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Therapeutic targeting novelties in microRNAs-based treatment for atherosclerosis- A brief review

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It is known that several microRNAs (miRNAs) have an important regulatory role during different stages of atheroma plaque formation. In preclinical studies, regulation of miRNA expression has shown benefits in the treatment of atherosclerosis. Thus, therapeutic targeting of miRNAs represents an attractive approach for the treatment of atherosclerosis in preclinical and clinical studies. Different approaches have been undertaken to determine the therapeutic potential of miRNA. To repress pathological miRNAs or over-express protective miRNAs, miRNA inhibitors or miRNA mimics, respectively, have been employed. Despite significant achievements in the field, cellular uptake, the potential need for multiple doses to achieve the desired effect, biodistribution, the ability to target a specific tissue or cell, the unpredictable and unwanted side effects, and toxicity still remain the major limitations for miRNA-targeting therapies. All of these challenges have emphasized the need to develop more efficient delivery systems for miRNA therapeutics in the context of atherosclerosis. Thanks to the development of nanotechnology in the field of molecular biology, novel delivery miRNA-base therapies have emerged. In the present review, we focus on new tools that have been recently used to deliver miRNA-base treatment to strengthen vascular defenses and delay atherosclerosis development. We discuss the future perspective and challenges that are facing the scientific community.

Keywords: MicroRNAs, Atherosclerosis, Therapeutics, Nanoparticles, pH-Low Insertion Peptide, Exosome

Introduction

Noncoding RNAs (ncRNAs) are RNA molecules transcribed from the genome that do not encode proteins but produce noncoding transcripts that regulate gene expression and protein function. The two major classes of ncRNAs are microRNAs (miRNAs) and long noncoding RNAs (lncRNAs). It was in 1998 when miRNAs, 18-to 22-nucleotide ncRNA, were first described in the nematode Caenorhabditis elegans as developmental regulators (Fire et al., 1998). Thousands of miRNAs have been described since then in both, healthy and pathological states, being involved in nearly every biological pathway. It is now known that miRNAs regulate and control gene expression in most organisms, and they have the ability to target multiple genes, generally within the same or in a related pathway. miRNAs function as post-transcriptional regulators, providing a ubiquitous mechanism for control of gene expression either via cleavage and degradation of target mRNA or by inhibition of the translation process. The biogenesis and function of miRNA have been widely described in reviews by Bartel, Ardekanni, Ha, and Krol (Bartel, 2009; Ardekani, 2010; Ha and Kim, 2014) among others. Interestingly, a number of miRNAs are involved in the modulation of several key processes in every stage of plaque development, and the regulation of miRNA expression could prove beneficial in the treatment of atherosclerosis. A single miRNA has the ability to coordinately regulate the expression of numerous genes across different signaling pathways to execute a particular phenotypic trait. Therefore, alterations in the expression of one specific miRNA involved in different aspects of atherogenesis can enhance its impact on plaque formation.

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LncRNAs are larger transcripts, typically more than 200 nucleotides in size, that are synthesized similarly to mRNAs, but not translated into proteins (Cabili et al., 2011). It has been described that lncRNAs have two types of functional elements, the ones that directly interact physically with other nucleic acids, proteins, or lipids, and the structural elements, which direct their functional interactions, leading to the occurrence of secondary and/or tertiary RNA structures (Fabbri et al., 2019). As miRNAs, lncRNAs are mechanistically involved in many biological processes (Long et al., 2017). LncRNAs have several gene-regulatory roles by influencing transcription binding or epigenetic marks. For instance, interactions with mRNAs or proteins, may influence their stability, rate of translation, activity, or localization (Guttman and Rinn, 2012). Specifically, lncRNAs regulate the progression of atherosclerosis (Freedman and Miano, 2017; Haemmig et al., 2017), although this regulation is less characterized due to their low expression and conservation. Specifically, lncRNAs can regulate gene expression by acting as signals, guides, decoys, and scaffolds, in addition to acting as repressors or activators to modulate the process of gene transcription and translation (Wang and Chang, 2011).

Atherosclerosis is a chronic vascular inflammatory disease characterized by the accumulation of lipids in large arteries, which leads to plaque formation and narrowing of the vessel lumen. Typically, it is driven by the dysregulation of endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) and the infiltration of monocyte-derived macrophages in response to a pro-atherogenic environment. Over the last two decades many different miRNAs have been implicated in the different processes of atheroma plaque formation, such as cellular adhesion, proliferation, lipid uptake, and efflux, among others. Moreover, miRNAs can also regulate atherosclerosis development by influencing other risk factors and numerous related diseases. It is known that some miRNAs regulate blood pressure and hypertension (Xin et al., 2009; Sequeira-Lopez et al., 2010; Santulli, 2016). Other miRNAs are involved in diabetes mellitus pathogenesis due to an impairment of glucose metabolism (Dey et al., 2011) or by affecting pancreatic B-cells through the regulation of cell survival, proliferation, differentiation, or insulin secretion (Feng et al., 2016). How and which miRNAs regulate vascular macrophage, endothelial, and smooth muscle cells dysfunctions have been extensively described elsewhere and are not the focus of this brief review (Madrigal-Matute et al., 2013; Aryal et al., 2014; Feinberg and Moore, 2016; Laffont and Rayner, 2017; Vartak et al., 2022).

Different types of RNA-targeting therapeutics are used to induce miRNA-like functions, deplete or restore the levels of a specific miRNA, or to inhibit interaction with its targets. They are typically known as miRNA mimics, if you want to overexpress a miRNA, or miRNA antagonists (antagomiRs), if you want to inhibit a miRNA. Due to their chemical negative charge, miRNAs are unstable and unable to cross cell membranes. Therefore, several chemical modifications have been developed in order to improve their pharmacokinetics and pharmacodynamics. First-generation modification was the antisense oligonucleotide (ASO). ASOs are single-stranded DNA molecules with full complementarity to one specific target mRNA. These molecules may act by blocking protein translation, degrading mRNA or changing pre-mRNA splicing. Second-generation modifications known as "gapmers" improve bioavailability and efficacy, while reducing toxicity and immunostimulation by replacing the 2'-O-alkyl group for 2'-O-Me, 2'-MOE or 2'-F. Third-generation chemical modifications apply changes to the furanose ring to create locked nucleic acids (LNAs), peptide nucleic acids (PNAs), or phosphorodiamidate morpholino oligomers (PMOs). LNAs are modified nucleotides in which the 2′ oxygen and 4′ carbon atoms are joined by an extra bridge. Oligonucleotides that include LNAs show enhanced specificity, sensitivity, and hybridization stability. Thus, LNA probes are resistant to degradation by nucleases. PNAs are artificial oligonucleotides mimetics with a peptidic backbone in lieu of a phosphoribosyl backbone that makes a more stable oligonucleotide, but it has less cellular permeability. PMOs are short single-stranded DNA analogs that are built upon a backbone of morpholine rings connected by phosphorodiamidate linkages with high efficacy and safety but their intracellular delivery needs improving (Winkle et al., 2021). Moreover, several lncRNAs have been shown to regulate miRNAs that are key for the atheroma plaque formation, but an atherosclerosis analysis has been not specifically performed. Finally, miRNA sponges are artificial transcripts that contain multiple miRNA binding sites to sequester them, which can be quite useful when targeting a whole miRNA seed family. Thus, there are several types of RNA-targeting therapeutics. However, the in vivo efficacy of current anti-miRNA technologies has been hindered by physiological and cellular barriers to deliver miRNA mimics/inhibitors into targeted cells, and the broad regulatory nature of miRNAs, which can lead to deleterious off-target effects. The target preferences and impact of miRNAs can vary dramatically in different tissues and cell types. Therefore, further research must be committed to improve the method of miRNA administration to ensure precise miRNA delivery to the intended site of atherosclerosis. Here, we will discuss the recent findings in the field, highlighting the novel therapeutic uses of miRNAs-base therapies for treating atherosclerosis.

It is important to note that the pathophysiology of atherosclerosis in small and large animals differs at some degree from that observed in humans (Getz and Reardon, 2012; Emini Veseli et al., 2017). The majority of atherosclerosis animal models do not spontaneously develop the complications seen in humans such as plaque rupture, myocardial infarction, stroke, or sudden death. Although numerous animals develop atherosclerotic plaques after a cholesterol rich diet, the morphology and topography of the lesions is not always similar as compared in humans. Lesions occur more frequently in humans in the coronary arteries, carotids and peripheral vessels, and in mice more frequently in the aortic root and aortic arch. However, it is also known that the typical small animal model used in atherosclerosis such as mice, are easy to acquire, easy to handle, not expensive to maintain, and have well-defined genetic characteristics and ease of genetic manipulation (Getz and Reardon, 2012; Emini Veseli et al., 2017). Thus, we need to be aware of these limitations.

MiRNAs-base therapies in atherosclerosis

Several approaches have been undertaken to decipher the potential of miRNA therapeutics. This fact has been enhanced thanks to the emergence of precision nanomedicine. Thus, the different types of RNA targeting therapeutics can be delivered using different approaches (see Figure 1 as a schematic representation of the recent microRNA delivery systems):

Viral vectors

Viral vectors can deliver nucleic acid into targeted cells with high efficiency. Different types of viral vectors can be used, such as adenoviruses, adeno-associated viruses (AAVs), retroviruses, or lentiviruses (LVs). This approach has been used in multiple preclinical studies (Gentner et al., 2009; Rayner et al., 2010; Soh et al., 2013). Interestingly, Lovren et al. (2012) demonstrated that VSMCS-specific overexpression of miR-145 not only limits plaque burden in atherosclerosis-prone *ApoE*$^{-/-}$ mice but also reduces plaque inflammation and increases features of plaque stability in a fashion consistent with the promotion of the VSMC contractile phenotype. The authors use the mouse miR-145 LV under the control of the mouse SMC-

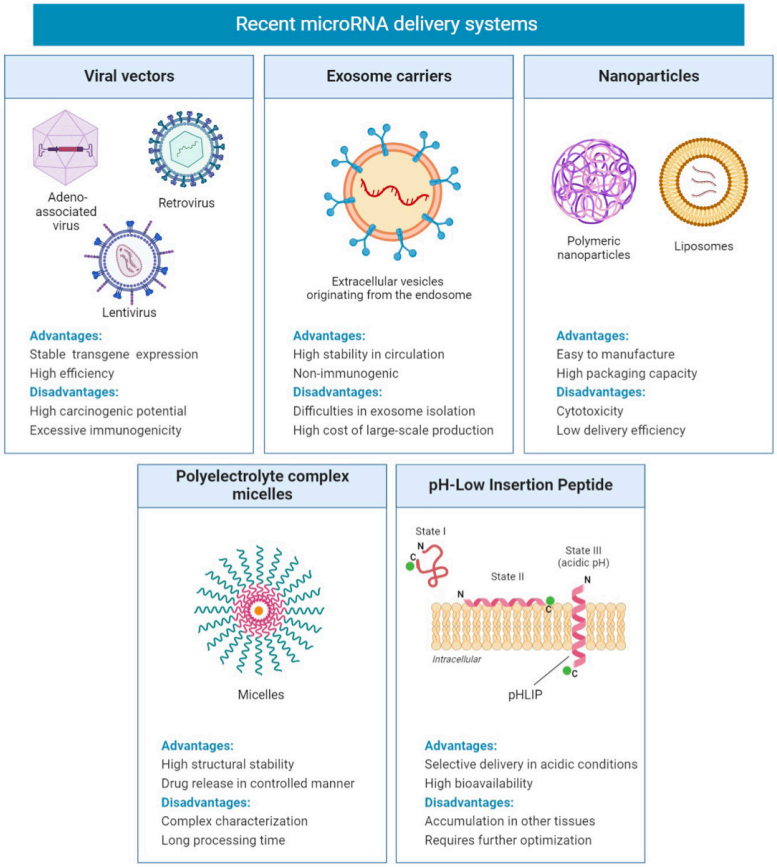

Figure 1. Schematic representation of recent microRNA delivery systems with their advantatges and disadvantatges

specific promoter *SM22*α generate the LV, they cloned the miR-145 of the human EF1α promoter region and the human EF1α promoter was replaced with the VSMC-specific minimal mouse *SM22*α promoter in a self-inactivated lentiviral vector (Lovren et al., 2012). However, for clinical use, viral delivery systems will require careful scrutiny. The safety of genomic integration of LVs, which may trigger the expression of oncogenes or excessive immunogenicity, and the transient nature of miRNAs expression in the case of adenoviruses and AAVs needs to be taken into consideration.

MiRNA-exosome carriers

Exosomes, which are extracellular vesicles originating from the endosome with a diameter of about 40-160 nm, can carry miRNAs among other cargos and participate in intracellular communication regulating pathological processes (Kalluri and LeBleu, 2020). It is known that ECs, macrophages, and SMCs communicate with each other through exosomes during different stages of atherosclerotic plaque formation. Exosomes are promising drug delivery carriers because of their ability to cross a wide range of biological barriers and their stability in circulation (Liu and Su, 2019; Kalluri and LeBleu, 2020). Moreover, if you compare nanoparticles and exosomes, the latter are less immunogenic (Elsharkasy et al., 2020). There are many in vitro exosomes related studies that focus on the different processes that affect the initiation of atherosclerotic plaque formation, but only a few in vivo studies. For example, a recent study by Bu et al. (2021) nicely showed an exosome-based delivery of an engineered interleukin (IL)-10 mRNA that alleviated atherosclerosis in an *ApoE*$^{-/-}$ mice model. Specifically, they engineered a miR-155-responsive IL-10 mRNA using the internal ribosome entry site (IRES) sequence of the hepatitis C virus that recognized miR-122 and changed to the sequence complementary to miR-155, which is a well-known inflammation-associated miRNA. Then, they transfected the IRES-IL-10 plasmid into HEK293T cells and obtained exosomes Exo$^{\text{IRES-IL-10}}$ that were used to prove the therapeutic efficacy of these exosomes on atherosclerosis in vivo. Interestingly, plaque formation was mitigated without off-target effects (Bu et al., 2021). In another study, exosomes from Krüppel-like factor 2-transduced ECs reduced atherosclerotic lesion formation by inducing decreased levels of proinflammatory M1 macrophages and increased levels of anti-inflammatory M2 macrophages in mice, and it was at least partly due to decreased expression of inflammation-associated miR-155 (He et al., 2018). Exosomes derived from mesenchymal stem cells (MSCs) exert immunomodulatory and immunosuppressive effects, although few studies have addressed their effects on atherosclerosis in vivo. Exosomal miR-100-5p derived from human umbilical cord mesenchymal stem cells (hUCMSC-Ex) has been shown to inhibit cell migration, to promote apoptosis, and to inhibit the inflammatory response in eosinophils via frizzed class receptor 5 (FZD5)/Wnt/β-catenin pathway, in addition to reducing the atherosclerotic plaque area in mice (Gao et al., 2021). Li et al. (2019) demonstrate that MSC-exosomes ameliorated atherosclerosis in a mice model and promoted M2 macrophage polarization in plaques through the miR-let7/high-mobility group AT-hook 2 (HMGA2)/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway (Li et al., 2019). In another preclinical study, the authors identified that exosomal-miR-125b-5p from mouse bone marrow-derived MSCs suppressed atherosclerotic plaque formation by inhibiting mitogen-activated protein 4 kinase 4 expression (Lin et al., 2021). Thus, these studies show the potential of using exosomes as a therapeutic tool for atherosclerosis. However, technical

issues with current exosome isolation methods, strategies to enrich target cargos, or standards for loading efficiency, need to be improved before clinical translation. Moreover, large-scale production of exosomes is not easily attainable.

Nanoparticles (NPs)

An alternative to using exosomes as a drug delivery system for bioactive miRNAs, which does not suffer the same mass production and quality control limitations, is the design of nano-sized particles mimicking the properties of natural extravesicular particles. There are numerous engineered nanoparticles (NPs) delivery platforms such as chitosan-based NPs (chNPs), polymeric NPs, and liposomes. Most of these nanoparticles have been developed as a tool to deliver siRNAs or miRNAs and have been tested for various cancer models, but not too many studies related to atherosclerosis have been pursued. In one of these studies, Nguyen et al. (2019) developed NPs using chitosan-polyethylene glycol and tripolyphosphate crosslinking via an ionic gelation method and encapsulated miR-33 mimics (miR-33-chNPs). Functional miR-33 mimics delivered to macrophages via this method evaded endolysosomal degradation and reduced ABCA1 expression, which is a well-known target of miR-33. Cholesterol efflux and reverse cholesterol transport (RCT) in vivo were altered as well (Nguyen et al., 2019). Moreover, other experiments with efflux-promoting miRNAs, such as miR-206 and miR-223, were also delivered via chNPs, and cholesterol efflux and the RCT pathway were improved, suggesting that atherosclerosis might be affected. However, they did not specifically perform atherosclerosis experiments to elucidate if those miR-chNPs were able to affect plaque formation (Nguyen et al., 2019). Importantly, NPs tend to accumulate in other tissues including the liver and spleen. Thus, there is a need to conjugate these NPs with ligands with specific affinity for those important cells related to atherosclerosis plaque formation. For instance, to achieve vascular EC-specific delivery of miRNA-chNPs, a short peptide REDV (Arg-Glu-Asp-Val) was used (Zhou et al., 2016). The delivery of miRNA-chNPs specifically to macrophages can be improved using external peptides like hyaluronan (Beldman et al., 2017), fibronectin (Yu et al., 2018), or folate, which is highly expressed in activated macrophages (Zhao et al., 2008).

Polyelectrolyte complex micelles

Polyelectrolyte complex micelles have emerged as a novel therapeutic gene-delivery vehicle due to their ability to encapsulate negatively charged nucleic acids forming a core by neutralizing the charge and protecting the nucleic acids from non-specific interactions and enzymatic degradation (Kuo et al., 2014). Interestingly, Zhou et al. (2021) have recently proved the therapeutic effectiveness of the vascular cell adhesions protein-1-targeting polyelectrolyte complex micelle to deliver miR-92 inhibitors and treated pathological vascular remodeling in vivo (Zhou et al., 2021).

pH-Low Insertion Peptide

In a very recent study, we used the pH-Low Insertion Peptide (pHLIP) to deliver anti-sense oligonucleotides specifically to atherosclerotic lesions. The pHLIP delivery system is a family of soluble approximately 36 amino acid peptides that binds to the membrane surface, forms an inducible transmembrane α-helix under acidic conditions, and has the ability to translocate membrane-impermeable molecules into cells via non-endocytic route. This peptide targets areas of high acidity at the surface of cells and it was employed for the first time to deliver miRNA inhibitors to the acidic environment of tumors (Cheng et al., 2015). In a second study, Sahraei and colleagues (2019) used the anti-miR-21 oligonucleotides conjugated with pHLIP to directly target tumor-associated macrophages and showed that the progression of the tumor was attenuated. As the kidney also has an acidic microenvironment, we also proved pHLIP could be used to delivery anti-miR treatment to this tissue (Price et al., 2019). Given the hypoxia in macrophage foam cells and the acidic environment of the lipid core in atherosclerosis, we explored the ability of this peptide to deliver anti-miR-33 therapy into macrophages in the atheroma plaque (Zhang et al., 2022). With different in vitro, ex-vivo, and in vivo studies we were able to deliver the anti-miR-33 therapy specifically to macrophages of the vascular lesions, avoiding potential off target effects that the chemically modified antisense oligonucleotides anti-miR therapy alone may cause in other tissues (Zhang et al., 2022). Thus, pHLIP technology can also be used to selectively delivery other protective miRNAs to macrophages of atherosclerotic plaques for therapeutic intervention in atherosclerosis-associated cardiovascular diseases.

MiRNA-based therapeutics in other fields and clinical applications

There are several diseases where miRNA-based therapeutics have been develop as well, such as cancer, hepatitis C, and other cardiovascular diseases. For instance, as mentioned before, pHLIP was used for the first time to inhibit miR-155 in a mouse model of lymphoma (Cheng et al., 2015). More recently, a next-generation of chemically modified triplex PNAs that bound miR-155 has shown superior therapeutic efficacy in treating lymphoma in a preclinical study (Dhuri et al., 2021). In another study, the authors described a nanoparticle-complexed antimiRs for inhibiting tumor growth and metastasis in prostate carcinoma and melanoma. Specifically, the authors use a nanoparticle formation with polyethylenimine (PEI F25-LMW) with different antimiRs (Kunz et al., 2020). In non-human primates miR-122 inhibition resulted in non-lasting suppression of hepatitis C virus without liver toxicity or adverse immune effects (Lanford et al., 2010). Moreover, preclinical studies in porcine models of heart failure (HF) treated with an antisense locked nucleic acid (LNA) oligonucleotide (CDR132L) targeting miRNA-132-3p, showed a favorable safety profile, efficient drug delivery to cardiac tissue, and potent, dose-dependent target reduction in myocardium and plasma (Foinquinos et al., 2020; Batkai et al., 2021)

Translation of this miRNA-based therapeutics into the clinic has been hampered by different issues associated with delivery, tolerability, specificity, and efficacy. Due to these issues, most of the clinical trials had been terminated. However, there are a few clinical trials that had positive results. A first-in-human, phase I study assessed the safety, maximum tolerance dose, pharmacokinetics, and clinical activity of a liposomal miR-34a mimic (MRX34) in patients with advanced solid tumors (Beg et al., 2017), but the trial was closed early due to adverse reactions in four patients that died (Hong et al., 2020). Another phase I clinical trial for cancer treatment is the MesomiR-1, where the miR-16 mimic was used in patients with malignant pleural mesothelioma using TargomiRs (van Zandwijk et al., 2017). Moreover, the first clinical trial antisense drug in HF patients was recently describe using CDR132L and targeting miRN-132-3p. The study was safe and well tolerated, and a reduction of miR-132 in plasma was described. Although the number of patients in the study was small, the pharmacodynamic findings were encouraging (Täubel et al., 2021).

There are currently nine ongoing clinical trials using various miRNA-based therapies as follows: AMT-130, a pri-miR-451 AAV5 for Huntington disease (NCT04120493), AZD4076, anti-miR103/107 using the GalNAc-conjugated antagomiR to treat type II diabetes and nonacoholic fatty liver disease (NCT03225846 and NCT04617860), MRG-201 or Remlarsen, a miR-29 mimic using a cholesterol conjugated for the treatment of keloid (pathological fibrosis) (NCT02603224

and NCT03601052), and lastly, SPC3649 or Miravirsen, an anti-miR-122 (PS-β-D-oxy-LNA gapmer ODN) for hepatitis C virus infection (NCT01646489, NCT01727934, NCT01872936, NCT01200420). No lncRNA- targeting therapeutics have entered clinical development so far.

Future perspectives and challenges of novel therapeutics using miRNAs

There are other tools that might be used for the delivery of miRNAs, but they have not been tested in atherosclerosis studies yet. It is known that high-density lipoproteins (HDLs) can transport endogenous and exogenous miRNAs and can deliver them to target cells and particular messenger RNA sequences (Vickers et al., 2011). Nakayama et al. (2012) showed an effective functional delivery of cholesterol-siRNA to mouse liver by an ApoE-containing particle. Uptake of cholesterol-siRNA occurred similarly to native HDL (scavenger receptor BI-dependent) when ApoA-liposomes were used. Similarly, an ApoE-containing particle was able to deliver miRNAs to cancer cells after cell internalization through CXC chemokine receptor 4-stimulated micropinocytosis (Jiang et al., 2020). What is more interesting is the incorporation of active compounds into synthetic HDLs (sHDLs) with therapeutic purposes, since sHDLs have drug-delivery nanocarriers properties. The combination of sHDL with miRNAs has a profound synergistic effect, as it improves the efficacy compared to treatment with bare sHDL (Uribe et al., 2021). Thus, the use of these optimized ApoA-1-sHDL particles or recombinants HDL particles, in combination with LNAs might be extremely useful since it combines the features of HDLs as nanocarriers and its anti-atherosclerotic properties with the inhibition of specific miRNAs. Nowadays, the new generation of tailored sHDL is expected to entail enhanced functionalities with strong prospects for industrial scale-up that could lead to personalized medicine for vulnerable populations with clinical atherosclerosis in which, for example, an intensive statin therapy could not halt plaque progression or be prescribed. LncRNA-based therapeutics are being actively explored as biomarkers, but they might also be as a therapeutic tool since they can regulate miRNAs expression as well. However, no lncRNA-targeting therapeutic studies in the atherosclerosis field have been developed so far. Importantly, these novel therapeutic delivery systems should be tested together with other therapies that are already on the market, such as statins, to elucidate if working together, they are more effective in ameliorating or even regressing plaque formation. Thus, co-deliver multiple therapeutics may be of interest.

Conclusions

Atherosclerosis contributes to cardiovascular risk, which is still the number one cause of death in western societies and is becoming a global epidemic. The study of molecular biology mechanisms of atherosclerosis has provided insights into the process that leads to atheroma plaque formation. miRNAs are without doubt important biological players in this physiopathological process. Therapeutics to regulate miRNA activity are emerging as the next frontier in treatment options for atherosclerosis. The translation of miRNA-based therapies into clinical applications is still in its infancy and requires further investigation in order to overcome some challenges such as dosage adjustment and duration of the treatment. A balance between therapeutic and side effects needs to be determined for every miRNA and studies in non-human primates and future clinical studies should provide key data to evaluate the prospective use of miRNA modulation in atherosclerosis. Most of the significant development has been made in the utility of miRNAs as a biomarker of disease status and response to therapy. Diverse types of RNA targeting therapeutics can be delivered using several approaches such as viral vectors, NP, exosome, pHLIP, or polyelectrolyte complex micelles, but they have some limitations. The challenge of undesired effects and a better delivery system has perhaps been the greatest hurdle that miRNA-base therapies must overcome. In this new era of nanomedicine, the improvement and the development of new in vivo delivery systems would be vital for the delivery of miRNAs to the targeted site in order to stop or regress atherosclerotic plaque formation. Different disciplines such as molecular biology, pharmacology, immunology, chemistry, and nanotechnology must converge in order to improve tolerance, specificity, and delivery of miRNA-based therapeutics for atherosclerosis treatment, as well as other diseases.

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Disclosures

NA

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