are exposed to collagens, von Willebrand factor and tissue factor derived from the subendothelium and get activated. This leads to platelet aggregation, a process that triggers the secretion of various cytokines and miRNA-containing microvesicles [82, 84, 85]. The notion that microvesicles and platelet-derived microvesicles can serve as a major source of circulating miRNAs for uptake by either ECs or other circulating cells [44, 86] further support the notion that miRNA-126 does not exclusively exert its effects in ECs, but could potentially function as a critical mediator of vascular homeostasis.

Concluding remarks

MiRNA-126 is abundantly expressed in ECs and plays an important role in neovascularisation by regulating the expression of various proteins driving both angiogenesis and vasculogenesis [43, 57-59, 66, 76]. Furthermore, a role for miRNA-126 in adjusting the expression and microvascular location of VCAM-1 in ECs upon inflammation has been demonstrated [23, 71]. In addition, miRNA-126 is expressed in bone marrow derived cells where it can determine the erythroid and hematopoietic fate of the cell [36-38].

The notion that on one side increased levels of miRNA-126 play a facilitating

role in angiogenesis and on the other side a lowered expression of miRNA-126 supports vasculogenesis may underline the importance of this vascular miRNA as a vasculogenic switch. This hypothesis is supported by the fact that lowered levels of miRNA-126 lead to the increased expression of VCAM-1 that may facilitate homing of leukocytes to the endothelium.

A major gap in current understanding of miRNA-126 biology is knowledge about the molecular mechanisms underlying the regulation of this miRNA in ECs. So far it has been demonstrated that binding of Ets-1 or Ets-2 to the EBS and induction of flow are needed to govern the expression of the EGFL7/miRNA-126 gene [53, 62]. Furthermore, it has been shown that EC-derived apoptotic bodies can increase the levels of miRNA-126 in ECs [43]. Despite these studies, a real understanding whether there are any extracellular factors that may contribute to an altered expression of miRNA-126 is unknown. For instance, cytokines like VEGF and TNFα that mediate endothelial activation, lead to an up regulation of a distinct subset of miRNAs, but not miRNA-126 [87]. It is therefore interesting to design studies to unravel the mechanisms that lead to an increase or abrogation of miRNA-126.

To date the source of miRNA-126 in the circulation is unknown. ECs, circulating cells and platelets can be considered as the major sources that can release miRNA-126 into the periphery. It is likely that these three cell types, and potentially other cell types contribute to the total miRNA-126 content observed in the circulation.

At present no molecular mechanisms has been linked to circulating miRNAs (including miRNA-126) and cardiovascular disease. Whether the source of circulating miRNA-126 is endothelium, circulating cells or platelets, the involvement of miRNA-126 in vascular biology will make it a key component to investigate in patients with cardiovascular risk factors. To date, the use of circulating miRNAs as predictive and/or monitory biomarkers is still in an early phase. However, in the future a spectrum of circulating miRNAs, miRNAs in urine samples [88] or other bodily fluids [89] will be highly informative about the disease status of a patient in the clinic.

Currently no clinical trials to enhance or antagonize miRNA-126 function are, to our knowledge, undertaken. Nevertheless, subjects with cardiovascular risk factors have decreased levels of miRNA-126 in their plasma [46-48], suggesting that mechanisms whereby miRNA-126 could be administered to these subjects could be an effective modality in the prevention of cardiovascular disease.

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