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MicroRNAs in the progression of atherosclerosis: rise and fall of the atherosclerotic plaque

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ABSTRACT

Atherosclerosis is the main cause of mortality globally, being at the basis of most cardiovascular diseases. It is a multifactorial disease, arising from complex interactions comprising changes in lipid metabolism, inflammation and oxidative stress. These factors contribute to endothelial damage and dysfunction, the accumulation of immune cells and smooth muscle cells in the intima, ultimately leading to the formation of atherosclerotic plaques, which restricts blood flow through the vessels. Much progress has been made in the last decades in debunking the underlying mechanisms of atherosclerosis development, especially concerning the evaluation and prediction of plaque stability and the understanding of the roles played by each of the involved cell types. As yet, mechanisms that drive plaque development toward specific 'vulnerable' phenotypes remain undiscovered. Based on recent advancements in RNA therapeutics, this review aims to illustrate a comprehensive overview of miRNAs relevant to various aspects of atherosclerosis and emphasizes their theranostic potential, highlighting their dual role as both drug targets and biomarkers.

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Introduction

In 2020, cardiovascular diseases (CVDs) caused 1.69 million deaths in the EU, making CVDs the leading cause of mortality [1]. The majority of CVDs come as a result of atherosclerosis, the thickening and stenosis of the arterial walls in response to an insult to the endothelial layer (EL) and the accumulation of oxidized low-density lipoproteins (oxLDL) within the tunica intima [2]. Major risk factors for atherosclerosis are hypertension, smoking, diabetes, and dyslipidemia [3]. Biological sex also plays a role as a risk factor for atherosclerosis. In fact, in the EU, total deaths by CVDs in 2020 were 35.3% in female and 30.2% in male populations, and standardized death rates per 100,000 inhabitants were 288.9 and 413.7 for females and males respectively, meaning that sex differences are markedly age-dependent. Moreover, in 2010, a meta-analysis including 23,706 participants reported a sex- and age-dependent prevalence for severe and moderate asymptomatic carotid artery stenosis, with men bearing the highest incidence within all the considered age groups [3]. Such differences could at least in part be explained by the sex-specific regulation of cytokines, transcription factors, and non-coding RNAs (ncRNAs) that has been observed in patients suffering from coronary artery disease (CAD) [4, 5].

ncRNAs are functional RNA molecules that do not encode proteins. Genome-wide association studies (GWAS) are unravelling

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numerous genetic mutations associated with non-coding regions, also affecting atherosclerosis. This review focuses on the roles of micro-RNAs (miRNAs) in the development of atherosclerosis. miRNAs are short single strands of RNA (usually 22 nt long) that can negatively regulate the translation of multiple mRNAs through pairing with target sequences located within their 3' untranslated region (3'UTR). miRNAs are often located within intronic regions of genes and are initially transcribed as pri-miRNAs and processed into pre-miRNAs and mature miRNAs by a protein machinery [6], and can reach out for their target mRNAs either autocrinally, paracrinally or systemically through extracellular vescicles-mediated cell signalling [7, 8]. The burgeoning field of RNA therapeutics demonstrates increasing interest in exploring RNA theranostic potential, merging therapy and diagnostics into a single platform. Theranostic approach aims at simultaneously treating conditions and monitoring therapeutic responses using the same molecular agents. Hence, with this review, we describe the miRNAs known to play significant roles in the different stages of plaque development.

The stages of atherosclerosis

The natural course of an untreated atherosclerotic plaque evolves towards its expansion and subsequently results in arterial stenosis or occlusion. Atherosclerotic lesions tend to develop in supra-aortic trunks (especially carotid arteries), lower limbs (resulting in peripheral artery disease, or PAD), and coronary arteries [9]. Atherosclerosis development involves the participation of macrophages, B- and T-cells, and the secretion of pro-inflammatory cytokines. However, the critical initiating event in atherosclerosis is–the binding of an LDL particle to the basal membrane (BM) of the endothelium [10]. This binding is mediated by the positively charged residues on the outer N-terminal of apolipoprotein B-100 (apo B-100)–the sole apolipoprotein component of LDLs–and the negatively charged glycosaminoglycans of the BM. Once bound to the basal lamina, LDLs become exposed to the oxidising action of the resident lipoprotein lipases (LPL) and platelets [11, 12]. ECs exposed to oxLDL up-regulate their surface expression of cell adhesion molecules, including E-selectin, P-selectin, vascular- and inter-cellular adhesion molecule-1 (VCAM-1 and ICAM-1) [13], thus recruiting monocytes, which transmigrate through the EL, differentiate to macrophages and begin to internalize oxLDL by scavenger receptors-mediated recognition of their oxidized phospholipids [14]. *In vitro* differentiated, PMA-activated macrophages were also demonstrated to internalize native LDLs by macropinocytosis [15]. At this stage, elevated intracellular cholesterol levels activate the liver X receptor alpha (LXRα) transcription factor, master regulator of ATP-binding cassette transporter 1 (ABCA1) and ATP-binding cassette subfamily G member 1 (ABCG1), in turn mediators of cholesterol esters binding to apolipoprotein A-I (apo A-I) in nascent high-density lipoproteins (HDL) for reverse cholesterol transport (RCT) to the liver [16, 17]. However, cholesterol accumulation within the cytoplasm triggers macrophages differentiation towards M1 phenotype, proliferation, and eventually necrotic, apoptotic, or pyroptotic cell death, feeding the necrotic core of the plaque. Pyroptosis, a form of programmed cell death, involves NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome-mediated caspase 1/4/5 activation and gasdermin D-mediated pore formation, resulting in the leakage of cytoplasmic contents and the release of pro-inflammatory cytokines [18]. The disease then progresses via the recruitment of T-cells and vSMCs, which contribute to the growth of the lipidic/necrotic core as well as, in the case of vSMCs, to the fibrotic cap formation.

miRNAs in atheroma development

LDL binding to the intima and oxidation

Retention of LDLs within the intima can be considered the kick-start of atherosclerotic plaque deposition [19]. In physiological conditions, the intact EL and the minimal presence of highly atherosclerotic small dense LDLs prevent such interaction [20]. However, upon the development of endothelial dysfunction (ED) and dyslipidemia, prevalently in arterial regions subject to perturbed blood flow such as bifurcations, BM can be transiently exposed to the blood flow, attracting LDLs with a frequency that is dependent on LDL-C. Several ncRNAs influence the build-up of the atherosclerotic plaque either by regulating LDL-C or by playing a role in ED (**Figure 1**).

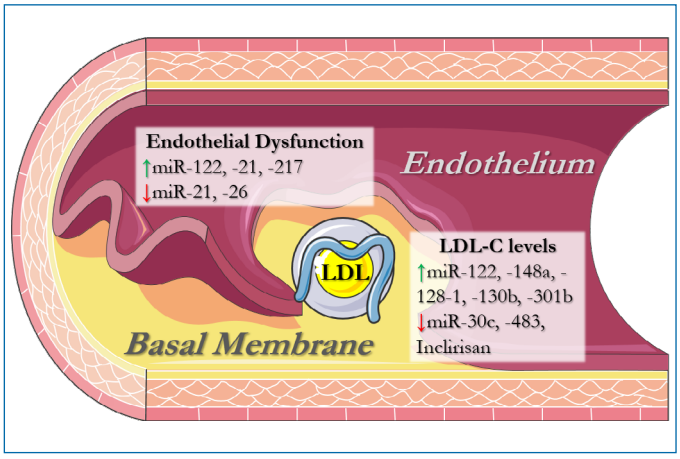

Figure 1 | microRNAs in lipoprotein adhesion to the basal membrane. *Schematic overview of the miRNAs involved in the regulation of endothelial dysfunction, LDL synthesis and uptake. Green arrow: endothelial dysfunction/LDL-C enhancement; red arrows: endothelial dysfunction/LDL-C modulation. Created with Servier Medical Art (https://smart.servier.com), licensed under CC BY 4.0.*

miRNAs regulating LDL synthesis

miR-122 accounts for 70% of total liver-secreted miRNA [21]. In the Bruneck Study, proteomics data from human serum unravelled a linear correlation between miR-122-5p levels and apo B-100, apo C-II, apo C-III, apo E, and apo L-I, and inverse correlations with apo A-IV and apo D [22]. Plasma levels of miR-122 were also shown to correlate with atherosclerosis severity in two independent studies [23, 24], suggesting that it could serve as a useful biomarker. mmu-miR-122a-5p knockout [25] and inhibition [22, 26] in mice resulted in a significant reduction of total cholesterol (TC) levels. Liver-secreted miR-122-5p is able to reach target cells within the liver as well as in muscle and adipose tissues, inhibiting triglyceride synthesis by acting on its putative targets diacylglycerol O-acyltransferase 1 and 1-Acylglycerol-3-Phosphate O-Acyltransferase 1. The authors also observed a concomitant increase in carnitine palmitoyltransferase 1a, which catalyses a limiting-step reaction in β-oxidation, which explains at least in part the correlation between miR-122 and atherosclerosis progression [27]. However, miR-122 is pivotal for several molecular pathways of paramount importance for liver function, making it a poor therapeutic target. Indeed, liver-specific and germline knockout of *miR-122* in mice resulted in reduced plasma TC but increased lipid and cholesterol synthesis in the liver, leading to hepatic steatosis, inflammation, and increased vulnerability to hepatic cancer [25, 28-30].

miR-30c genetic locus resides in intron 5 of the nuclear factor Y subunit (NFY-C) transcript, however, even though pri-miR-30c is ubiquitously expressed where NFY-C is detected, miR-30c-5p is mainly expressed in heart, skeletal muscle and kidney [31]. miR-30c-5p targets MTP, which is responsible for the lipidation of nascent apo B, a critical step for the biosynthesis of very low density lipoproteins (vLDL) and LDL [32, 33], and its reduction in plasma of patients predicted carotid plaque formation by up to 11 years [34]. Moreover, miR-30c-5p overexpression in *Apoe*$^{-/-}$ mice reduced lipoprotein secretion, plasma cholesterol, and triglycerides, and finally the insurgence of atherosclerosis [31, 32]. Intriguingly, in human arterial ECs (HAECs) undergoing oxLDL-induced, forkhead box O3 (FOXO3)/NLRP3-driven pyroptosis, miR-30c-5p expression was dose-dependently reduced by oxLDL treatment. miR-30c-5p transfection in oxLDL-treated HAECs prevented pyroptosis through direct targeting of FOXO3 and consequent inhibition of NLRP3 inflammasome activity [35]. Collectively these data suggest that miR-30c-5p has the potential to represent a relevant target for the development of atherosclerosis therapies. Indeed, in a recent publication, a series of synthetic, more stable miR-30c analogs were tested *in vitro* on HuH7 cells for their ability to inhibit apo B but not apo A-I secretion, with the purpose of future vector-free clinical application [36]. Though MTP inhibition has been associated with hepatic steatosis, the above-mentioned studies confirm that miR-30c-5p-mediated MTP inhibition did not lead to hepatic steatosis in mice models. Still, MTP is also responsible for the lipidation of the CD1 antigen-presenting protein family [31, 37], which should be taken into account when systemically administering miR-30c-5p mimics or analogues. On the other hand, FOXO3 activity has been associated with several cardioprotective functions [38], including atheroprotective roles like the ability to regulate LDL-C homeostasis via control of PCSK9 gene expression [39], therefore careful evaluations are required for miR-30c-based therapeutic strategies to become available for use.

miRNAs modulating LDL and vLDL uptake

Elevated circulating levels of vLDL and LDL represent a key risk factor for the insurgence of atherosclerotic plaque [40]. Lipoproteins can be classified based on their protein content, which has been diligently examined in the last decades, and the picture that we now have depicts a fascinating complexity orchestrating lipoprotein metabolism, with profound implications on their role in the onset of atherosclerosis and consequent CVDs. miRNAs are emerging as key factors in the regulation of several actors of lipoprotein metabolism; in the following section, we provide an overview of ncRNA-based LDL-receptor (LDLR) modulation (**Figure 1**).

In 2015 two independent GWAS were published supporting the role of miR-148a-3p in the regulation of LDLR and ABCA1. Hepatic expression of miR-148a-3p was located under the transcriptional control of SREBP1, in a pathway downregulating LDLR expression in mice [41]. Data from more than 188,000 individuals were compared and miR-148a-3p locus was found to locate nearby several SNPs associated with LDL-C, HDL-C, and TC abnormalities, together with miR-128-1-3p, miR-130b, and miR-131b [42]. Furthermore, all four miRNAs were able to regulate both LDLR and ABCA1 expression *in vitro*, however, only antimiR-148a-3p and -128-1-3p increased HDL-C in *Apoe*$^{-/-}$ mice fed with a western diet, and only antimiR-148a concomitantly decreased LDL-C. Apolipoprotein B mRNA editing enzyme, catalytic polypeptide (Apobec), is responsible for converting Apo B-100 to Apo B-48, which is crucial for the clearance of Apo B-100 from plasma. Recently, miR-148a-3p targeting was evaluated in an *APOB*^{TG} (transgenic) *Apobec*$^{-/-}$ *Ldlr*$^{+/-}$ mice model of atherosclerosis in which no significant effect was observed by miR-148a-3p on circulating LDL-C levels [43]. Although these results might seem in conflict, this could be due to the specific genotype selected for the study. Indeed, we might expect to observe a reduced effect on circulating LDL-C levels when indirectly increasing the expression of *Ldlr* in an *Ldlr*$^{+/-}$ animal model compared to an *Ldlr*$^{+/+}$ counterpart.

miR-483-5p is a miRNA ubiquitously expressed in human tissues which has among its direct targets two strategic molecules for cholesterol metabolism: aldehyde dehydrogenase family 1, subfamily A3 (Aldh1a3) and PCSK9 [44, 45]. By targeting Aldh1a3, miR-483-5p helps maintain pancreatic β cells activity, while miR-483-5p loss in the onset of diabetes results in β cells de-differentiation and loss of insulin expression. Consequently, a statistically significant increase in LDL and a decrease in HDL and triglycerides were observed, along with hyperglycemia [44].

The other key target for miR-483-5p in atherosclerosis, PCSK9, plays a critical role in LDL uptake by binding to LDLR resulting in its lysosomal degradation [46]. In hyperlipidemic mice and humans, serum levels of miR-483 inversely correlate with LDL-C and TC. In mice models and human hepatocytic cell lines, overexpression of miR-483- increased LDLR expression at the protein level, sensibly lowering TC and LDL-C [45]. Notably, EMA recently approved two novel PCSK9 targeting monoclonal antibodies as drugs for the treatment of primary hypercholesterolemia [47, 48], and Inclirisan, a highly durable, liver-specific RNAi therapeutic inhibitor of PCSK9, for the treatment of hypercholesterolemia in combination with the maximum tolerated dose of statins and/or other lipid-lowering agents [49].

miRNAs regulating endothelial dysfunction in atherosclerosis

ED is a critical factor in the onset of atherosclerosis, as it determines the accessibility of the intima to lipoproteins. Its insurgence has been associated with both oxidative and shear stresses. Shear stress is critical for vascular homeostasis, as it regulates remodelling through signalling cascades initiated by integrin and cytoskeletal complexes [50]. However, excessive shear stress can cause the accumulation of oxidative damage, ultimately leading to ED. Research involving multimodal imaging and wall shear stress signatures from 37 patients undergoing computed tomography angiography, determined that luminal exposure to high shear stress, either alone or combined with a lipid-rich plaque phenotype, was associated with accelerated plaque progression at 1-year follow-up [51]. Several miRNAs play a role in the development of these conditions (**Figure 1**). In *Apoe*$^{-/-}$ mice, fed a normal vs high-fat diet, it was shown that oxLDL induced EC apoptosis by upregulating miR-122 and reducing the expression of its target, X-linked inhibitor of apoptosis (XIAP). This effect was confirmed at both mRNA and protein levels [52]. miR-122-5p regulation of XIAP activity was also described in pancreatic cancer patients, where the downregulation in macrophage-derived exosomes of miR-122-5p inhibitor lncRNA SBF2-AS1 reduced XIAP activity in pancreatic cancer cells, therefore, enhancing apoptosis [52]. *In vitro* evidence on Huh7 cells suggests that cholesterol mediates miR-122-5p-loaded exosome release from hepatocytes through induction of lysosome dysfunction [53]. This evidence delineates a leading role for miR-122-5p in lipoprotein metabolism as well as in the deleterious effect of oxLDL accumulation in the intima on the endothelium.

By targeting PTEN, miR-21-5p plays an anti-apoptotic role on ECs subject to shear stress stimulus [54]. On the other hand, an inverse correlation was found between plasma levels of endothelial nitric oxide synthase (eNOS) and

i) miR-21 in preclinical atherosclerotic patients with hypertension [55];
ii) monocytes miR-21-5p expression levels in patients with CAD [56].

Although eNOS does not happen to be its direct target, miR-

21-5p indirectly regulates its activity by targeting dimethylarginine dimethylaminohydrolase-1, thus crippling the degradation rate of eNOS inhibitor asymmetrical dimethylarginine [57].

In a similar manner to miR-21, miR-26-5p was found to have anti-atherosclerotic, endothelium protective functions on account of its ability to directly target PTEN in ECs. Indeed, while its silencing increased atherosclerosis-related gene expression, its overexpression resulted in the opposite outcome. Interestingly, eNOS mRNA expression followed the opposite trend. Furthermore, miR-26-5p was downregulated in CAD patients and the *Ldlr*$^{-/-}$ *Apoe*$^{-/-}$ mice model [58].

miR-217 was also found to indirectly inhibit eNOS expression in a mice model of atherosclerosis [59]. As a result of miR-217 overexpression, NO production was reduced, and with it, the relaxation of aortic arc walls and the lumen of the aorta and carotid arteries led to increased blood pressure, which translates into shear stress and exacerbated ED. On the contrary, inhibition of endogenous vascular miR-217 in *Apoe*$^{-/-}$ mice ameliorated vascular contractility and reduced atherosclerosis. Furthermore, miR-217 was suggested as a biomarker of vascular aging and cardiovascular risk, though further studies with broader cohorts are needed.

miRNAs involved in cholesterol processing by macrophages

Accumulation of lipids within macrophages results in foam cell formation, proliferation, and atherosclerotic plaque maturation through the widening of the lipidic core. Several miRNAs are associated with this stage of atherosclerosis (**Figure 2**).

A computational study implied that miR-155-5p together with miR-33 have among their direct targets the cholesterol transporter ABCA1 [60]. miR-155 plays a role in determining macrophage polarisation by inhibiting the M2 phenotype [60], and miR-155-5p is upregulated in plasma and plaques of atherosclerotic patients [61]. Moreover, miR-155-5p targets LXRα, a transcriptional activator of ABCA1 [62], and transcription factor HMG-Box Transcription Factor 1, inducing an increase in lipid uptake and reactive oxygen species (ROS) formation [63]. In the case of miR-33, two different studies inquired about its activity in relation to cholesterol in mice and human macrophages. Interestingly, acetylated (AcLDL) but not oxLDL stimulated miR-33 expression. In a mice macrophage cell line treated with AcLDL, miR-33 was shown to favour foam cell formation by targeting not only ABCA1 but also ABCG1 and the endolysosomal transport protein Niemann-Pick disease, type C1 (NPC1), reducing apo A-I and HDL cholesterol efflux [64]. However, miR-33 inhibition of ABCG1 was not confirmed in humans, as in human THP1 macrophages, only ABCA1 and NPC1 regulation by miR-33 was observed. Furthermore, miR-33-5p modulates fatty acid oxidation by targeting peroxisome proliferator-activated receptor coactivator-1α (PGC-1α) and several of its downstream effectors [65, 66]. Quite similarly, miR-486-5p overexpression in foam cells indirectly inhibits cholesterol efflux, though by targeting histone acyl-transferase 1, resulting in decreased *ABCA1* expression [67]. Moreover, the well-studied miR-27a and b miRNA couple targets foam cell formation by directly hitting on two central players, namely LPL and again ABCA1, hence inhibiting both cholesterol uptake and HDL secretion by foam cells. Overall, their effect has been evaluated as atheroprotective [68]. LPL, a 52-kDa glycoprotein, is the primary enzyme responsible for the hydrolysis of triglycerides in chylomicrons and vLDL, resulting in the production of chylomicron remnants and IDL, and is expressed by macrophages, muscle and adipose cells [69, 70]. miR-10-5p exerts a protective role on foam cells by targeting ligand-dependent nuclear receptor corepressor (Lcor) translation, resulting in upregulation of PGC-1, which in turn enhances the transcription of genes involved in the oxidation of fatty acids [71]. miR144-3p also inhibits cholesterol efflux by directly targeting ABCA1, and in addition, it was associated with an increased expression of inflammatory cytokines IL-1β, IL6, and TNFα [72]. miR-320b-3p was found to inhibit ABCA1 and ABCG1 both directly and indirectly by LXRα inhibition, and it was also found to directly target endonuclease-exonuclease-phosphatase family domain containing 1, which also supports cholesterol efflux [73]. miR-148a-3p was shown to ameliorate macrophage cholesterol efflux and inflammatory secretome profile, effectively reducing the insurgence of atherosclerosis in *Apob*^{TG} *Ldlr*$^{+/-}$ *Apobec*$^{-/-}$ mice [43]. Hydrolysed triglycerides are potent macrophage recruiters, consequently, LPL genetic knockout in mice macrophages dramatically reduced foam cells-driven atherosclerotic plaque development [70]. In line with this, miR-590-3p-mediated LPL targeting in human THP1 macrophages indirectly modulates plaque lipid accumulation *in vitro* [74]. miR-34a-5p targets ABCA1 and ABCG1 in macrophages and is highly abundant in atherosclerotic lesions [75]. Consistently, miR-34a-5p conditional knockout in myeloid cells as well as in bone marrow cells reduces atherosclerosis in *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice, respectively [75].

A strong correlation was found in diabetic patients between low serum adiponectin levels and impaired RCT, while only in macrophages from diabetic patients, adiponectin administration *in vitro* led to AdpR1/LXRα-dependent increase in ABCG1 expression, resulting in enhanced cholesterol efflux and reduced foam cell formation [76]. Intriguingly, miR-150-5p targeting of adiponectin receptor-2 (AdpR2 increases cholesterol efflux by enhancing ABCA1 and ABCG1 expression in oxLDL-treated THP-1 macrophages

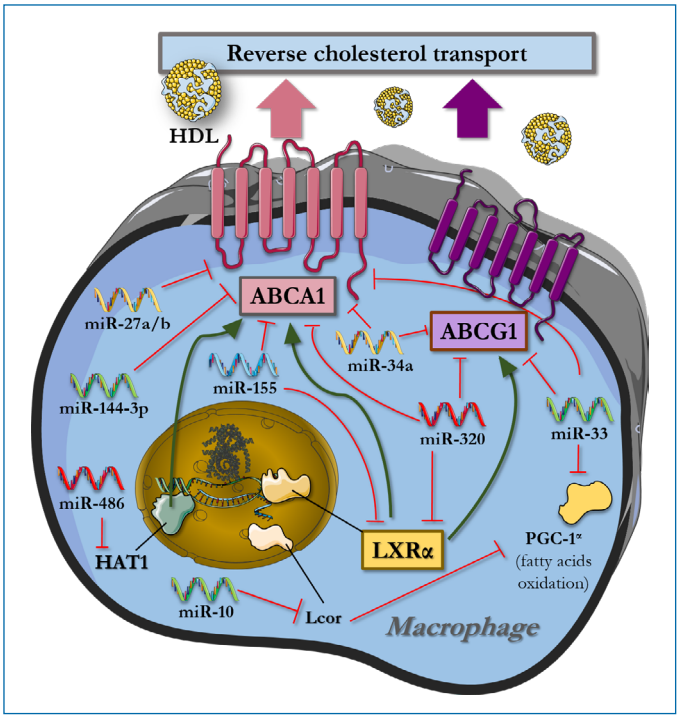

Figure 2 | Role of microRNAs in reverse cholesterol transport regulation within the macrophage. *Schematic representation of the miRNAs known to affect HDL nucleation and foam cell formation within plaque resident macrophages. Green arrows: positive transcriptional regulation; red connectors: negative translational regulation. Created with Servier Medical Art (https://smart.servier.com), licensed under CC BY 4.0.*

[77]. One plausible explanation for this seemingly dual effect of adiponectin on RCT in macrophages, left aside the different experimental models used, might reside in the differential expression of the two receptors for adiponectin in M1 and M2 macrophages. Indeed, adiponectin has pro- and anti-inflammatory effects in M1 and M2 macrophages, respectively, due to the activation of two different signalling pathways (p38 mitogen-activated protein kinase and peroxisome proliferator-activated receptor alpha, respectively) [77]. Considering that macrophage priming with oxLDL leads to M1 polarisation, a phenotype characterized by a high AdoR1/2 ratio, in which adiponectin administration induces LXRα expression and upregulation of cholesterol efflux. We can conclude that AdpR2-dependent upregulation of cholesterol efflux by miR-150-5p in M1 macrophages could represent a promising target for therapeutic purposes in atherosclerosis, thus further studies are needed to better elucidate its mechanism of action.

The role of miRNAs in neointima expansion

After macrophages infiltrate the intima and differentiate into foam cells, the process of neointima formation begins [78]. Besides the well-characterized role of foam cells, B- and T-cells, it has been demonstrated that other effectors of innate immunity play a role in atherosclerosis. oxLDL have been reported to modulate macrophage-natural killer (NK) cell interaction in the plaque [79, 80]. Indeed, oxLDL induce IL-12 production by macrophages, activating resident NK cells. Anti-phosphorylcholine-opsonized oxLDL can instruct dendritic cell (DC)-NK cell interactions, leading to the exacerbated generation of interferon gamma (IFNγ) by NK cells [81]. IFNγ released by macrophages and DCs, activate NK cells, increasing their pro-apoptotic activity against SMCs and generating pro-inflammatory M1-like macrophages in the plaque, finally contributing to plaque rupture [81]. Mast cells (MCs) have been also found to be involved in plaque growth and destabilization [82-86]. MC-released tryptase and chymase trigger foam cell collapse generating a catastrophic line of events [82]. Activated SMCs secrete extracellular matrix components, forming a fibrous cap enveloping the plaque necrotic/lipidic core, the robustness of which determines plaque stability. Several miRNAs have been revealed to modulate key aspects of this phase as detailed in the following sections.

Macrophages-T cells crosstalk and inflammation

Systemic inhibition of miR-148a-3p polarized macrophages toward an M2-like phenotype, therefore inhibiting the expression of pro-inflammatory cytokines such as TNFα and IL-6, inducible-NOS, and cyclooxygenase-2, ultimately resulting in the formation of more stable plaques, as assessed by fibrotic cap thickness and necrotic core evaluation [43]. miR-155 is highly expressed in macrophages, especially by the M1 subtype, where it has been shown to act downstream of toll-like receptor (TLR), by inhibiting B-cell lymphoma 6 (BCL-6), therefore upregulating TNFα and chemokine (C-C motif) ligand 2 (CCL2,) that are key activators of M1 polarization [87]. On the other hand, hyperglycaemic mice transplanted with *miR-155*$^{-/-}$ vs wild-type bone marrow developed more severe atherosclerosis, characterized by the presence of more pro-inflammatory macrophages and granulocytes, fewer T-regs, and less stable plaques [88]. The most relevant difference between the two reported studies resides in the genotype of the mice models used to induce atherosclerosis, as while the former study used *Apoe*$^{-/-}$ donor and recipient mice, the latter settled the matter just using *Ldlr*$^{-/-}$ recipient mice. Consequently, the impaired RCT was observed only in the first study, suggesting that the miR-155 net effect is pro-atherosclerotic when RCT is impaired in macrophages, however, the opposite is true in a closer to physiological *Apoe*$^{+/+}$ design. design. Notably, miR-155-5p was also shown to directly target inositol phosphatase, responsible for the hydrolyzation of the 5′ phosphate of Phosphatidylinositol (PI)-3, 4, 5-P3 to generate PI-3,4-P2 (89). This process impedes PI3K-mediated membrane localisation of signaling molecules such as protein kinase B and phosphoinositide phospholipase Cγ, with serious implications for the differentiation of leukocytes and their subpopulations. Collectively, these findings imply that both *APOE* and miR-155 genotypes should be carefully evaluated in the interpretation of miR-155 involvement in atherosclerosis, with preference given to conditional knockout approaches.

A group of miRNAs was shown to inhibit nuclear factor kappa-light-chain-enhancer of activated B cells (Nf-Kβ) pro-inflammatory signalling in macrophages, namely miR-147, miR-21, and miR-146a/b. Interestingly, miR-147-3p is part of a negative feedback loop upon pro-inflammatory activation of TLRs 2, 3, and particularly 4, where it downregulates the expression of TNFα and IL-6 [90]. Similarly, miR-21-5p implemented its negative feedback activity by targeting PDCD4 upon lipopolysaccharide (LPS) stimulation in macrophages, resulting in reduced IL-6 and augmented IL-10 production [91]. miR-21-5p upregulation was also observed in CD34+ peripheral blood mononuclear cells (PB-MNCs) of diabetic patients affected by severe PAD (92). miR-146a-5p and b-5p both were shown to inhibit key players in TLR signalling: TNF receptor-associated factor 6, and IL-1 receptor associated kinase 1, in a TLR-NF-kβ-dependent manner [93].

Inflammation, therefore, plays a central role in atherosclerosis, and sex differences in the transcriptional regulation of inflammatory pathways in CVDs have been previously reported [94]. In mouse-derived splenic lymphocytes, plasma levels of anti-inflammatory miR-146a are negatively regulated by estrogen [95]. Consistently, in patients below 55 years of age plasma miR-146a is significantly higher in men, though with ageing it decreases significantly faster in men than in women [96]. Moreover, mouse studies described a male-specific induction of miR-23a-3p, miR-27b-3p, miR-130a-3p, miR-133a-3p, miR-143-3p, and let-7e-5p, coupled with a corresponding downregulation of their molecular targets involved in mitochondrial metabolism, hence contributing to sex-related differences in cardiac remodeling [4]. These results imply that sex represents a critical variable that necessitates consideration when selecting miRNAs as biomarkers or therapeutic targets in atherosclerosis. Thus, further studies are imperative to comprehensively elucidate sex-specific differnces in atherosclerosis.

miRNAs in CD34^{+} *HSPCs participation to atherosclerosis*

Recent data increasingly elucidate the involvement of bone marrow (BM)-derived CD34^{+} hematopoietic stem/progenitor cells (HSPCs) in atherosclerosis. Specifically, studies have indicated a positive correlation between levels of total- and LDL-C with the mobilisation of CD34^{+} HSPCs; suggesting that the release of HPCs within the bloodstream may represent an early reaction to the development of atheroma [97] (**Figure 3**). Alternatively, patients with CAD exhibited reduced levels of CD34^{+} HSPCs in their bloodstream in comparison with healthy individuals [98]. Although these findings might appear contradictory, it must be noted that while the former study identifies HSPCs as CD45^{dim}CD34^{+}, the latter only describes them as CD34^{+} buffy coat cells, therefore likely including CD45^{+}CD34^{+} cells with potentially different characteristics. Notably, it was demonstrated that CD34^{+} HSPCs mobilization has a negative effect on the atherosclerotic plaque environment, exacerbating inflammation on account of their differentiation into macrophages and eventually foam cells [99], a process shown to be modulated by ncRNAs.

A monocytic lncRNA, was suggested to enhance atherosclerosis progression by promoting CD34+ HSPCs differentiation to monocytes/macrophages by sequestering miR-199a-5p, thus inducing the expression of activin A receptor type B (ACVR1B) [100, 101]. Transplantation of miR-155$^{-/-}$ BM-HSCs in BM-depleted *Ldlr*$^{-/-}$ mice caused increased M1 macrophages, reduced T-helpers, and plaque destabilization [88].

CD34+ HSPCs support microvasculature growth paracrinally and can differentiate to ECs (102-104). miR-378 modulates the proangiogenic potential of CD34+HSPCs with beneficial effects on ECs in patients with myocardial infarction [105]. Moreover, patients with PAD and diabetes mellitus (DM) show a decreased mobilization of CD34+HSPCs characterized by a dysregulated angiogenic activity [106, 107].

Such alterations may arise from an altered miRNA expression within diabetic CD34+HSPCs. Notably, miR-155-5p and miR-21-5p downregulation were observed in diabetic CD34+HSPCs, resulting in poor cell survival and increased apoptotic induction [92, 107].

Moreover, miR-21-5p downregulation in BM-derived CD34+ HSPCs is associated with an increased expression of its target tumor suppressor programmed cell death protein 4, and that this pro-apoptotic signal can be paracrinally transferred to ECs through taurine upregulated gene 1, a lncRNA sponging miR-21-5p [92]. Furthermore, serum and CD34+HSPCs from patients with DM and PAD had elevated levels of miR15a and miR16 impairing CD34+HSPCs migration and adhesion [108].

miRNAs in SMC recruitment and differentiation

Two recent studies showed that approximately 40-70% of the mature plaque resident cells originate from migrating vSMCs [109-111]. Indeed, during atherogenesis contractile vSMCs in the media transition into mesenchymal-like cells, then migrate into the intima, and subsequently differentiate into synthetic, macrophage/foam cell-like, or osteogenic phenotype, eventually supporting lesion expansion [112, 113]. Traditionally, the participation of SMCs in plaque formation was associated with the sole synthesis of the fibrous cap. However, their ability to differentiate to a plethora of phenotypes has represented a paradigm shift [114]. Several ncRNAs play roles in these processes.

Recent findings uncovered BCL2 [B-cell lymphoma 2]-associated transcription factor 1 (BCLAF1) expression in SMCs to correlate with plaque stability. Downregulated BCLAF1 was indicative of high lipid content, low SMC de-differentiation, and reduced plaque infiltration [115]. Relevant to the purpose of this review, BCLAF1 expression

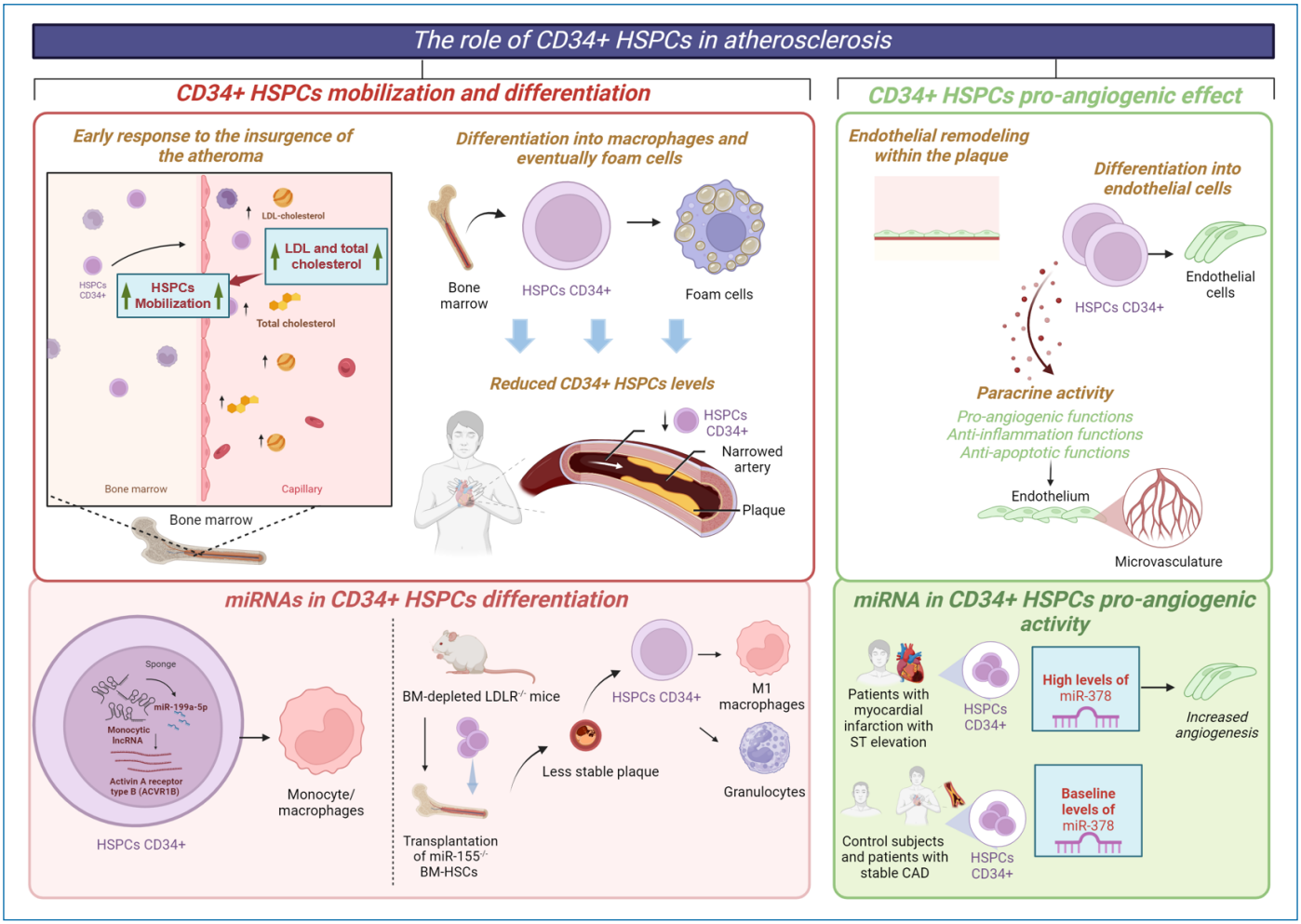

Figure 3 | miRNAs and the role of CD34+ HSPCs in atherosclerosis. *The role of miRNAs in the mobilization, differentiation (left panel), and function (right panel) of CD34+ HSPCs in the development of atherosclerosis. HSPCs: hematopoietic stem and progenitor cells; LDL: low-density lipoprotein; BM-HSCs: bone marrow hematopoietic stem cells; LDLR: LDL receptor; ST: S-T electrocardiogram segment; CAD: coronary artery disease. Created with BioRender.com*

(and subcellular relocation) was associated with the maintenance of an undifferentiated state in hematopoietic progenitors [116]. Interestingly, in this particular context, its expression was modulated by miR-194-5p, which overexpression in a mice model of abdominal aortic aneurism increased the rate of vSMCs apoptosis [117]. Finally, lnc-SOX2OT was found to sponge miR-194-5p *in vitro* and *in vivo* resulting in reduced apoptosis of gastric cancer cells [118]. Hence, BCLAF1 is emerging as a key modulator of vSMC differentiation in atherosclerosis. However, due to the association of BCLAF1 expression to SMCs role in atherosclerosis being observed only recently, direct inquiries within the lnc-SOX2OT/miR-194/BCLAF1 axis in the context of atherosclerosis are still lacking, which could yet unravel important breakthroughs.

Lnc-SOX2OT also targets miR-145-5p, another miRNA expressed in SMCs, and plays a role in vascular diseases [119]. In cultured vSMCs, silencing of lncRNA-SOX2OT inhibited Angiotensin II-mediated induction of oxidative stress and inflammation. lncRNA-SOX2OT was shown to act by sponging miR145-5p, thus upregulating its target early growth response factor-1 (EGR1). miR-145 is emerging as a key modulator of vSMC de-differentiation. Indeed, miR-145-5p is the most abundant miRNA in vSMCs and its expression is quickly downregulated upon de-differentiation [120, 121]. Moreover, miR-145 overexpression in embryonic stem cells leads to their differentiation towards SMC phenotype, through the downregulation of its direct target Kruppel like factor-4 (KLF-4) and subsequent enhancement of myocardin expression [122]. These findings well correlate with the findings by Cordes and colleagues, showing that miR-145-5p is not expressed by vSMCs throughout arterial development, only to be observed in post-natal, completely developed arteries [121]. Moreover, *in vitro* under platelet-derived growth factor β (PDGFβ) stimulation as well as in balloon-injured arteries, de-differentiated SMCs expressed significantly lower levels of miR-145-5p compared to PDGFβ untreated- and uninjured- controls respectively [120]. Interestingly, LPS was found to repress *miR-145* transcription in PB-MNCs and to drive vSMCs dedifferentiation in vascular diseases suggesting a role for bacteria in inflammation-driven vSMCs recruitment [123-125]. Notably, low amounts of LPS constantly flow out of the intestine, become inactivated through loading into vLDL and LDL, and are reactivated by the chemical modification of LDLs happening in the atherosclerotic plaque, resulting in macrophage activation upon interaction with TLRs [126, 127]. Consistently, plasma LPS is a risk factor in the development of atherosclerosis and is present in atherosclerotic plaques [128]. miR-145 was also shown to target two other key factors in the determination of vSMCs fate: KLF4 and -5 [129]. Brilliant work by Deborah Chin et al. shows miR-145-5p was successfully delivered to proliferative vSMCs onsite by using C–C chemokine receptor-2-targeting micelles in an *APOE*$^{-/-}$ atherosclerosis mice model, resulting in significant mitigation of the disease progress both in early and mid-stages [130]. Specifically, targeted delivery of has-miR-145-5p-carrying micelles resulted in significantly reduced plaque lesion size and necrotic core area, while, of note, collagen I content was increased, therefore preserving plaque stability. Of note, hsa-mir-145-5p shares a 100% identity with mmu-miR-145a-5p. These results were a confirmation of what was observed by others in the same mice model but using lentiviral-mediated, SMC-specific *miR-145* overexpression, where it reduced macrophage plaque infiltration and lower serum CCL2 levels were also observed [130]. Consistent with these data, lentiviral expression of miR-145 antisense oligonucleotide resulted in increased expression of pro-inflammatory cytokines including CCL2 in tissues, leading to increased macrophage infiltration and proliferation [123]. Moreover, another study reported elevated expression levels of *miR-143/5* in saphenous vein vSMCs from patients with type 2 diabetes. This peculiar phenotype was induced by the diabetic milieu through TGFβ stimulation and resulted in reduced proliferative potential and plasticity of vSMCs [131]. These results suggest that miR-145 is central to the process of trans-differentiation of vSMCs towards their proliferative, synthetic, osteogenic, and macrophage/foam cell-like phenotypes fuelling atherosclerotic plaque progression. Therefore, miR-145 could represent a strategic therapeutic tool for the treatment of atherosclerosis. However, a recently completed single-centered interventional study proved a positive correlation exists between the miR-145-5p plasma levels in atherosclerotic patients and cardiovascular risk calculated with the American College of Cardiology/American Heart Association (ACC/AHA) index (NCT03855891, [132]). Another study, using knockout of *mir-143/145* in *Ldlr*$^{-/-}$ mice reported a reduction in plaque size and increased macrophage infiltration [133]. Therefore, we may conclude that careful attention must be paid to finely target the delivery of miR-145.

miR-143 is co-transcribed with *miR-145* as they both reside in the same bicistronic precursor in human chromosome 5, under the transcription control of serum response factor (SRF), myocardin and NK2 transcription factor related, locus 5 (Nkx2-5) [121, 134]. Furthermore, miR-145-5p and miR-143-3p cooperatively target KLF4 and ETS Like-1 to promote differentiation and repress the proliferation of SMCs. Plasma from patients with unstable atherosclerotic plaques contained significantly lower levels of miR-143-3p compared with plaque-free controls and showed a non-significant downregulation trend compared to patients with stable plaques [132].

miR-181a-5p/b-5p are both involved in vSMCs differentiation toward a synthetic phenotype through targeting of SRF, upstream of the afore-described *miR-143/145* cluster, also playing a role in promoting SMCs proliferation and migration [135]. Consistently, miR-181b-5p was found overexpressed in the plasma of patients with stable plaques compared to unstable plaques and plaque-free patients [132]. Moreover, its expression was enhanced in response to pro-inflammatory stimuli in plaque-derived SMCs, but not in SMCs from healthy donors [136]. Interestingly, angiotensin-II promotes atherosclerosis at least in part by enhancing the expression of osteopontin, which in turn enhances vSMCs migration. Osteopontin is negatively regulated by miR-181a-5p, and miR-181a-5p overexpression attenuated angiotensin-II-induced increase in vSMCs migration on collagen fibres [137].

The role of miRNAs in determining plaque stability

A vulnerable, or unstable, plaque is defined as a plaque containing a large necrotic core, a thin fibrous cap, and elevated levels of apoptosis, necrosis, pyroptosis, and pro-inflammatory cells [138]. In this respect, vSMCs traditionally play a pivotal role, as they are considered responsible for the synthesis of the fibrous cap. miR-126 treatment of mice arteries increased the sub-intimal relocation of vSMCs, which was associated with a concomitant increase in fibrous cap thickness. However, vSMCs mobilisation is not sufficient to guarantee plaque stability, as vSMCs progenitors can transdifferentiate towards de-stabilizing phenotypes such as macrophage/foam cell-like. Therefore, maintenance of a pro-synthetic, contractile phenotype must also be addressed. Within this context, lentiviral as well as targeted micelle-based delivery of miR-145-5p achieved vSMCs differentiated phenotype maintenance while improving extracellular matrix deposition and fibrous cap thickness in *Apoe*$^{-/-}$ atherosclerotic mice model [130, 139]. DNA topoisomerase II inhibitor teniposide was shown to prevent phenotypic switch of

vSMCs both *in vitro* in human aortic SMCs and *in vivo* in mice, and its effects were shown to be at least in part due to the transcriptional activation of *miR-21* [140]. Moreover, *miR-21* overexpression in vSMCs reduced ROS-induced apoptosis [139], increased proliferation, and differentiated vSMCs towards a synthetic phenotype [141]. IFNγ secreted by T-cells hinders vSMCs proliferation and ability to differentiate towards a synthetic phenotype [142]. miR-29 was shown to directly target IFNγ mRNA in immune cells [143], however, miR-29a-3p also targets genes for extracellular matrix proteins in vSMCs, while treatment of aortic wall with miR-29a-3p inhibitors enhanced matrix synthesis [144]. Therefore its role in fibrous cap modulation is still not completely elucidated. Expression of miR-24-3p in foam cells inversely correlates with plaque stability. Accordingly, miR-24 directly targets matrix metalloproteinase-14 (MMP-14), resulting in reduced invasiveness by macrophages and plaque instability [145]. miR-210-3p plasma concentration was shown to positively correlate with plaque stability in patients with carotid plaque [146]. Moreover, the same study showed that miR-210 enhances plaque stability in mice by targeting APC and Wnt signalling, therefore promoting vSMCs survival and pro-fibrotic differentiation.

Pyroptotic and necrotic death of macrophages exacerbate inflammation and undermine plaque stability. In this contest, miR-210-3p reduces ATP and increases ROS levels by targeting 2,4-dienoyl-CoA reductase1 (Decr1), which is pivotal in the β oxidation of unsaturated fatty acids, acting under the transcriptional activation operated by HIF-1α, enhancing macrophages necroptosis [147]. Recently, miR-21-5p expression in macrophages after efferocytosis was associated with a protective effect, as it blocked LPS-induced overexpression of TNF-α, thus reducing inflammation [148]. Such observation was carried out into blood monocyte-derived, *in vitro* differentiated macrophage models, therefore more studies are needed to ascertain whether it applies to the *in vivo* atherosclerotic contest. Interestingly, miR-223-3p was shown to directly target the NLRP3 inflammasome, silencing inflammation in macrophages, therefore showing promise as a therapeutic tool to improve plaque stability [149].

miRNAs as biomarkers for cardiovascular risk in atherosclerotic patients

Atherosclerosis can remain a latent and elusive pathology up until the manifestation of major clinical symptoms, such as stroke or myocardial infarction. Moreover, atherosclerotic plaques can either develop into stable or unstable plaques, depending on their inner composition. Hence, the identification of novel biomarkers to easily assess cardiovascular risk in atherosclerotic patients is of paramount importance. The role of several miRNAs has been proven pivotal in the development of artherosclerosis, and many of these miRNAs are secreted in the bloodstream by producer cells before they can reach their targets, suggesting that the detection of a peculiar miRNA pattern within the bloodstream might be descriptive of a corresponding, quantifyiable cardiovascular risk in atherosclerotic patients. Indeed, the concept of using circulating miRNA patterns as diagnostic biomarkers has been largely considered in the last decades for several pathologies, including though not limited to, colorectal cancer, nervous system, kidney, liver, and cardiovascular diseases [150-152], miRNAs need a stabilizing carrier to circulate within the bloodstream, as they would otherwise be readily degraded by plasma RNases [153]. Such a carrier system has been identifyied and sorted in two different modalities, referred to as extracellular vescicles (EVs)- and argonaute-2 (Ago2)-mediated transportation [154]. Both of these transportation systems offer a chance to detect miRNAs as biomarkers to define the cardiovascular risk for an atherosclerotic patient. Bloodstream circulating miRNA detection has been carried out by examining whole blood, PB-MNCs, EVs, serum, or plasma, and by using RNA sequencing or PCR-based readout systems [150]. Importantly, it was demonstrated that EVs- and Ago2-based carrying systems are only partly redoundant, hence miRNAs are selectively sorted into each carrying system upon secretion [152]. To selectively inquire EVs-derived miRNA, an initial purification step by either ultracentrifugation, ultrafiltration, size-exclusion chromatography, immunoaffinity, or a growing number of alternative methods is needed and followed by vescicles lysis, miRNA isolation an detection [152].

In a mice model of atherosclerosis, a miRNA signature was associated with plaque formation that included miR-378d, miR-181b-5p, miR-146a-5p, miR-421-3p, miR-350-3p, and miR-184-3p deregulation, by using Illumina deep sequencing and Taq-Man Real Time RT-PCR [155]. In patients, it was shown that the combination of EV-derived miR-17-5p, miR-126-5p, and miR-145-3p can indeed improve diagnostic accuracy for myocardial infarction [156]. The clinical interventional study entitled "microRNAs in the Diagnosis of Atherosclerotic Plaque Instability (NCT05680935)" is currently recruiting participants and aims at identifying miRNAs as novel circulating biomarkers for atherosclerosis progression [157].

The ever-growing number of candidate biomarkers, in association with the development of smarter, solid, and cost-effective techniques for miRNA detection and sequencing is currently unlocking a new era in theranostics, which represents a promise in the quest for novel diagnostic biomarkers for cardiovascular risk determination in atherosclerotic patients.

Conclusion and future perspectives for miRNA-based theranostics in atherosclerosis

A growing body of research highlights the pivotal role of miRNAs in the progression of atherosclerosis, spanning from the sub-clinical appearance of ED and dyslipidaemia, to the expansion of atherosclerotic lesions, thinning, and rupture of the fibrous cap. The presence of miRNAs in various bodily fluids, their stability, and their capacity to reflect dynamic changes during disease progression underscore their potential as disease biomarkers, However, significant challenges persist in both therapeutic and diagnostic applications that need to be addressed [158].

Currently, several RNA therapeutics targeting lipid components of atherosclerosis are in development [159]. Yet, there is a growing focus on identifying druggable miRNA targets related to inflammation or vSMCs. A notable limitation in this pursuit is the often low tissue and cell specificity of miRNAs, which complicates their use as precise drug targets [158]. Addressing this challenge requires the development of RNA delivery systems capable of specifically targeting miRNAs to the affected cells, thereby minimizing systemic side effects [159].

Diagnostic and prognostic applications of miRNAs in atherosclerosis have been substantiated by several studies. However, only a limited number of these biomarkers have been successfully validated across diverse cohorts. An important challenge in analyzing miRNAs in bodily fluids is the influence of sample type and various pre-analytical factors, such as lipemia and hemolysis [160]. To advance miRNAs from basic research to their clinical application, it is imperative to standardize procedures across pre-analytical, analytical, and post-analytical stages of miRNA quantification. Such standardization is essential for translating miRNA research into practical clinical tools.

Conflict of interests

The authors declare no conflict of interest exists.

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Author contribution

Conception and supervision: Gaia Spinetti; writing-original draft preparation: Andrea Rampin, Martina Mutoli, Miron Sopic, Antonino Bruno, Massimiliano Martelli, Alberto M. Settembrini; writing-review & editing: Andrea Rampin, Gaia Spinetti, Miron Sopic, Tijana Mitic, Fabio Martelli .; preparation of figures: Andrea Rampin and Martina Mutoli. Preparation of figure legends: Andrea Rampin. All authors have read and agreed to the published version of the manuscript.

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