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Epigenetic regulation as a promising tool for treatment of atherosclerosis

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1. ABSTRACT

Atherosclerosis is one of the leading causes of death from cardiovascular disease (CVD) that primarily involves mid size and large arteries. Atherosclerosis is associated with disruption of lipid metabolism and chronic inflammatory processes. One approach for treatment of atherosclerosis is by virtue of epigenetic control by noncoding RNAs (ncRNA) including miRNA, siRNA and lncRNA, commonly employing miRNA antagonists and mimic compounds. Here, we review such usages as well as other approaches for correcting the molecular lesions of atherosclerosis including specific activation of atheroprotective miRNAs, as well as use of siRNAs and lcRNA to control aberrant lipid metabolism. We also discuss some of these technologies that have already shown to be effective in clinical trials and are likely to enter the clinical arena.

2. INTRODUCTION

Cardiovascular diseases (CVDs) are the leading causes of death of 17.9 million people or 31% of all deaths worldwide annually (1). Among these diseases, atherosclerosis ranks highest, being a chronic inflammatory disease of the middle and large arteries that results from disruption of lipid metabolism and functional disorders in the arterial wall (2). The disease is initiated in the intima by accumulation of low-density lipoproteins (LDL), and consequent activation of endothelial cells, and the recruitment of monocytes to the intima, where they differentiate into macrophages, intake of modified lipoproteins and formation of foam cells (3). Atherosclerotic plaque are characterized by a fibrous cap that covers a necrotic center, rich in lipids and bordering by white blood cells in the marginal zones (3). This results in conversion of the endothelial cells

and proteolytic degradation of the the extracellular matrix. Such unstable lesions can rupture and lead to myocardial infarction or stroke.

The use of noncoding RNA (ncRNA) has emerged as a promising approach in the treatment of atherosclerosis (4). MicroRNAs (miRNAs) are small noncoding single-stranded molecules comprised of 19-22 nucleotides that posttranscriptionally regulate gene expression (2-5). Another class of noncoding RNAs are interfering RNAs (siRNAs), small RNAs comprised of 21-23 bp. These RNAs are double-stranded with one inactive sense strand matching the target mRNA and an antisense active strand (5). The mechanism of action of many miRNAs is similar to the action of siRNAs; however, the latter act exclusively on one gene (5). In this review, we discuss the new therapeutic approaches that lend themselves in correcting genetic regulation of transcription of key genes involved in the development of atherosclerosis through an epigenetic approach.

3. LIPID METABOLISM IN ATHEROSCLEROSIS

Atherosclerosis is a chronic disease characterized by the activation of innate and adaptive immunity (7). The main cause of the development of atherosclerosis is a disruption of lipid metabolism associated with dysfunction of apolipoprotein B (apoB) (6-7). The latter is a cofactor of enzymes, receptor ligands and lipid transporters that regulate lipoprotein metabolism and tissue uptake (8). Numerous studies have demonstrated the involvement of LDL, apoB, and very low-density lipoproteins (VLDL) and their residues in the formation of atherosclerosis (9-10).

The key event in the initiation of atherosclerotic lesions is the retention and accumulation of cholesterol-rich apoB-containing lipoproteins in the intima of the arteries which as a consequence leads to the formation of atherosclerotic plaques (11). LDL and other apoB-containing lipoproteins with a diameter of less than 70 nm freely pass through the arterial intima (10).

The physiological level of LDL cholesterol (LDL-C) is considered to be in the range of 0.5–1.0 mmol/L (20–40 mg / dL) for LDL-C (10). Higher levels of LDL-C increase the chance of internal retention of LDL which is the initial event in initiation and progression of atherosclersis (8, 10).

The terminology “cholesterol”, and “LDL cholesterol (LDL-C)” is often used interchangeably (13). Cholesterol is an important component of the cell membrane, the precursor of bile acids and steroid hormones. LDL particles make up an approximately 90% of the entire circulating apoB-containing fraction of lipoproteins in the fasting blood in most people (10, 12). However, in clinical practice, plasma LDL is usually not measured directly but is measured by the concentration of its LDL cholesterol, an indicator of the total amount of cholesterol that is contained in LDL particles. Thus, the level of LDL in plasma has become an important parameter for assessing the risk of developing CVDs and in obtaining a therapeutic effect.

3.1. Therapeutic approaches based on the use of miRNAs aimed at the disruption of lipid metabolism

Today, there are many highly effective substances that reduce the level of LDL (13). Currently, new long-acting drugs are being developed to reduce lipid levels (13). Based on the importance of reducing LDL, the role of miRNA in the regulation of gene expression that leads to dysfunction of endothelial cells and the development of atherosclerosis is being actively pursued (14). Large-scale global studies have identified numerous miRNAs, which are important regulators of lipid metabolism (15). Given that microRNAs have the potential to regulate several genes and molecular pathways, there is a great interest to examine and identify atheroprotective miRNAs and discriminate these from those that are proatherogenic (Table 1)(16).

The miRNAs of the RNA-induced silencing complex (RISC) can bind to a complementary sequence in the 3’ untranslated region of the target messenger RNAs (mRNA). This leads to posttranscriptional cleavage by endonucleases and degradation of the sequence complementary to the target mRNA (17). miRNA can also induce the deadenylation and decapping of mRNA (18).

Table 1. The mechanism of action of non-coding RNA

ncRNA	Impact targets	Impact level	Therapeutic strategies based on ncRNA	
			Downregulation	Upregulation
miRNA	Regulate the expression of several genes	Repression translation degradation of mRNA	Anti miRNA: Inhibition endogenous miRNA; miR sponges; Target site blocker; CRISPR	Mimics miRNA; re-introduction of miRNA ORN
siRNA	Highly specific, complementary to the target gene	Endonucleolytic cleavage of the target mRNA	–	–
lncRNA	Regulate the expression of several genes	Regulation of gene expression from the start of transcription to protein translation	Inhibition LNCRNA: LNA-GapmeR; Short hairpin RNA; CRISPR	–
Note: ncRNA - non-coding RNA; ORN - synthetic oligoribonucleotides.				

Studies in mice have shown that overexpression of miR-30c inhibits microsomal triglyceride transfer protein by reducing VLDL production (Figure 1) (19). The accumulation of low-density lipoprotein (LDL) leads to the activation of endothelial cells (19). The study of Sodi R. *et al.* (2017) showed a positive correlation between miR-30c expression with total cholesterol and LDL in cases of hypercholesterolemia (20). miR-30c inhibits lipid synthesis in the liver by acting on lysophosphatidylglycerol acyltransferase-1 (LPGAT1), an enzyme which is involved in the synthesis of phospholipids (21). Increased expression of miR-30c is a compensatory mechanism aimed at reducing lipid secretion in hypercholesterolemia (20). Thus, the increased expression of miR-30c can be used in the treatment of hyperlipidemia and atherosclerosis.

miR-33 inhibits a cluster of genes that control cellular energy metabolism and cholesterol outflow from macrophages (Figure 1) (22). The miR-33 family consists of miR-33a and miR-33b encoded in introns of the genes, sterol regulatory element-binding proteins 1 (SREBP1) and sterol regulatory element-binding proteins 2 (SREBP2) (22). Both miR-33 isoforms have the same sequence, but in the 3' region, there are differences of two nucleotides (23). The role of miR-33 in sterol metabolism was originally reported as a regulator of cholesterol transporter expression (ABCA1 and ABCG1) in hepatocytes and macrophages in mice (24, 25). ATP-binding cassette A 1 (ABCA1) is the main regulator of reverse cholesterol transport (26). ATP-binding cassette subfamily G member 1 (ABCG1) is involved in the transport of cholesterol and phospholipids in macrophages and can regulate cellular lipid homeostasis in other types of cells (27).

Overexpression of miR-33 suppresses the expression of ABCA1 and ABCG1 genes in the liver and contributes to a decrease in the level of HDL in the blood plasma of mice (Figure 1) (24). However, a decrease in miR-33 expression using antisense oligonucleotides (ASO-33) leads to an increase in the expression of the ABCA1/ABCG1 genes and plasma HDL levels (24). In addition, the inhibition of miR-33 increased mitochondrial respiration and ATP production by activating miR-33 target genes, such as peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α) and pyruvate dehydrogenase lipoamide kinase isozyme 4 (PDK4) (22).

Currently, miRNA therapy more commonly employs compounds that act as miRNA antagonist (28) rather than miRNA mimics. The introduction of miR-33 antagonists coordinates a network of metabolic processes that increase ATP-dependent cholesterol outflow and contributes to antiatherogenic effects in macrophages, emphasizing a new therapeutic pathway to stimulate cholesterol outflow and to reduce atherosclerosis (28).

An increase in circulating apoB-containing lipoproteins (VLDL and LDL) leads to infiltration and retention of these lipoproteins in the arterial wall,

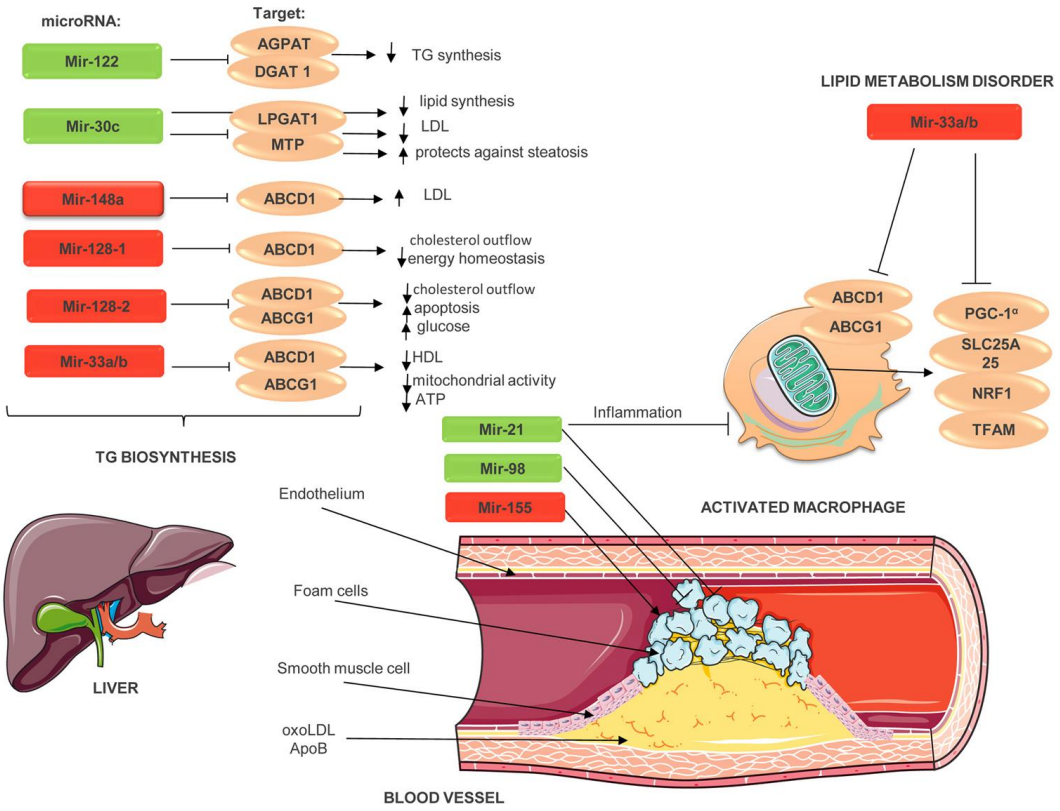

Figure 1. MicroRNAs effect on cellular mechanisms of atherosclerosis. Activation → ; Suppression ⊣ ; Upregulation ↑ ; Downregulation ↓, TG - triglycerides; LDL - low-density lipoprotein; ABCA1 - ATP-binding cassette A 1; ABCG1 - ATP-binding cassette subfamily G member 1; LPGAT1 - lysophosphatidylglycerol acyltransferase-1; MTP - Microsomal triglyceride transfer protein; AGPAT – 1-acylglycerol-3-phosphate-O-acyltransferase; DGAT1 - Diacylglycerol O-acyltransferase 1; PGC-1α - peroxisome proliferator-activated receptor gamma coactivator 1-alpha; SLC25A25 - Solute Carrier Family 25 Member 25; NRF1 - nuclear respiratory factor; TFAM: transcription factor A, mitochondrial; OxoLDL - oxidized low-density lipoprotein; ApoB - apolipoprotein B.

which is a critical event in the development of atherosclerosis (15). The microRNA, miR-122, is highly expressed in the liver and is involved in fatty acid metabolism (9). Inhibition of miR-122 expression contributes to lower plasma cholesterol and triglyceride levels in animals, and elevated miR-122 levels have been reported in patients with nonalcoholic fatty liver disease, obesity, and type 2 diabetes (29-30). MiR-122 influences lipid metabolism, making it a promising biomarker of cardiovascular and metabolic disorders. Statins reduce miR-122 levels in the blood circulation, while other types of drugs, such as platelet inhibitors, do not affect this expression (31). However, the molecular mechanisms and mRNA targets that mediate this effect remain unknown. Wang Y. *et al.* (2018) proved that serum miR-122 can be used as a biomarker for non-invasive diagnosis of atherosclerosis and assessment of the extent of atherosclerotic lesions in the arteries (32).

miR-148a may control an extensive network of lipid metabolism regulators, including LDL (26). Inhibition of miR-148a increases the expression of LDLR (LDL) in the liver and decreases plasma LDL-C in mice (Figure 1) (26). MiR-148a is also expressed in adipose tissue and hematopoietic cells (33). Genome-wide association studies (GWAS) revealed that SNPs in the miR-148a locus are associated with obesity (34).

In humans, miR-128 is encoded in the intron of the R3H domain containing 1 gene (R3HDM1) on chromosome 2 and is coexpressed

with its host gene in many tissues (33). Some authors have shown that miR-128-1 plays a key role in the regulation of lipid cholesterol and energy homeostasis of both the proapoptotic molecule and as the regulator of cholesterol homeostasis (35). Activation of miR-128-2 reduces cholesterol efflux by inhibiting the activity of the ABCA1, ABCG1 and retinoid X receptor alpha (RXRα) genes in human cell lines (35).

miR-148a and miR-128-1 control lipoprotein metabolism in the blood by directly targeting the 3' UTR of the LDL receptors and ABCA1 genes (Figure 1) (33). Antagonists of miR-128-1 also improve the clearance of glucose and increase the sensitivity of the liver to insulin. In addition to regulating lipoprotein metabolism, miR-128-1 regulates ABCA1 expression in macrophages and improves cholesterol outflow from them (33). These studies demonstrate that antagonism of miR-148 and miR-128-1 may be a promising therapeutic approach for the treatment of dyslipidemia, obesity, and CVDs.

Atherogenic components of the lipid and proteome appear to play a role in atherogenesis. The role of macrophages in lipid metabolism which includes: cholesterol absorption, esterification and outflow, offers the opportunity to disrupt and hence to prevention of formation of "foam cells" (36). Oxidized or otherwise altered LDL accumulates in the subendothelial space and is absorbed by macrophages, forming foamy cells, which lead to the development of fatty subendothelial arterial bands of precursors of atheroma (37). Small, dense LDL particles are more susceptible to oxidation and accumulate more easily due to prolonged postprandial hypertriglyceridemia and low HDL cholesterol, resulting in an inflammatory reaction that is atherogenenic in nature, which forms the basis of so-called "response-to-injury" model of atherosclerosis.

miR-148a, along with its participation in lipid metabolism, together with DNA methyltransferase 1 (DNMT1), regulates the expression of genes involved in the pathogenesis of atherosclerosis (39). DNMT1 is a target gene for miR-148a / 152. Overexpression of miR-148a / 152 leads to suppression of the expression of DNMT1, and suppression of miR-148a / 152 contributes to increased expression of DNMT1 (39). Mutual regulation between miR-148a / 152 and DNMT1 in foam cells probably plays a critical role in the pathogenesis of atherosclerosis.

Thus, metabolism of lipoproteins is an important therapeutic target in the treatment of atherosclerosis. Increasing the expression of miR-30c and inhibiting the expression of a host of other miRNA namely, miR-33, miR-122, miR-128-1, miR-128-2 and miR-148 have all been proposed as therapeutic appraoches for disorders of lipid metabolism and atherosclerosis (Figure 1). Currently, several patents on the use of miR-33 inhibitors (US8859519B2) (40) and mir-27b and mir-148a (WO2014201301A1) (41) mir-128 (WO2012097261A2) (42) for the treatment of dyslipidemia have been filed. There are also ongoing preclinical trials for the treatment of atherosclerosis based on targeting miRNAs such as anti-miR-148a, anti-miR-122, anti-miR-33, anti-miR-92a, anti-miR-33, and anti-miR155 (43).

3.1.1. Technologies for miRNA delivery

There are two main approaches for using miRNAs as targets. They include use of antisense oligonucleotides (ASOs), and inhibitors, miR sponges, target site blockers (TSB)), and miRNA mimics.

miRNA mimics are RNA molecules that mimic endogenous molecules and help enhance their function. The goal of this approach is to reintroduce miRNA, that their expression has been decreased in a pathological process. MiRNAs are delivered to cells via nanoparticles, encapsulation in liposomes, or miRNA expression vectors (44).

Antagonists of miRNAs are used to inhibit endogenous miRNAs that demonstrate an enhanced function in a pathological context (Table 1). These treatments are similar to methods that utilize siRNA. The miRNA antagonists bind to mature miRNA targets with strong affinity, forming a duplex that is ultimately destroyed. Since miRNAs regulate the expression of several genes, inhibition of miRNAs may have side effects. Target site blockers are

antisense oligonucleotides designed to bind to a 3' UTR region which are complementary to miRNAs. In view of the development of methods and chemical modifications that can stably inhibit miRNAs and optimize their delivery, the utilization of miRNAs has increased in recent years. These techniques block nucleic acids (LNA), peptide nucleic acids (PNA), phosphorothioate groups (phosphorothioate oligonucleotide), miRNA sponges and nanoparticles (45, 46).

The base constituting the LNA is a nucleic acid analog in which the ribose ring is chemically modified by the introduction of a methylene bridge. This chemical modification provides the molecule with a greater thermodynamic stability and prevents its destruction by nucleases, enhancing its affinity for its target (47). The effective method for suppressing miRNA functions is accomplished by the so-called “miRNA sponges”. This method directly “adsorbs” miRNAs so that miRNA molecules can not further bind to their natural mRNA targets. (45). This technology improves the understanding of the functions of miRNAs and has the potential to be used clinically for the treatment of remedying the dysregulation of miRNAs in atherosclerosis.

The oligonucleotides for systemic use are introduced intravenously or by subcutaneous injection. After systemic administration, single-stranded phosphorothioate-modified antisense oligonucleotides are rapidly transferred from the blood to the tissues. The uptake of oligonucleotides by cells is predominantly mediated by endocytosis. Thus, improvements of use of miRNAs greatly hinges upon development of methods that stably inhibit miRNAs and optimizing their delivery.

3.2. Therapeutic approaches based on the use of siRNAs aimed at the disruption of lipid metabolism

Use of siRNAs as therapeutic agents is much ahead of the technology in using miRNAs (48). In contrast to miRNAs that target several related genes, siRNAs are highly specific as they target a single gene. siRNAs in the protein complex are responsible for the specific cleavage of the target RNA because they are completely complementary to these target sequences (Table 1) (46). miRNAs are encoded by their own genes and are cut out from the hairpin formed by the precursor. siRNAs are not coded by genes, rather they represent fragments of longer RNA (49).

There are two fundamentally different strategies for the treatment of atherosclerosis using miRNA: increasing miRNA levels by overexpressing them using synthetic oligoribonucleotides (ORN) or via targeted inhibition of miRNA using single-chain antisense oligonucleotides (anti-miRs) (50). Inclisiran is a chemically synthesized siRNA molecule that insures sustained specific silencing of the RNA transcription factor of proprotein convertase subtilisin/kexin type 9 (PCSK9) in hepatocytes. PCSK9 contributes to the degradation of the LDL receptor to control plasma LDL cholesterol levels (51). Thus, PCSK9 causes a steady decrease in the level of low-density lipoprotein cholesterol (LDL) in patients who are at a high risk for developing CVDs (52). It appears that Inclisiran can be safely and efficiently be used for lowering LDL cholesterol (52).

Profiles of circulating miRNA in patients that are at various stages of atherosclerosis have been indexed at ClinicalTrials.gov (NCT03855891) (53). The effectivenss of several siRNA-based drugs have proven effective as promising treatments for CVDs have passed clinical trials (54). Clinical trials are currently underway to evaluate the effectiveness and safety of Inclisiran (ORION-4, NCT03705234 (52); ORION-10, NCT03399370) (55).

3.2.1. Technologies for siRNA delivery

Therapeutic approaches based on the use of siRNA include the introduction of synthetic siRNA into target cells to induce RNA interference (RNAi), thereby inhibiting and silencing the expression of specific messenger RNA (mRNA) (56). Although siRNA and microRNA have similar physico-chemical properties, they markedly differ in respect of their regulation property. siRNA, leads to the endonucleolytic cleavage of the mRNA while microRNA, repress translation and caused mRNA degradation (56).

At present, the most common method of delivery for these RNAs is to use lipid-based

nanocarriers of siRNA or miRNAthat traverse cell membrane (57-58). Another potential therapeutic approach is to block miRNA using siRNA and to suppress the synthesis of many proteins (56).

The method of delivery of miRNAs requires further research, because although these are small molecules, they are still too large to penetrate the plasma membrane on their own. Thus, the success of new therapies using small RNAs all depends on the development of efficient mechanisms tha allow delivery of these molecules to target cells. In addition, while circulating in the bloodstream, these molecules are unstable and are rapidly destroyed.

Despite a great promise, such strategies impose disadvantages, and may be associated with side effects. For example, the use of some miRNAs can cause dyslipidemia, obesity, liver steatosis, or hepatocellular carcinoma pointing to the fact that further research is needed to develop ways to prevent such complications.

4. INFLAMMATION AS A MAJOR RISK FACTOR FOR ATHEROSCLEROSIS

Epidemiological studies have shown that chronic stress plays an important role in the development of atherosclerosis, yet all the risk factors for the disease have yet to be fully characterized (59). Chronic stress has a significant effect on the accumulation of macrophages in the intima and in acceleration of damage to the vascular endothelial cells. (59). In addition, chronic oxidative stress is an independent risk factor for the development of atherosclerosis and intimal functioning. Foam cells perform proatherogenic functions and increase the activity of enzymes that destroy the matrix. This increases the likelihood of plaque rupture and occlusion of blood vessels (1).

In a mouse model of atherosclerosis, miR-155 was expressed in plasma and macrophages. Inhibition of miR-155 in mice significantly reduced lipid accumulation in macrophages and reduced the size of the atherosclerotic plaques (60). The increased expression of miR-98 also inhibited the formation of foam cells (61). Search for new targets aimed at a heterogeneous population of foam cells may provide novel therapeutic approaches.

The formation of neointimal injuries is one of the causes of atherosclerosis that is regulated by miR-21. Consistent with this, suppression of miR-21 expression contributed to a reduction in neointima formation in the rat carotid artery after angioplasty (62). It has been shown that shear stress induces miR-21 which, in turn, modulates apoptosis and eNOS activity (63). miR-21 directly impacts NF1B and CDC25A, a cyclin-dependent kinase regulator of cell proliferation and apoptosis. Stress also induces miR-21 in vascular smooth muscle cells in rats and activation of miR-21 has been shown to inhibit apoptosis and proliferation of vascular smooth muscle cells, which contributes to the thickening of neointima *in vivo* (64).

5. EPIGENETIC REGULATION IN THE TREATMENT OF ATHEROSCLEROSIS

In the last decade, increasing evidence has helped to characterize the role of abnormal epigenetic modulation in the development of cardiovascular diseases. The traditional view that chronic inflammatory and lipid disorders are the main immediate causes of atherosclerosis has graduallty shifted to consider this disease to be due to epigenetic changes. For this reason, epigenetic modifications, such as DNA methylation and post-translational modifications of histones, are now accepted as promising approcahes for the treatment of many diseases including atherosclerosis (Figure 2). Patients with cardiovascular diseases show differential DNA methylation and acetylation profiles in tissues and cells including aortic lesions, vascular endothelium, and monocytes (65), suggesting that alteration of histone methylation or acetylation can be effectively used for the regulation of epigenetic processes that lead to the disease (Figure 2). Posttranslational modifications regulate gene expression by remodeling og chromatin structure from a tightly packed condensed state (heterochromatin) to an open conformational state (euchromatin), which allows nuclear transcription factors or DNA-binding proteins to access DNA and thus change gene expression (Figure 2) (66). These modifications include DNA methylation (Me) and acetylation (Ac) of histone tails. DNA

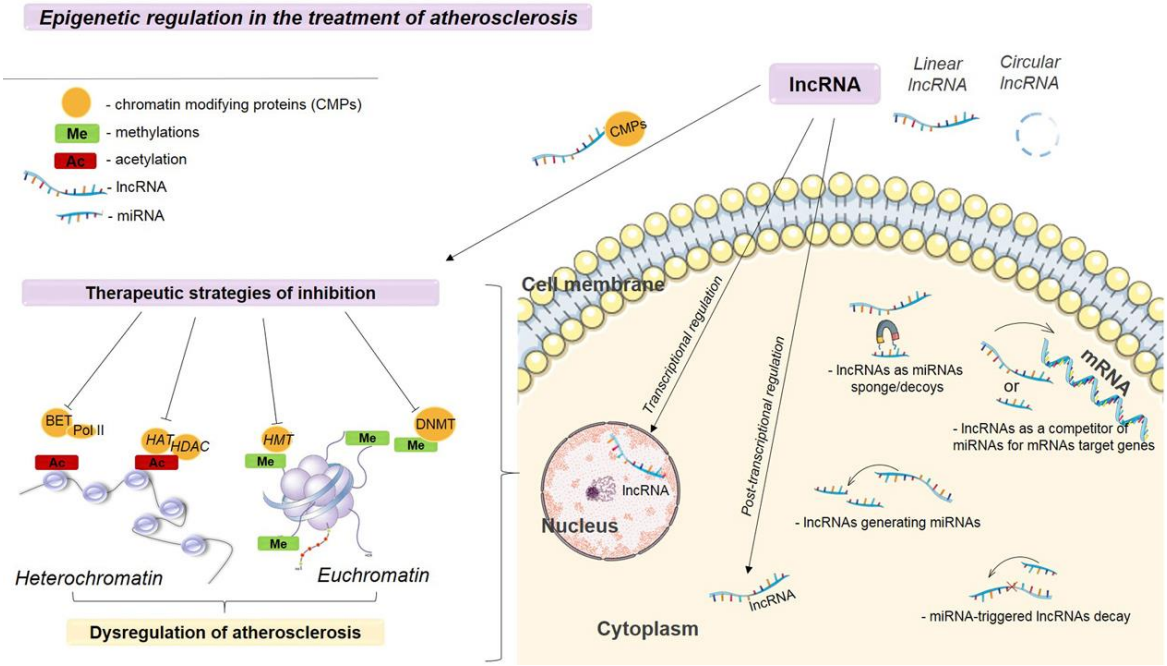

Figure 2. Epigenetic regulation in the treatment of atherosclerosis. lncRNA - long non-coding RNA; Ac - acetylation; Bet - bromodomain and extraterminal protein; Pol II - Polymerase II; HMT - histone methyltransferases; HDAC - histone deacetylase; DNMT: - DNA methyltransferase; HATs - histone acetyltransferases.

methyltransferase (DNMT) by adding methyl groups to the C5 position of cytosine in CpG dinucleotides in the regulatory regions of genes, leads to the formation of heterochromatin, which suppresses transcription, preventing the binding of transcriptional complexes to the promoters of genes (66-67). Studies have shown that atherosclerosis is associated with both hypomethylation and hypermethylation of DNA that consequently affect genes and pathways that regulate the normal function of the endothelium and smooth muscle cells (66-67).

DNMT1 has been implicated in the progression of atherosclerosis and for this reason attempts have been made to inhibit atherosclearosis by modulation of this target enzyme (68). Hydralazine, a nonnucleoside inhibitor of DNMT, with demethylation and proinflammatory properties has been approved by the Food and Drug Administration (FDA) as an antihypertensive drug (69). Administration of hydralazine suppressed angiotensin II inducible fibrosis (Ang II) and decreased infiltration of Mac-$^{2+}$ inflammatory cells and reduced expression of proinflammatory cytokines, such as IL-1β and IL-6 (69). Another candiate for the treatment of atherscloerosis is, a nonnucleoside inhibitor that directly binds to the active site of DNMT1 and inhibits its activity (70). Due to its low toxicity, RG108 might be useful in atherosclerosis (71). RG108 also shows an inhibitory effect on DNA methyltransferase 3a (DNMT3A), which has been implicated in the coronary heart disease (72). In addition to these synthetic inhibitors of DNMT, natural, food-derived inhibitors of DNA methylation have been studied (Figure 2) (73). Among these, resveratrol has a wide range of functions, including cardioprotective, atheroprotective, and vascular protective activity and for this reason resveratrol is useful for prevention of various cardiovascular and metabolic diseases (74-76).

Histone methyltransferases (HMTs), histone deacetylases (HDACs) and histone acetyltransferases (HATs) affect gene expression, depending on the site and number of modifications (Figure 2) (65). Histone acetyltransferase (HAT) and

histone deacetylase (HDAC) are the main enzymes that play an important role in determining the state of histone acetylation (77). The acetylation and methylation of histones have been incriminated both in the induction of inflammation and the development of CVDs (77). For these reasons, treatment of atherosclerosis are aimed only at modifying histones without altering the genetic code in the cells. Several HDAC inhibitors that are currently being used in the clinic for treatment inhibit various HDACs (I, II, and IV bind to Zn^{2+}-containing domains, and III binds to NAD^{+}-dependent enzymes) (78). Dihydrocoumarin, naftopyranone, 2-hydroxynaphalehyde and other sirtuin inhibitors belong to the class III inhibitors (79).

Bromodomain and extra terminal (BET) proteins regulate the transcription of lipoproteins and regulate some inflammatory pathways which are involved in induction of atherosclerosis (80). RVX 208 (apabetalon) is a new and unique BET protein inhibitor for the treatment of atherosclerosis. RVX 208 is an oral inhibitor of the BET protein with some anti-inflammatory properties, which increases the transcription of apo A-I, the major HDL receptor (81). RVX 208 also helps to delay the onset of type 2 diabetes (81). RVX 208 increases the production of apo AI in the liver and intestines, thereby increasing the level of apo AI in the plasma (81). RVX 208 transports more free cholesterol and phospholipids from peripheral cells and assists in the maturation of HDL (82). Subsequently HDL cholesterol esters are transported to the liver promoting reverse cholesterol transport. RVX 208 enhances this reverse cholesterol transport and inhibits inflammatory pathways that are associated with atherosclerosis (65). However, phase I and phase II trials showed that the action of this inhibitor is short-lived. In addition, there is as yet no evidence that this inhibitor protects against the development of cardiovascular pathologies (81). A phase III trial should establish the relative risk reduction in major adverse cardiac events (MACEs) including myocardial infarction (MI) and stroke (Resverlogix Corp).

5.1. Treatment of atherosclerosis based on the use of long noncoding RNA (lncRNAs)

Long noncoding RNAs (lncRNAs), which regulate gene expression from transcription to protein translation are considered to be of high therapeutic potential (83-84). Recent studies have indicated that miRNAs, along with lncRNAs, are involved in both DNA methylation and various histone modifications (85). Noncoding microRNAs and various types of lncRNAs, form complex molecular networks within the cell, and closely interact with each other to regulate the processes of cellular homeostasis (86). LncRNAs coordinate many epigenetic regulatory processes, including the chromatin dynamics, DNA methylation, mRNA stability and noncoding RNAs (Figure 2- 3) (85). In particular, nuclear lncRNAs mainly act on transcription, and control the epigenetic state of certain genes, participate in transcriptional regulation, alternative splicing, and form subnuclear compartments (85,87). On the other hand, the cytoplasmic lncRNAs modulate post-transcriptional gene expression (85). Cyclic forms of lncRNA which have been shown to exist in various organisms that interact with miRNAs. (88). lncRNAs are classified according to the region of the genome from which they are synthesized. It has been proposed to classify lncRNAs as intergenic, intragenic and those that overlap with genes (89). Cyclic RNAs are not susceptible to exonucleases and can more effectively perform the role of endogenous competitive RNA (90-91). These transcripts compete with mRNAs for microRNA binding and reduce the detrimental effect of miRNA on transcriptional and post-transcriptional regulation of gene expression (86). lncRNAs with the help of proteins, regulate gene expression in the cis or trans position of the gene (93-94). lncRNAs direct chromatin-modifying proteins, which can activate or, in the case of tritorax, to suppress the gene expression ny epigenetic modification of histones or DNA (95). The interaction between lncRNAs and miRNAs encompasses miRNA-triggered lncRNA decay, lncRNAs acting as miRNA sponges/decoys, lncRNAs as competitors of miRNAs for mRNAs of target genes and finally lncRNAs that lead to generation of miRNAs (96) (Figure 2). Thus, miRNAs and lncRNAs, acting alone or together, control the gene expression through various posttranscriptional mechanisms, thus contributing to a reliable regulation of expressed proteins.

The CHROME (cholesterol homeostasis regulator of miRNA expression) is a lncRNA that

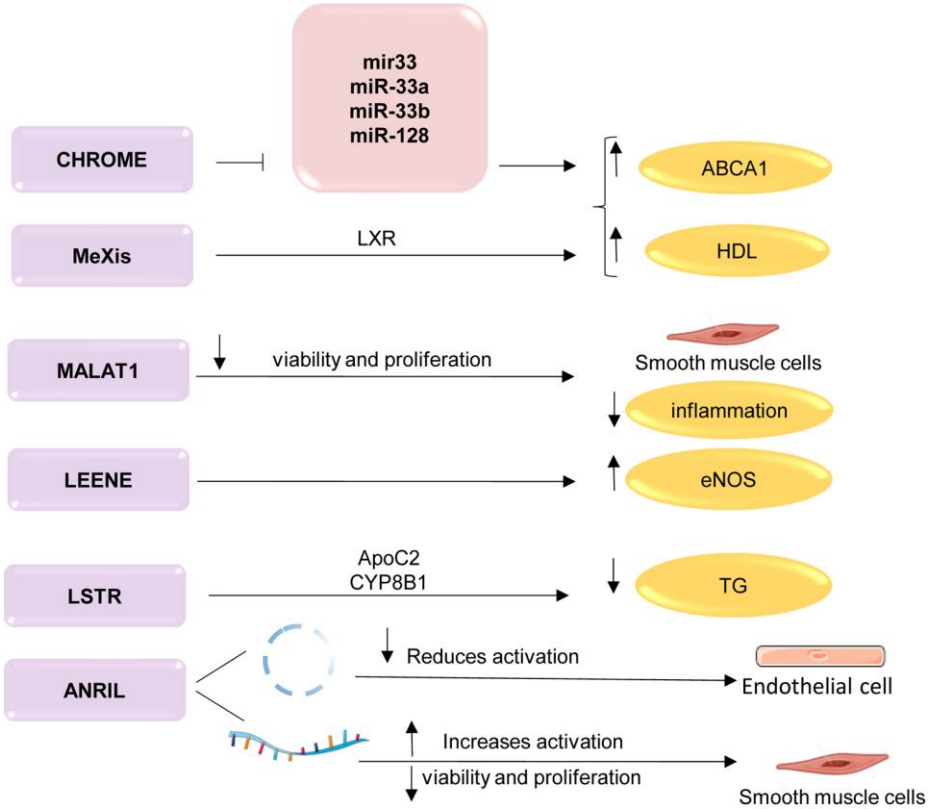

Figure 3. Long non-coding RNAs and their targets that regulate various processes of atherosclerosis. Activation → ; Suppression ⊣; Upregulation ↑ ; Downregulation ↓, TG - triglycerides; LXR - liver receptors; ApoC2 - apolipoprotein C2; CYP8B1 - cytochrome P450 family 8 subfamily B, polypeptide 1; ABCA1 - ATP-binding cassette A; HDL - high-density lipoprotein.

regulates systemic cholesterol homeostasis in the liver and macrophages by inhibiting miRNAs such as miR-33 (Figure 3) (97). The knockdown of CHROME in human hepatocytes and macrophages increases the miR-27b, miR-33a, miR-33b and miR-128 levels. As a result, the expression of their overlapping target gene networks and their associated biological functions are reduced. In particular, cells without CHROME showed a reduced expression of ABCA1, which regulates cholesterol outflow and the formation of nascent HDL particles. Thus, CHROME is one of the key noncoding RNAs that control cholesterol homeostasis in humans, and it can have protective properties against atherosclerosis (97).

The lncRNA MeXis is an enhancer of the ABCA1 gene which interacts with liver receptors (LXRs) (Figure 3). LXR receptors are activated by sterol transcription factors related to the nuclear receptor superfamily (98). These factors also play an important role in the pathology of atherosclerosis as key gene regulators which are involved in cholesterol transport (99). MeXis interacts with and controls the binding of the transcription co-activator promoter DDX17. Thus, a knockout of MeXis resulted in impaired cholesterol outflow and accelerated atherosclerosis in mice (100).

MeXis enhances the transcription of the ABCA1 gene in an LXR-dependent manner. LXRs regulate the expression of genes involved in macrophage responses to cholesterol and inflammation (101). LXR activation supports reverse cholesterol transport by induction of a number of genes, including Abca1, which encodes the plasma membrane transporter ABCA1. This ATP-dependent transport is critical for the formation of high-density lipoprotein (HDL) (102). In mice with a knockout of

the MeXis gene, the level of ABCA1 was reduced. In mouse bone marrow cells, the inhibition of MeXis altered the chromosome architecture at the ABCA1 locus, impaired cellular responses to cholesterol overload, and accelerated atherosclerosis. Thus, MeXis regulates the expression of ABCA1 through LXR (100). An impact on the LXR-MeXis-Abca1 axis can enhance the reverse transport of cholesterol in macrophages. Exposure to MeXis is a potential therapeutic targeting strategy for the regulation of macrophage cholesterol efflux.

LSTR (hepatic triglyceride regulator) regulates the clearance of plasma triglyceride by apolipoprotein C2 (APOC2) and lipoprotein lipase. Blocking of lncLSTR could reduce triglyceride levels in a mouse model with hyperlipidemia (Figure 3) (103). lncLSTR regulates the TDP-43/FXR/apoC2-dependent pathway to maintain systemic lipid homeostasis.

The ANRIL noncoding RNA is a key molecule of atherogenesis, located at the Chr9p21 locus. ANRIL affects several cell types related to the development of cardiovascular diseases (104). ANRIL in a cis position leads to high levels of linear ANRIL but reduces the annular level of ANRIL (104). The balance of the linear and circular RNA ANRIL, defined by the Chr9p21 genotype, regulates the molecular pathways and cellular functions involved in atherogenesis (104). ANRIL reduces the viability and proliferation of smooth muscle cells and activates inflammation and apoptosis in endothelial cells (105-106). In addition to acting in the cis-conformation, ANRIL acts in the trans-conformation (through Alu elements) to regulate other genes that are involved in proatherogenic pathways (Figure 3) (107).

A commonly expressed and evolutionarily conserved lncRNA, MALAT1, is less actively expressed in atherosclerotic plaques (108). Reduced expression of MALAT1 in hematopoietic cells contributes to the development of atherosclerosis and inflammation in mice *in vivo* (109). ApoE$^{-/-}$ heterozygous mice with MALAT1 deficiency, exhibited an increased level of inflammation and showed susceptibility to the development of atherosclerosis (110). The knockdown of MALAT1 in vascular smooth muscle cells (VSMCs) and endothelial cells (ECs) led to cell cycle arrest and the reduction in cell proliferation (Figure 3) (111). In addition, the silencing of Malat1 with LNA-GapmeR inhibited the proliferation and formation of primary endothelial cells (SMMECs) *in vitro* (112). The mechanism of action resulting in the inhibition of proliferation is associated with MALAT1 binding to the VEGFR2 gene (113). Taken together, these results suggest that MALAT1 may play a critical role in the development of angiogenesis (113).

lincRNA-p21 is reduced in patients with coronary heart disease and in mice with atherosclerosis (114). lincRNA-p21 regulates the p53-dependent proliferation and apoptosis of smooth muscle cells (114).

Endothelial nitric oxide synthase (eNOS) is an important element of endothelial homeostasis and vascular function (115). Transcription of eNOS is regulated by two key transcription factors namely Krüppel-like factors 2 (KLF2) and 4 (KLF4) (116). Whereas the lncRNA LEENE enhances expression of eNOS, its inhibition at the level of transcription suppresses eNOS, while the overexpression of LEENE increases the level of eNOS and its bioavailability of NO (117). In addition to the regulation of eNOS, LEENE can interact with genomic loci that encode certain sets of genes (118). These genes are involved in multiple pathways, which are critical to endothelial homeostasis, for example, cell adhesion and VEGF signaling (118).

Identifying the functions of lncRNAs in atherosclerosis may be the key to revealing the mechanisms that contribute to the development of this pathology. Together, the available evidence suggests that increasing the levels of CHROME, MeXis, Malat1, lincRNA-p21, or LEENE and inhibiting the activity of LSTR or ANRIL can be used in the treatment of disorders of lipid metabolism and atherosclerosis (Figure 3). Further studies are required to show whether lncRNAs act synergistically and whether they play redundant and/or compensatory roles with other un-regulated lncRNAs and/or mRNAs associated with the development of atherosclerosis.

The most common mechanism of epigenetic regulation is methylation directed at long non-coding RNA (lncRNA). Thus, methylation of histones or DNA in the CpG sequences using methyltransferase 3, histone H3, lysine 9, methyltransferase, and polycomb repressive complex 2 (PRC2) lead to the stable repression of genes (119). The manipulation of lncRNAs is based on the introduction of oligonucleotides by injection or inhibition of expression of lncRNA (120). Currently, there are two pre-clinical models that suppress lncRNA expression. One approach is based on RNAi-based methods, such as siRNA and LNA-GapmeR antisense oligonucleotides (ASO), which induce RNAase dependent cleavage (121). Another approach is based on the RNAi method, using siRNA and a short hairpin RNA (shRNA) which are delivered via a viral vector, and target lncRNA, which are primarily localized in the cytoplasm (122). GapmeR can be used for core-localized lncRNA because it induces degradation with RNase-H and is RISC-independent (123). GapmeR can be used to modulate the expression of lncRNA *in vivo*. The LNA-GapmeR-mediated silence of MALAT1 in the endothelial cells of skeletal muscle effectively reduces the formation, migration, and proliferation of endothelial cells (87). Genome editing with CRISPR/Cas9 which acts as “molecular scissors” is a new tool for modulating gene expression associated with ncRNA, including the manipulation of lncRNA (123-124). CRISPR inhibition (CRISPRi) suppresses the expression of miR-21 and lncRNAs, including GAS5, H19, MALAT1, NEAT1, TERC, XIST (126), UCA1, and lncRNA-21A (125). Thus, in the context of atherosclerosis, genetic manipulation of lncRNAs via antisense oligonucleotides or CRISPR/Cas9 can be used to remove or activate/repress the expression of lncRNA as an effective therapeutic approach towards the treatment of cardiovascular diseases.

6. DRUG DELIVERY USING NANOPARTICLES

Despite significant progress in creating systems that model atherosclerosis, accurately testing potential epigenetic inhibitors for the treatment of atherosclerosis requires developing more robust systems. Delivery of compounds to the site of injury remains a problem that can be solved by the development of nanoparticles. The effectiveness of nano drugs has been shown in the prevention, diagnosis, and treatment of various diseases, including atherosclerosis. Nanoparticles already are being used as visualization tools to detect vulnerable atherosclerotic plaques and similar theranostic strategies have already demonstrated the potential for identifying diseases such as cancer and neurodegenerative disorders using a variety of imaging methods, including optical imaging, magnetic resonance imaging (MRI), ultrasound and photoacoustics, computed tomography (CT), and nuclear imaging based on single-photon and positron emission tomography (127). Molecular markers namely VCAM-1, ICAM-1, P-selectin, E-selectin, and $\alpha_v\beta_3$-integrin over-expressed on activated endothelium are the main targets for targeted treatment with nanoparticles (128). High-density lipoproteins (HDLs) are responsible for modulating inflammation, and are involved in the reverse transport of cholesterol, therefore, these are also potential targets. Intimal macrophages are critical cells in the development of atherosclerosis and can absorb nanoparticles by phagocytosis, so they are potential targets for nanoparticles too (129). It should be noted that the use of nanosystems significantly reduces the risk of side effects. Targeted drug delivery to atherosclerotic foci and plaques will be a much more effective method than classical treatment methods.

Nanoscale drug delivery systems are obtained using various organic, inorganic, lipid, and polymeric biomaterials. Numerous studies have shown that the structural and physico-chemical characteristics of nanoparticles can affect their performance *in vitro* and *in vivo*. The large surface to volume ratio facilitates the design of multi-functional nanoparticles, i.e., the shape and surface charge of the nanoparticles which are shown to affect the penetration of nanoparticles through the blood-brain barrier, biodistribution in organs, and cellular absorption. Nanoparticles are classified based on various properties including their morphology or source. For example, based on their morphology these are divided into nanospheres, nanotubes, and dendrimers and linear, block, and graft grafted structures (grafts). Nanoparticles can also be

categorized, based on their source namely natural, synthetic, hybrid, or metallic sources.

The unique characteristics of nanomaterials (for example, shape, size, and charge) make them promising tools for both diagnostics and therapeutic approaches, but today there are still many limitations and shortcomings that has hampered their clinical. Currently, out of 51 FDA-approved nano-medicines and 77 products undergoing testing, only few have been identified as potential therapy for atherosclerosis. Among these are

1. Tricor (Lupine Atlantis, 2004) with nanocrystalline fenofibrate
2. Rapamune sirolimus immunosuppressant (Wyeth Pharmaceuticals, 2000)
3. plasmon immunosuppressant sirolimus (2000)
4. plasmonic immunosuppressant sirolimus (2000)
5. plasmon-containing immunosuppressant sirolimus Rapamune (plastophage)

In addition, atherosclerosis is being treated with stem cells (NCT01270139), as well as MRI with iron and enhanced ferumoxitol for the assessment of myocardial infarction (NCT01995799, NCT01323296), including Feridex/Endorem superparamagnetic imagers (superparamagnetic ferric oxide of nanocephanol) dextran-coated (SPION) (AMAG Pharmaceuticals, 1996, 2008) and GastroMARK; Lumirem (SPION, coated with silicone; AMAG Pharmaceuticals, 2001, 2009) (130-131). However, still 98.83% of these approaches are still in a pre-clinical stage (132).

Removal of nanoparticles from body has been a major concern since nanoparticles accumulate in the reticuloendothelial system (RES), due to their polydispersity and/or the complex reproducibility of their preparation, or because of the difficulty of their scaling and high production costs, especially when particles are multifunctional in nature (133).

7. SUMMARY AND PERSPECTIVE

CVD is one of the key causes of mortality, and to date, there are many methods of correcting specific aspects of this disease. Epigenetic-based atherosclerosis hypotheses have improved the understanding of the molecular mechanisms of atherosclerosis, which has been traditionally regarded as a chronic inflammatory and lipid disorder, with genetic codes being a key determinant. Therapy based on miRNA is a new area for research with a significant promise. Studies in mice, primates, and early human trials all have clearly demonstrated the potential of using miRNAs as valuable therapeutic agents. In the treatment of atherosclerosis, a number of non-coding RNAs such as miRNA, siRNA, and lncRNA, have been identified, to contribute to the pathogenesis of the disease.

Presently, some features of the regulation of gene expression using ncRNAs have been investigated, but their enormous therapeutic potential is already coming to focus. Noncoding RNAs are involved in many epigenetic regulatory processes of atherosclerosis. Long non-coding RNAs possess therapeutic potential by coordinating many epigenetic regulatory processes, including chromatin dynamics, DNA methylation, and the stability of mRNA and other non-coding RNAs.

However, there are several unsolved problems regarding the safe and effective delivery methods for epigenetic modifiers, their long-term effectiveness, as well as side effects that might emerge upon long-term. It is also currently still not entirely clear how to achieve the desired specificity of miRNAs which are aimed at a specific metabolic pathway. Given the relatively short period of time since the discovery of miRNAs, progress seems to be sufficient to justify an optimism regarding the development of new therapeutic agents based on targeting miRNAs.

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Abbreviations: CVD: Cardiovascular disease; ncRNA:non-coding RNA; siRNA:small interfering RNA; LDL:low-density lipoprotein; Lnc:RNA - Long non-coding RNA; VLDL:low density lipoproteins; LPGAT1: lysophosphatidylglycerol acyltransferase-1; ABCA1:ATP-binding cassette A; ABCG1; ATP-binding cassette sub-family G member 1, SREBP:Sterol regulatory element-binding proteins; HDL: High-density lipoprotein; PGC1-α:Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PDK4-Pyruvate dehydrogenase lipoamide kinase isozyme 4; RXRα:Retinoid X receptor alpha; DNMT: DNA methyltransferase; ASOs:Antisense oligonucleotides; LNA: locked nucleic acid; ORN: synthetic oligoribonucleotides; PCSK9:Proprotein convertase subtilisin/kexin type 9; FDA:Food and Drug Administration in the USA; HAT:Histone acetyltransferase; HDAC:histone deacetylase; LXR:liver receptors; VSMC:vascular smooth muscle cells; EC:endothelial cells; eNOS:Endothelial synthase nitric oxide; KLF:Krüppel-like factors 2; VEGF:Vascular endothelial growth factor; ASOs:Antisense oligonucleotides; shRNA: short hairpin RNA

Key Words: Atherosclerosis, miRNA, siRNA, lncRNA, Epigenetic, Review

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