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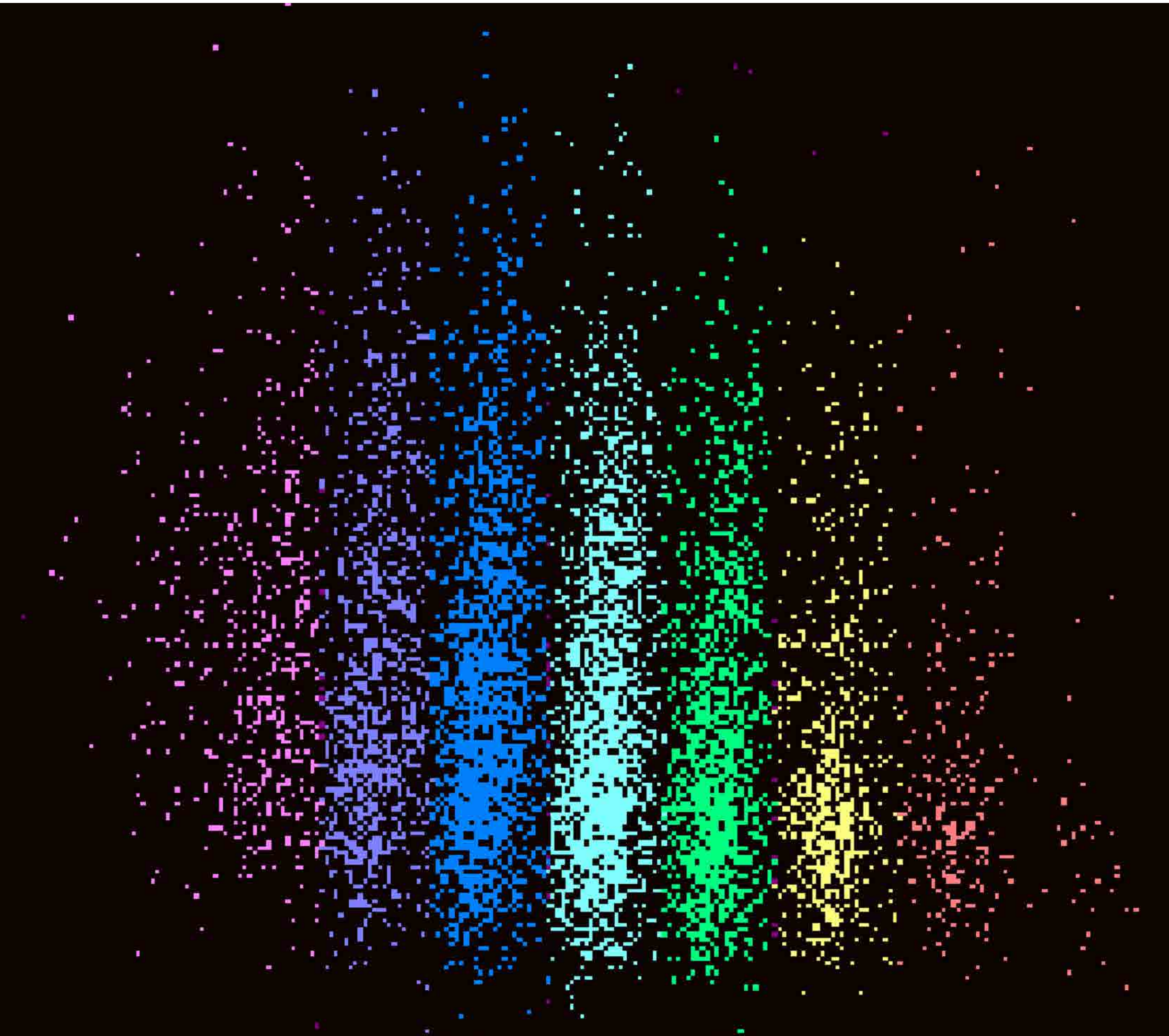

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Contents

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Atherosclerosis associated with *Chlamydia pneumoniae*: Dissecting the etiology

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ABSTRACT

Keywords
Atherosclerosis;
Chlamydia pneumoniae;
foam cells;
inflammation;
endothelium;
infection

Chlamydia pneumoniae related infections and atherosclerosis are both common entities. Today, the literature presents an increasing amount of data regarding the role of C. pneumoniae in the development and sustainment of atherosclerosis and allowing us to comprehend the molecular mechanisms behind better. The implications of C. pneumoniae in atherogenesis include altered platelet function, hypercoagulability, macrophage dysfunction, vascular smooth muscle proliferation, and increased neutrophilic migration. Therefore, it would not be wrong to implicate that, C. pneumoniae plays important roles in almost every stage of atherogenesis. Furthermore, various serological markers suggestive of active or past C. pneumoniae infection are known to be associated with multiple clinical presentations, such as abdominal aortic aneurysms, subclinical atherosclerosis in young individuals, aggravated atherosclerosis in heterozygous familial hypercholesterolemia. This review, aims to provide detailed insights into the pathophysiological mechanisms of atherogenesis associated with C. pneumoniae and its clinical implications.

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Introduction

Atherosclerosis is a public health issue leading to numerous clinical syndromes and diseases affecting a great majority of the population, including but not limited to coronary heart disease, peripheral artery disease, ischemic stroke, vascular dementia and mesenterial ischemia, constituting the greatest contribution to morbidity and mortality [1]. While the recent developments have shown that atherosclerosis is a complex process, in which many factors acting on the endothelium play various roles, whereby the immune system is responsible for a majority of irreversible changes or widespread evolution of this condition throughout the body, which led to the efforts of identifying specific cell types inhabiting the atherosclerotic niche, which includes but not limited to single-cell approaches [2].

The latest research has identified many antigens implicated in the pathogenesis of atherosclerosis, with the majority of these being modified innate molecules such as oxidized low-density lipoproteins (oxLDLs), beta-2-glycoprotein-1 (Beta2GP1), lipoprotein (a) (Lp(a)), with some other foreign antigens stemming from bacteria such as some *Porphyromonas spp.* and *Chlamydia pneumoniae* (Cp) [3].

Among these, Cp is an obligate intracellular implicated Gram-negative and exists in two morphological forms: elementary and reticulate bodies. The elementary bodies play an important role in the transmission of the pathogen, which are not metabolically active. Whereas reticulate bodies are active forms with no role in transmissions [4]. Cp is known to induce various epigenetic changes in the cell, including deoxyribonucleic acid (DNA) methylation, gene silencing via microRNAs, and post-translational histone modifications [5].

Cp is a well-recognized cause of atypical community-acquired pneumonia (CAP), responsible for 10% of all CAPs, therefore a common pathogen to encounter in the clinical wards, and it has been of interest as a potential suspect in several immune-mediated diseases, spanning from multiple sclerosis [6] and reactive arthritis to asthma [7]. The exact mode of transmission for Cp is still uncertain but transmission through respiratory secretions, in form of droplet aerosols, has been suspected. This organism is known to survive on laminated countertops for 30 hours and small-particle aerosolization. Enclosed populations, including military personnel, prisoners and nursing home residents constitute significant risk groups for infection with Cp [8].

The role of Cp in the immune changes associated with atherosclerosis has lately become a topic of interest, with some authors suggesting that Cp might act as both an initiator and a driver of chronic inflammation in the atherosclerotic plaque niche [4].

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Cp has been discovered to be involved in a series of atherosclerotic cardiovascular diseases (ASCVD) including coronary artery disease (CAD), carotid artery stenosis, stroke, peripheral artery disease, and aortic aneurysms. This pathogen is proven to exist preferentially in the atherosclerotic lesions both in humans and animal models [4], however the data on how it contributes to the atheroma development or progression is controversial.

Nevertheless, three possible roles can be hypothetically attributed to Cp: first, it might persist in vascular cells as a bystander, therefore, not contributing to the pathogenesis; second, it might initiate atherosclerotic changes; third, it might contribute to the severity of the disease, all of which, however, requiring further investigation and validation [4].

In this review, we aim to summarize the latest literature in order to provide insights into the etiological role of Cp in the development and sustainment of the atherosclerosis, along with emphasizing the molecular pathways implicated. The role of Cp in the development of atherosclerotic lesions is summarized in **Table 1** and depicted in **Figure 1**.

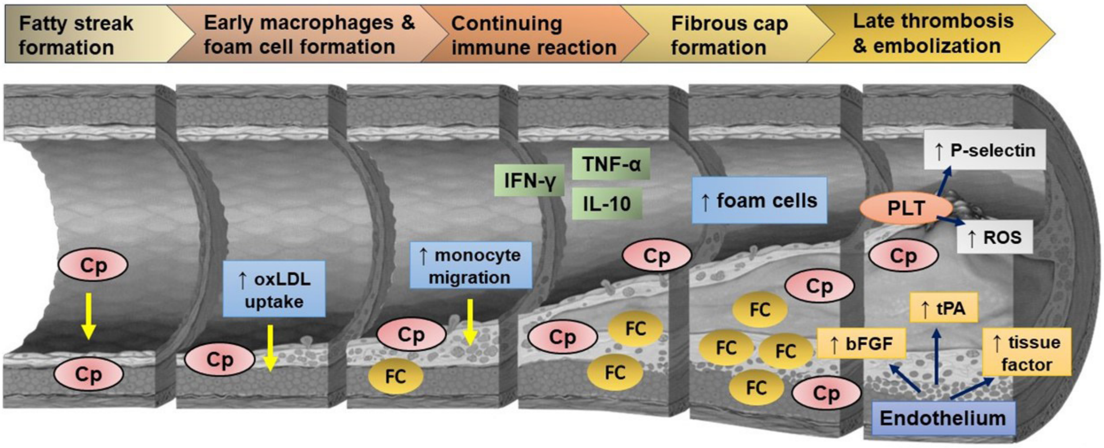
C. pneumoanie as a mere bystander? Probably not

Some authors agree that the relationship between Cp and atherosclerosis might not be as straightforward as often presented, therefore adhering to the fact that it might only play the role of a bystander,

Table 1 | Roles attributed to Chlamydia pneumonia in atherosclerosis.

Stages of atherosclerotic lesion	Chlamydia pneumonia associated changes
Fatty streak formation	Increased uptake of oxLDL by endothelium, by upregulation of LOX-1 [17-19]
Early macrophage reaction and foam cell formation	Promoting trans endothelial migration of monocytes through phosphorylation of tyrosine in vascular endothelial cadherin [25] Induction of foam cell formation from monocytes when LDL-C is present, dependent on TLR2 [12-14] Firmer monocyte adhesion to endothelium, through redistribution of CD44 [27] Increased uptake of oxLDL by macrophages, by upregulation of LOX-1 [17-19]
Continuing immune reaction	Increased macrophage presence and faster lesion progression through activation of IL-17 [47-49] Increased serum levels of IFN-γ, IL-10, TNF-α, sVCAM-1 and soluble E-selectin [51, 54-55] Increased expressions of SAA and VCAM -1 [50]
Fibrous cap formation	Promoting vascular smooth muscle cell migration through JunB-Fra-1/MMP2 pathway [24]
Late thrombosis and embolization	Increased tissue factor formation from the endothelium [31] Increased chemokine expressions on platelets [32] Increased platelet aggregation through, increased P-selection expressions on platelets [33] Stimulation of platelets, leading to ROS production and migration via bloodstream [35] Increased tPA expressions, leading to reduced thrombosis [39] Leading to increased bFGF expressions, associated with symptomatic plaques [51-52]

OxLDL: oxidized low-density-lipoprotein, **LOX-1:** oxidized low-density lipoprotein receptor 1, **LDL-C:** low-density lipoprotein cholesterol, **TLR2:** toll-like receptor 2, **IL-17:** interleukin-17, **IFN- γ:** interferon gamma, **TNF-α:** tumor necrosis factor alpha, **sVCAM1:** soluble vascular cell adhesion molecule 1, **SAA:** serum amyloid A, **VCAM:** vascular cell adhesion molecule, **bFGF:** basic fibroblast growth factor, **ROS:** reactive oxygen species, **JunB:** transcription factor Jun-B, **Fra-1:** Fos-related antigen 1, **MMP2:** matrix metalloprotease 2.



Cp: Chlamydia pneumoniae, **FC:** foam cell, **IFN-γ:** interferon gamma, **TNF-α:** tumor necrosis factor alpha, **IL-10:** interleukin 10, **PLT:** platelets, **ROS:** reactive oxygen species, **oxLDL:** oxidized LDL, **bFGF:** basic fibroblast growth factor, **tPA:** tissue plasminogen factor

Figure 1 | Chlamydia pneumoniae and atherosclerosis formation.

therefore, not contributing significantly to the pathogenesis [4]. While the subjects included in the epidemiological studies, where Cp seropositivity has been detected, were also carrying many other possible confounding risk factors for atherosclerosis such as smoking, high-cholesterol diet, diabetes, and hypertension [4]. Additionally, the incidence of Cp and arterial disease differs significantly among countries, ranging from 71% in Canada [9] to 0% in Australia [10]. The role of the sample-size, detection methods used and the season of sample collection in contributing to this variety remains unknown [4].

On the other hand, data from basic and translational science have propesed various roles for Cp in the development of atherosclerosis, after spreading from the lungs to reach atherosclerotic lesions via vasculature. First, Cp is known to induce *in-vitro* vascular smooth muscle cell (VSMC) proliferation and lead to the release of atherogenic cytokines in cultured human aortic vascular smooth cells inoculated with Cp [11]. Second, Cp can induce foam cell formation from monocyte-derived macrophages in the presence of LDL-C. These macrophages can enhance cellular oxidation of LDL-C, a reaction independent of the presence of reactive oxygen species (ROS), when Cp is involved. The latter, however, could be prevented by the heat treatment of the bacterium, which rules out the bacterial lipopolysaccharide (LPS) as the culprit for the LDL oxidization, as previously acknowledged [12], leading to the identification of another possible culprit known as chlamydial heat shock protein 60 (HSP60), that is found to be present in 47% of human surgical atherosclerotic tissue specimens [12]. However, it is important to note that, bacterial lipopolysaccharide (LPS) still plays an important role in the formation of foam cells, a hallmark of atherosclerosis [11].

Pathophysiological mechanisms

C. pneumoniae infects monocytes and vascular smooth muscle cells

An earlier study proved that phagocytosed Cp leads to the increased survival of granulocytes, whereas normal granulocytes undergoing apoptosis within 10 hours, while infected ones surviving up to 90 hours, as a result, exploiting these as host cells for multiplication [13]. The internalization of Cp occurs in an opsonin-independent manner [13].

Cp can infect *in-vitro* VSMCs [14]. Approximately two hours after inoculation with Cp, VSMCs start to overexpress toll-like receptor (TLR) 2 mRNA, which belongs to a greater family known as pattern recognition receptors (PRR) [4], while TLR2 only recognizing the organism but not chlamydial LPS or chlamydial heat shock protein 60, which are involved in various atherogenic processes, the latter two are recognized by another TLR, TLR4 [15].

Cp leads to the induction of foam cell formation in monocyte-derived macrophages after internalization, a sign of the early stages of atherosclerosis, when LDL-C is also present. However, LPS of Cp leads to this change only in cells presenting TLR2. On the other hand, lack of TLR4, another toll-like-receptor indicated in the *Escherichia coli* LPS associated foam cell formation, does not interfere with Cp-associated foam cell formation, suggesting that foam cell formation due to Cp is dependent on TLR2 [16].

Both TLR2 and TLR4 have an adapter protein named MyD88 to be activated, delineating this protein as a therapeutic target against chlamydia-associated atherosclerotic changes [15]. Nevertheless, Cp is capable of inducing foam cell formation dependent on and independent of MyD88 pathways, which are both activated by TLR2 and TLR4 [17]. On the other hand, liver X receptors (LXR) are known to downregulate TLR-mediated inflammatory pathways, and their expressions are decreased by Cp, promoting the transformation of macrophages into foam cells [18]. An LXR agonist can, as a result, significantly reduce Cp associated foam cell formation [17].

In *in-vitro* LDL-treated macrophages, Cp upregulates the expressions of both scavenger receptor A1 (SR-A1) and acyl-coenzyme A: cholesterol acyltransferase 1 (ACAT1), additionally downregulating the expression of ATP binding cassette transporters (ABCA1 and ABCG1) by exploiting the TLR2-NFκB-miR-33 pathway [19], facilitating cholesterol accumulation and therefore foam cell formation. Peroxisome proliferator-activated receptor gamma (PPARγ) plays an important role in the regulation of the above-mentioned SR-A1, ACAT, ABCA!, and ABCG1. Agonism of PPARγ through rosiglitazone can attenuate Cp-related foam cell formation, whereas antagonism of PPARγ leads to increased foam cell formation. Similar positive effects are produced by PPARα agonist fenofibrate, suggesting PPARα's possible role [20, 21]. Additionally, retinoic acid can suppress foam cell formation due to Cp in hyperlipidemic mice, whereas having no effect on uninfected animals [22]. Another inhibitor of ABCA1 is Cp-induced extracellular IL-1β through exploiting NLRP3 inflammasome, leading to the accumulation of lipids inside cells, which might lead to the investigation of NLRP3 inhibitors as alleviators of Cp-associated accelerated atherosclerosis [23].

On the other hand, inhibition of C-Jun NH2 terminal kinase (JNK1/2) and extracellular signal-regulated kinase (ERK1/2) strongly inhibit foam cell formation due to Cp infection in cultured macrophages, as Cp can not only downregulate PPARγ and PPARα but also downregulate the expressions of JNK1/2, ERK1/2 and p38 mitogen associated protein kinase (MAPK) through phosphorylation, suggesting the role of MAPK-PPARα/γ signal transduction pathway in foam cell formation due to Cp [24, 25].

Infection with Cp leads to the accumulation of mitochondrial ROS, which activates downstream signaling of the JunB-Fra-1/MMP2 pathway, promoting VSMC migration. In TLR2$^{-/-}$ ApoE$^{-/-}$ animal models, downstream activation of this pathway is not possible and Cp infection cannot lead to atherosclerosis [26].

Cp promotes trans-endothelial migration (TEM) of the monocytes into the subendothelial intimal layer, as it increases the permeability of the vascular endothelium and the rate of monocyte TEM while increasing the phosphorylation of tyrosine in vascular endothelial cadherins [27].

Furthermore, lung infection with Cp directly targets white adipose tissue and results in lipolysis, which subsequently result in fatty acid binding protein 4 (FABP4) secretion via endoplasmic reticulum stress. Released FABP4 can be taken up by neighboring adipocytes and contribute to further spread of the inflammation [28].

In addition, infection of fresh monocytes with Cp leads to a slower, uniform and steady rolling on E-selectin and endothelium compared to controls. Infection with Cp leads to the redistribution of CD44, a modulator of rolling functions, resulting in firmer adhesion of these monocytes on endothelial cells [29].

Lectin-like ox-LDL receptor is activated by C. pneumoniae

Oxidized forms of LDL (oxLDL) are known to be very proatherogenic and are one of the main culprits in the atherosclerotic plaque formation. Their receptor-mediated uptake can lead to functional changes in various cells involved in atherosclerotic plaque development, as a result, presenting the oxLDL-LOX-1 axis as a potential future therapeutic target [30]. Cp is able to upregulate the expression of the LOX-1 mRNA in both macrophages and endothelial cells , resulting in an increased uptake of oxidized LDLs [31]. This upregulation requires activation of ERK1/2, however can be inhibited by PPARγ agonists such as rosiglitazone, as Cp is also known to decrease PPARγ expressions in the infected endothelium, eventually leading to apoptosis [32]. However, the role of statins on this mechanism activated by Cp remains a mystery, requiring further investigation, considering that LOX-1 is normally inhibited by statins [33].

On the other hand, chlamydial glycan plays a crucial role in the Cp-induced upregulation of LOX-1, as prior treatments of organisms with PNGase, which can remove the chlamydial glycan, abolishes this process [31]. Additionally, mitigation of LOX-1 upregulation can be achieved through preincubation of the cells to be inoculated with anti-LOX-1 antibodies, that leads to the prevention of increased adhesion protein expressions such as matrix metalloproteinases (MMP) 1 and 3, which is observed after inoculation with Cp [31].

C. pneumoniae promotes hypercoagulability and altered platelet functions

In-vitro studies conducted with human umbilical vein endothelial cells (HUVEC) infected with Cp showed that endothelial synthesis of tissue factor has been increased via chlamydial factor, which can explain thrombosis associated with Cp infections [34].

Platelets in atherosclerotic patients have significant expressions for various chemokines including CCL3, CCL5, CCL7 and CXCL8 and treatment of platelets with live Cp or chlamydial LPS is known to induce similar increases in the expressions of these chemokines. In addition, these patients had positive sera of anti-Cp antibodies [35].

Chlamydial LPS increases platelet aggregation, leading to thrombotic occlusion of the vessels, as it causes increased P-selection expression on platelets, a process which can be inhibited by interfering with glycoprotein IIb/IIIa via abciximab [36]. Cp IgM is associated with platelet activation and there has been a relationship between P-selectin levels and Cp IgM titers in patients with myocardial infarction with ST-segment elevation, who received thrombolysis [37].

On the other hand, the interaction of Cp with platelets leads to ROS production through protein kinase C. This interaction is eventually associated with the oxidation of LDL particles, which is known to play an important role in the development of ASCVD [38]. These stimulated platelets leading to lipid peroxidation can be released from atherosclerotic lesions into the circulation even after percutaneous coronary interventions [39]. Additionally, in patients undergoing carotid endarterectomy, Cp IgA seropositivity is associated with embolization due to thrombosis but not due to plaques [40].

In patients with ASCVD widely used cyclooxygenase (COX) inhibitors fail to affect platelets stimulated by Cp, however inhibitors of 12-lipoxygenase have effects on these stimulated platelets, antagonizing their activation [41].

Some earlier studies suggest that Cp infection may lead to increased tissue plasminogen activator (tPA) levels in patients with chronic heart disease, which is associated with lower aggregability of platelets, therefore highlighting the increased inflammatory response as the main culprit for the development of atherosclerosis in Cp infection. Nonetheless, treatment with azithromycin in these patients with known Cp infection can also lead to higher levels of tPA [42].

C. pneumonia and apolipoprotein B are likely associated

Apolipoprotein (Apo) B plays a role in the LDL receptor mediated clearance of the LDL-C, as a result, mutations leading to defective or absent ApoB can result in familial hypercholesterolemia [43]. Some studies suggest that patients with Cp IgG and IgM seropositivity have higher mean ApoB levels compared with Cp negative controls [44]. Human ApoB and chlamydial LPS share common antigenic epitopes, and therefore antibodies against Cp might cross-react with ApoB [45].

Another study assessing the treatment with a multi-antigenic construct consisting of ApoB100, HSP60 and outer membrane protein of Cp showed that this treatment is associated with plaque stabilization and reduction of necrosis in plaques, with decreased expressions of MMP9, leading to reduced macrophage apoptosis. The authors suggested that tolerance to these atherogenic peptides causes an increase in regulatory T cells activating M2 macrophages and preventing the proliferation of T lymphocytes, eventually reducing plaque destabilization and inflammation, in an animal model of established atherosclerosis [46]. This approach might be the basis of future vaccines of atherosclerosis, as it causes reduction of early atherosclerotic lesions [47].

IL-17 is a mediator of C. pneumonia associated vascular changes

Cp is known to induce a T-helper-1 (Th1) dominated response, including the induction of proinflammatory cytokines such as IFN-γ, IL-12, and TNF-α [48]. Interleukin 17 (IL-17) is another proinflammatory cytokine, which functions to maintain the integrity of the epithelium and modulate the activity of adipocytes [49]. IL-17$^{-/-}$ mice fed with high-fat are shown to have diminished lesions in aortic sinus plaques and aorta when compared with controls after infection with Cp. They also tend to have slower lesion progression and lesser macrophage presence in the atherosclerotic niche [50].

Infection with Cp leads to increased IL-17 expression in VSMCs in ApoE deficient mice model of hyperlipidemia, which results in VSMC migration via c-Fos/IL-17 signaling [51]. Resveratrol can decrease IL-17 expressions, superoxide anions and the number of foam cells [52].

Endothelial response to C. pneumoniae is multifaceted

Cp leads to increased concentrations of adhesion molecules such as intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM), whose levels correlate with measured serum amyloid A levels, potentially reflecting the extent of endothelial affection, however requiring further investigation [53].

Chronic infection with Cp, on the other hand, leads to the activation of endothelial cells, promoting the production of basic fibroblast growth factor (bFGF) [54], a growth factor implicated in unstable plaque rupture and thrombus formation, leading to symptomatic plaques associated with myocardial infarction [55]. On the contrary, some authors suggest that Cp leads to an in-vitro decrease of platelet derived growth factor receptor beta (PDGFR-β) expression by aortic smooth muscle cells, considering this as a possible inhibiting factor for the development and advancement of atherosclerotic plaques [56], whereas others suggesting that it leads to an increase in PDGF-β, promoting intimal thickening in rabbit models [57].

Seropositivity of IgA antibodies against Cp is associated with increased serum levels of IFN-γ, IL-10, TNF-α, soluble VCAM 1 (sVCAM-1) and soluble E-selectin, however, antibodies targeting Cp's major outer membrane protein are not associated with any increased inflammatory markers [58].

Possible Clinical implications

Chlamydial LPS circulating in serum, which is recognized by TLR4, correlates with the levels of LPS binding proteins, IL-6 and high C-reactive protein levels, in patients with established peripheral artery disease or abdominal aortic aneurysm [59]. Elevated chlamydial LPS levels also are associated with elevated body mass index [60].

In another study, which covered a 3.5 year follow-up, intimal medial thickness did not differ between patients who were seropositive and those who were seonegative for antibodies against chlamydial LPS [61]. In patients with anti-chlamydial IgA antibodies, the frequency of myocardial infarction was found to be lower, and the use of anti-chlamydial antibiotics had no effect on ischemic events [61]. Additionally, seropositivity for chlamydial LPS antibodies were more common in patients with abdominal aortic aneurysms (AAA) but not in those with thoracic aortic aneurysms. Presence of antibodies against chlamydia species cross react with vessel wall antigens in AAA [62].

The presence of IgG antibodies against Cp is associated with an increased risk for acute coronary syndrome (ACS) with an odds-ratio (OR) of 1.62, along with other factors such as diabetes (OR 1.91), hypertension (OR 1.46), prior myocardial infarction, (or 1.78), and elevated troponin-T (OR 12.44) etc. [63]. Circulating Cp DNA is also associated with CAD in men (odds ratio [OR] 3.2, 95% confidence interval [CI] 1.1-8.9), but not in women who were [64].

In patients with heterozygous familial hypercholesterolemia (HeFH), Cp infection is known to be associated with a risk of chronic heart disease, consistent with the animal models of this disease [65]. Atherogenic effects of Cp are potentiated by the high levels of serum cholesterol supporting the hypothesis that Cp plays an aggravating rather than an initiating role in CAD [65].

Lp(a) and fibrinogen levels were found to be higher in patients with Cp seropositivity [66]. Formation of immune complexes containing IgG antibodies against Cp can also enhance the proatherogenic effects of Lp(a) [67].

Circulating Cp DNA can be associated with asymptomatic atherosclerosis in younger normotensive individuals [68]. However Cp DNA is not a reliable marker in high-risk populations such as type 2 diabetic patients [69]. However, Cp seropositivity is not known to be associated with intima media thickness [70]. Additionally, an infection with Cp doesn't play a role in the development of diabetes [71], nevertheless, a positive association between Cp seropositivity and the occurrence of metabolic syndrome is known [72].

Rapamycin, an immunosuppressive and antiproliferative agent, can inhibit in-vivo growth of Cp if applied at the beginning of the chlamydial infection, therefore, rapamycin-eluting stents might be useful in preventing Cp-associated stent restenosis [73]. On the other hand, selective cyclooxygenase (COX) inhibitors have bacteriostatic effects on Cp in *in-vivo* studies, but they can neither prevent infection nor eradicate Cp in affected cells [74].

Although the effect of antibiotics is out of our scope for this review, there are some studies that deserve to be shortly mentioned. Therapy with antichlamydial antibiotics might prove itself useful in patients with peripheral artery disease, accompanied by Cp IgG seropositivity, which has been further associated with shorter walking distance and higher need for revascularization. Roxithromycin, a macrolide, has positive effects on these parameters in these patients [75]. Contrarily, Secondary prevention of atherosclerosis through chlamydia pneumoniae eradication (SPACE Trial) has shown that azithromycin has no significant effect in patients with peripheral artery disease [76]. In an Apo-E deficient animal model of hyperlipidemia, in which atherosclerosis had been accelerated via Cp, antibiotic treatment with azithromycin also had no significant effects [77]. Another microbial agent, rifalazil, provided no significant symptomatic improvement in patients with established peripheral artery disease [78]. However, in another study, including patients with established carotid atherosclerosis who had no improvement under probucol therapy for 24 months, a 12-month combination therapy with levofloxacin, a fluoroquinolone, and probucol, an antioxidant, proved beneficial [79].

Finally, another important concern is that even though Cp seropositivity has been shown in various autoimmune diseases involving many systems, confounding factors such as overall increased proinflammatory immune response might additionally explain the increased prevalence or acceleration of atherosclerosis in these patients, aside from Cp seropositivity [80].

Conclusion

Cp, as a common cause of respiratory tract infections, is a pathogen with which many practitioners are familiar. The effects of Cp on vascular system are of utmost importance due to Cp's enormous clinical impact. Cp gained the attention of many clinicians in the late 20th century through some pioneering work, however the data, as the methods of investigation, have significantly evolved ever since.

Today we know that Cp seems to play an important role in almost every stage of atherosclerotic lesion formation and might interfere with our therapeutic efforts and prove itself resistant to our therapeutic or prophylactic means. Even though an infection with Cp can easily be treated with antibiotics in short-term, the effects on the vascular bed still present itself as an important problem, while even IgG seropositivity, supporting old infections, is associated with vascular complications.

It's important to highlight that there are still many questions requiring answers, including but not limited to the following: Cp's interaction with Lp(a), another rediscovered risk factor for atherosclerosis, colchicine's secondary preventive effects on patients with ASCVD who are seropositive for Cp. As we have summarized here, these are the latest developments in this often overlooked field within atherosclerosis research.

Author contributions

All authors contributed equally and participated to the conception, design, data acquisition and analysis and data interpretation regarding the work presented. All authors contributed to drafting, reviewing and/or revisiting of the presented work and approved its publication.

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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**).

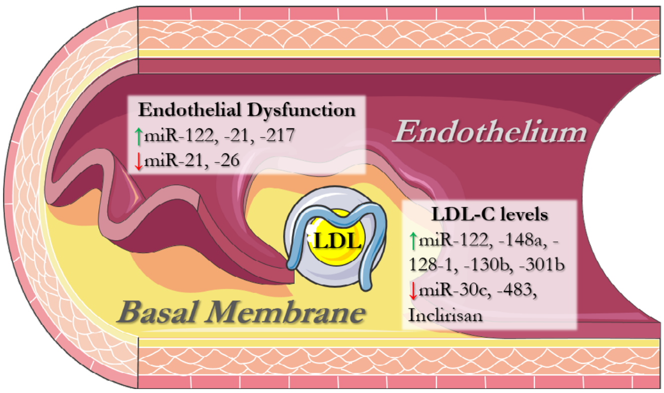

Figure 1 | microRNAs in lipoprotein adhesion to the basal membrane. *Schematic overview of the miRNAs involved in the regulation of endothelial dysfunction, LDL synthesis and uptake. Green arrow: endothelial dysfunction/LDL-C enhancement; red arrows: endothelial dysfunction/LDL-C modulation. Created 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].

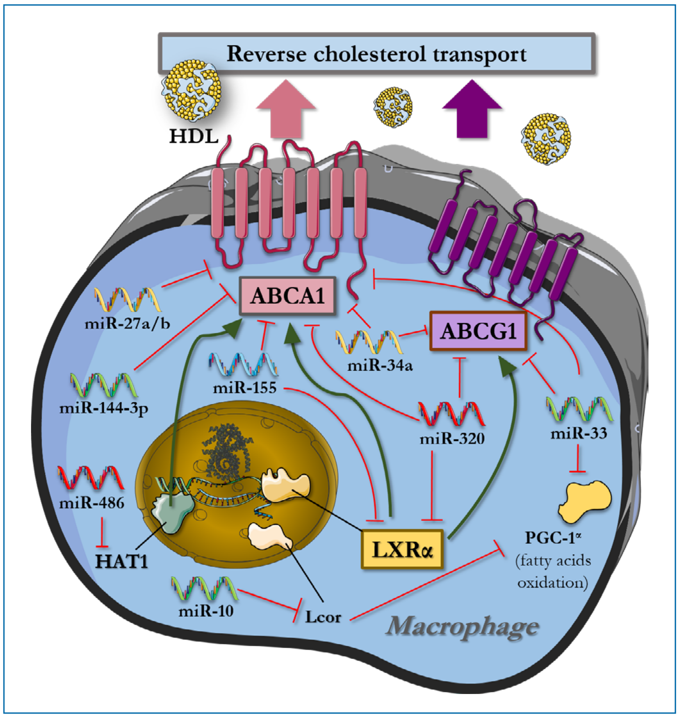

Figure 2 | Role of microRNAs in reverse cholesterol transport regulation within the macrophage. *Schematic representation of the miRNAs known to affect HDL nucleation and foam cell formation within plaque resident macrophages. Green arrows: positive transcriptional regulation; red connectors: negative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

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

[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

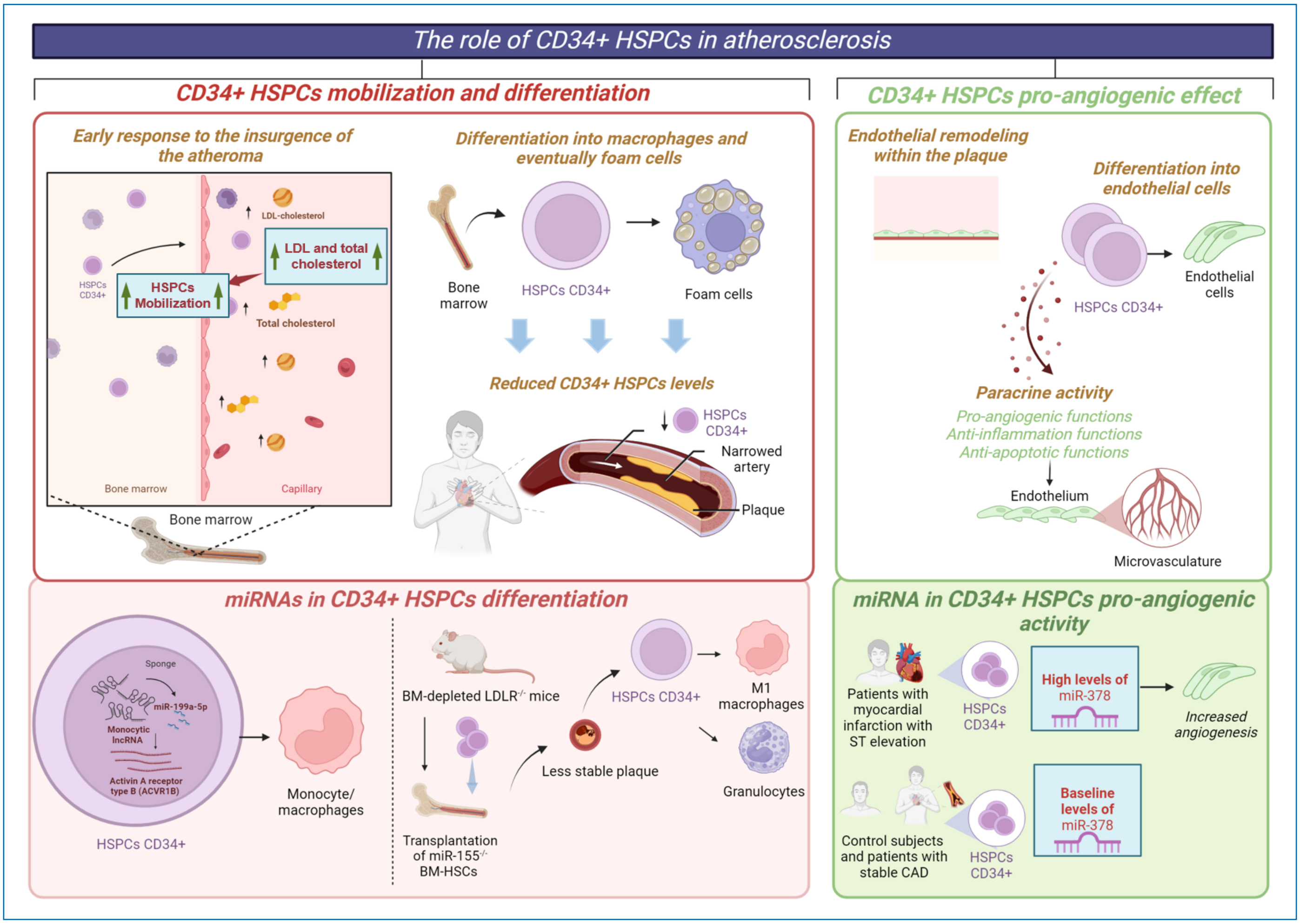

Figure 3 | miRNAs and the role of CD34+ HSPCs in atherosclerosis. *The role of miRNAs in the mobilization, differentiation (left panel), and function (right panel) of CD34+ HSPCs in the development of atherosclerosis. HSPCs: hematopoietic stem and progenitor cells; LDL: low-density lipoprotein; BM-HSCs: 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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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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Novel approaches to lipid lowering

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ABSTRACT

Lowering cardiovascular risk by reducing apoB-containing lipoproteins (primarily low-density lipoproteins, LDL) is the key step in cardiovascular prevention. Current treatments such as high-intensity statins, ezetimibe and proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors are effective, but some patients still experience cardiovascular events due to residual risks determined by factors beyond LDL-cholesterol (LDL-C) levels, including triglyceride levels and inflammation. New approaches are currently under investigation to further reduce cardiovascular risk. These include next-generation CETP inhibitors such as obicetrapib, which lowers LDL-C and increases HDL-C without the side effects of earlier drugs. Oral PCSK9 inhibitors (MK-0616 and AZD0780) show promise, potentially overcoming economic barriers. Efforts to reduce Lp(a) include antisense oligonucleotides, siRNAs and assembly inhibitors like muvalaplin, all showing significant Lp(a) reduction. PCSK9 gene editing using CRISPR-Cas9 technology has shown dramatic cholesterol-lowering effects in preclinical studies and thus offers potential for the future. These new approaches could significantly advance cardiovascular risk management.

Keywords
Lipid-lowering;
oral PCSK9 inhibitors;
antisense oligonucleotide;
small interfering RNA;
PCSK9 gene editing

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Introduction

This review focuses on novel and future approaches to reduce the cardiovascular (CV) residual risk by reducing the apoB-containing lipoprotein levels. We assume that in the clinical practice a combination therapy based on the use of high-intensity statins, ezetimibe, bempedoic acid, monoclonal antibodies (mAbs) targeting proprotein convertase subtilisin/kexin type 9 (PCSK9) or inclisiran may reduce most of the cardiovascular risk associated with the apoB containing lipoproteins and may contribute to achieve the LDL-cholesterol (LDL-C) goal in the majority of individuals at high and very high risk. However, we can assume that a proportion of well-treated patients will develop new cardiovascular events. Many factors such as the genetic background, the pre-existing burden of disease and the residual risk attributable to triglycerides, inflammation, coagulation and platelets may explain this recurrence of events. We discuss some novel options to reduce the residual risk due to the two main apoB-containing lipoproteins, LDL-C and Lp(a), that are (**Figure 1**):

- the newest-generation CETP (cholesteryl ester transfer protein) inhibitor, obicetrapib;
- the oral PCSK9 inhibitors;
- the novel approaches to reduce Lp(a) plasma levels (antisense oligonucleotides-ASO; small interfering RNA-siRNA; assembly inhibition);
- the PCSK9 gene editing.

Obicetrapib

CETP is a glycoprotein which regulates the two-way exchange of cholesteryl esters and triglycerides from high-density lipoprotein (HDL) particles to low-density and very low-density lipoproteins (LDL and VLDL) and also the transfer of triglycerides from LDL and VLDL to HDL particles. The human genetic model of CETP deficiency has shown that mutation carriers have no CETP activity and very high levels of HDL-cholesterol (HDL-C). Epidemiological data demonstrated that HDL-C is inversely correlated with the cardiovascular risk. It was obvious to design trials with drugs known to increase HDL-C such as fibrates and niacin to demonstrate a reduction of cardiovascular events due to the increase in HDL-C plasma levels [1].

The early CETP inhibitors, torcetrapib, dalcetrapib and evacetrapib were tested in clinical trials but, despite a significant increase

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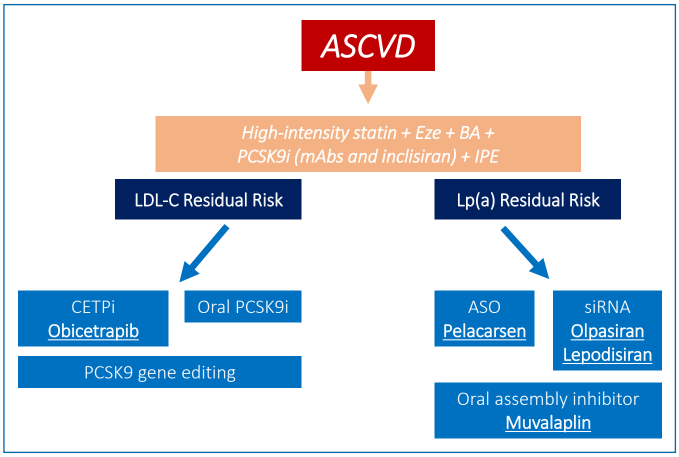

Figure 1 | Future approaches to apoB-containing lipoprotein-related residual risk. *ASCVD: atherosclerotic cardiovascular disease; Eze: ezetimibe; BA: bempedoic acid; PCSK9i: proprotein convertase subtilisin/kexin type 9 inhibitors; mAbs: monoclonal antibodies; IPE: icosapent ethyl; LDL-C: low-density lipoprotein cholesterol; Lp(a): lipoprotein (a); CETPi: cholesteryl ester transfer protein inhibitors; ASO: antisense oligonucleotide; siRNA: small interfering ribonucleic acid.*

in HDL-C (72%, 30% and 133%, respectively), torcetrapib increased cardiovascular events mainly for an off-target effect of blood pressure and dalcetrapib and evacetrapib trials were stopped for futility [2].

Anacetrapib, a CETP inhibitor, was tested in the REVEAL trial and showed a significant reduction in cardiovascular events attributable to the reduction in apoB-containing lipoproteins rather than the increase in HDL-C. The drug development was stopped because of the long-lasting accumulation of anacetrapib in the adipose tissue, but this trial paved the way for reversing the negative feelings regarding CETP inhibition as a cardiovascular prevention strategy [3]. In addition, recently the Mendelian randomization approach has shown that reducing LDL-C by CETP inhibition produces the same cardiovascular benefits as those achieved by statins, ezetimibe and PCSC9 inhibition, and there is also evidence of the ability of CETP inhibition to reduce the risk of new-onset diabetes since glucose tolerance and insulin sensitivity are improved [4]. Obicetrapib is the last-generation CETP inhibitor in the more advanced stage of development. The phase 1 and 2 studies have shown that obicetrapib significantly lowers all apoB-containing lipoproteins, including Lp(a) and the small LDL particles, and increases the levels of mature HDL as well as pre-beta HDL. In the phase 2 clinical trial ROSE2, obicetrapib in monotherapy or combination with ezetimibe reduced LDL-C by 43% and 63%, respectively, and increased HDL by 142%. Obicetrapib has been safe and well tolerated in thousands of patients enrolled in phase 1 to 3 clinical trials. None of the off-label or pharmacokinetic effects of torcetrapib and anacetrapib respectively have been seen in the thousands of patients enrolled in obicetrapib trials. The data from the outcomes study-PREVAIL will be known in 2026 and recently the results of the BROOKLIN trial have been released showing a significant reduction (41.5%) in LDL-C obtained in a difficult-to-treat population such as patients with heterozygous familial hypercholesterolemia (HeFH). In the ongoing TANDEM trial, the efficacy and safety of fixed-dose combination (FDC) of obicetrapib plus ezetimibe in adult patients with HeFH and/or atherosclerotic cardiovascular disease (ASCVD) or multiple risk factors for ASCVD are under evaluation [5]. Once approved for clinical use, obicetrapib will represent a novel LDL-receptor-independent approach to reduce LDL-C and cardiovascular risk.

Oral PCSK9 inhibition

The discovery of PCSK9 as a cause of familial hypercholesterolemia and the comprehension of its role in the LDL-receptor degradation pathway has opened a new era of LDL-C lowering pharmacology. Targeting PCSK9 by monoclonal antibodies, evolocumab and alirocumab, represents today a well-established therapeutic practice. Monoclonal antibodies to PCSK9 are safe, well tolerated and effectively reduce LDL-C up to 60%. The two outcomes trials, FOURIER and ODYSSEY Outcomes, have demonstrated significant reductions in cardiovascular events and mortality [6, 7]. In addition, the real-world data indicate a very high adherence and compliance. Recently the siRNA inclisiran, administered twice a year and targeting the PCSK9 gene expression in the liver, has been approved and entered into the clinical practice. However, some barriers are still limiting the use of PCSK9 mAbs, such as costs and prescription rules adopted in many countries. The RNA display screening technology led to the discovery of macrocyclic peptides that bind the PCSK9 with a monoclonal-like affinity. The two oral inhibitors in development are MK-0161 and AZD0780. MK-0161 has been evaluated in phase I and II trials. In the phase IIb trial, 381 adults with clinical ASCVD, intermediate/high ASCVD risk or borderline risk and LDL-C in a range between 70 mg/dL and 250 mg/dL according to the risk class were enrolled. The efficacy was dose-dependent: 41% LDL-C reduction with 6 mg daily, 56% LDL-C reduction with 12 mg daily, 59% LDL-C reduction with 18 mg daily, and 61% LDL-C reduction with 30 mg daily. The drug was safe and well tolerated. The results of the CORALreef Outcomes trial on cardiovascular benefits will be available at the end of 2029. AZD0780 is an oral small-molecule PCSK9 inhibitor; in the phase I trial the drug was administered on top of statin treatment and the efficacy results showed an LDL-C reduction of 52%. The development of oral, small molecules able to inhibit PCSK9 is promising and, if successful, would enrich our therapeutic armamentarium, potentially overcoming the economic issues and expanding the clinical settings of the PCSK9 targeting [8].

Novel approaches to reduce Lp(a)

Lp(a) was discovered 61 years ago by Kare Berg, but only in the last two decades a body of epidemiological and genetic studies have confirmed the role of Lp(a) as a cause of ASCVD and calcific aortic valve disease (CAVD). Lp(a) is the main carrier of oxidized phospholipids (oxPL) and plays a pivotal role in the residual CV risk; to date, lipid-lowering drugs are ineffective o poorly effective in reducing Lp(a) plasma levels. An ASO, a siRNA and a small molecule that interferes with the LDL-apo(a) binding are under development [9].

Antisense oligonucleotide-ASO

Pelacarsen is a N-acetylgalactosamine-conjugated oligonucleotide that was demonstrated in phase 1 and 2 studies to reduce Lp(a) plasma levels up to 80%, with up to 98% of patients achieving Lp(a) plasma levels <50 mg/dL [10]. The outcome trial, Lp(a) Horizon, was designed to measure the cardiovascular endpoints reduction in patients with myocardial infarction (MI) or ischemic stroke and clinically significant symptomatic peripheral artery disease and with entry-level of Lp(a) of 70 mg/dL. The endpoints-time to CV death, nonfatal MI, nonfatal stroke, and urgent coronary revascularization requiring hospitalization - will be evaluated in patients with baseline Lp(a) ≥70 mg/dL and ≥90 mg/dL. The results are expected in 2025 [11].

Small interfering RNA-siRNA

Olpasiran and lepodisiran are two siRNAs that potently lower Lp(a), according to the results of the phase 1 trials. Olpasiran has reduced Lp(a) levels up to 98% in individuals with entry levels >75mg/dL. The drug was well tolerated and safe. Lepodisiran reduced Lp(a) levels up to 97% in subjects with entry levels >30 mg/dL and was also safe and well tolerated. The outcome trials to establish the clinical effectiveness of reducing Lp(a) are ongoing [9]. For olpasiran the results of the OCEAN study are expected in 2026; OCEAN has enrolled 7,000 patients with very high CV risk with an entry-level of Lp(a) ≥90 mg/dL and the endpoint is a 3-point coronary heart disease MACE (cardiovascular death, myocardial infarction, and coronary revascularization) [12]. Lepodisiran outcome trial, ACCLAIM-Lp(a), will enrol 12,500 patients with ASCVD or high-risk patients including HeFH patients and an Lp(a) entry level of 80 mg/dL. The main expected outcome is the reduction of CV mortality and the results are estimated to be delivered in 2029 [13].

Assembly inhibition

Lp(a) is assembled following a noncovalent interaction between apo(a) kringle 7 and 8 domains and lysine residues of apoB100 in the hepatocyte. Muvalaplin is a small molecule that inhibits the formation of Lp(a) by blocking the formation of the covalent disulfide bond [14]. In the phase 1 trial muvalaplin reduced Lp(a) plasma levels by 65%, with up to 93% of enrolled individuals achieving Lp(a) levels <50 mg/dL. The drug was safe and well tolerated. Muvalaplin could represent a valid alternative to the other more expensive Lp(a)-lowering drugs.

PCSK9 gene editing

The study of the mechanisms of DNA repair including the discovery of the enzymes involved opened the way to the concept of gene editing. A crucial step has been the discovery of the CRISPR-CAS9 as a gene-editing tool. As a DNA-editing tool, CRISPR-Cas9 can abolish or increase the function of a given gene. The potential of this methodology will lead to the cure for many genetic diseases. Since the adenovirus cannot be used to deliver the CRISPR-CAS9 apparatus because it is too large for the vector capacity, lipid nanoparticles with N-acetylgalactosamine (GalNAc) are currently used. GalNAc is a high-affinity ligand for the asialoglycoprotein receptor which is located only on the hepatocytes and this allows selective delivery to the liver. PCSK9 gene is a good candidate for a gene editing approach: i-carriers of loss-of-function mutations in the PCSK9 gene have very low levels of LDL-C and this marked reduction from birth translates into a dramatic reduction in cardiovascular diseases; ii- the clinical use of mAbs anti-PCSK9 resulted in an effective reduction in CV events. In the mouse model targeting the PCSK9 gene by CRISPR-CAS9 gene editing produced a ~95% decrease in plasma PCSK9 and a ~40% total cholesterol levels decrease [15]. In non-human primates, the improvement of gene editing technology produced a ~90% decrease in plasma PCSK9 levels and a parallel 60% reduction in LDL-C levels [16]. The preclinical results opened the way to clinical studies and the efficacy of the PCSK9 gene editing is currently tested in heterozygous familial hypercholesterolemia patients (trial NCT05398029 by VERVE Therapeutics). The preliminary results of the VERVE-101 trial on HeFH patients with severe ASCVD are promising, as a stable reduction in LDL-C up to 55% was observed after 6 months. However, many issues remain unanswered including the ethical aspects, the long-term safety and the right target disease-population.

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Atherosclerosis and cholesterol: What we should know

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ABSTRACT

Keywords
Cholesterol;
atherosclerosis;
low-density lipoprotein;
lipid-lowering

Epidemiological studies consistently link high low-density lipoprotein cholesterol (LDL-C) levels with an increased risk in cardiovascular disease. This correlation remains strong across various populations. LDL-C plays a key role in atherosclerosis by transporting cholesterol to arterial walls, where it induces plaque formation. Lowering LDL-C levels has proven to reduce the risk of coronary heart disease, regardless of the drug used. Although high-density lipoprotein cholesterol (HDL-C) has long been considered protective, recent studies have suggested that increasing HDL-C alone may not reduce cardiovascular risk and that the function of HDL may be relevant, rather than the HDL-C plasma level. Genetic studies, such as Mendelian randomisation, have confirmed that LDL-C is a causal factor for heart disease. Triglyceride levels, which are transported by lipoproteins, also contribute to cardiovascular risk, although lowering apolipoprotein B is considered more crucial for reducing cardiovascular events. Overall, lowering LDL-C levels remains the cornerstone of cardiovascular disease prevention and treatment.

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Epidemiological studies have consistently shown a strong association between elevated LDL-C levels and increased cardiovascular risk. The well-established relationship between plasma cholesterol levels and the risk of cardiovascular events is continuous, regardless of whether total cholesterol or its fractions, such as LDL-C, are considered.

When analysing plasma cholesterol levels and integrating data from several studies, including the Pooling Project, the Framingham Heart Study and the Israeli Perspective Study, a consistent association between serum cholesterol levels and coronary events was confirmed worldwide [1]. This pattern was particularly clear in the Seven Countries Study, in which the relative risks of coronary heart disease (CHD) mortality as a function of serum cholesterol levels were similar in the different cohorts studied, although the absolute risks were different [2]. The observed differences in risk between different populations are largely attributable to baseline risk values, suggesting that other factors, such as diet, may play an important role. The Framingham Heart Study has shown that its results are applicable in any country when adjusted for baseline risk, suggesting a universal pattern in the relationship between cholesterol levels and cardiovascular risk. This has led to debate because the relationship has been oversimplified and presented as linear when it is not so in absolute terms. For example, a 0.5 mmol/L (about 20 mg/dL) increase in total cholesterol correlates with a 12% relative increase in CHD mortality risk. Consistent with this observation, data from the Cholesterol Treatment Trialists' (CTT) Collaboration showed that lowering low-density lipoprotein cholesterol (LDL-C) by 1 mmol/L reduces the risk of coronary heart disease by 22-23%, which is consistent with data from clinical trials [3]. A collaborative meta-analysis of ~900,000 individuals in 61 prospective observational studies has shown that age significantly attenuates the proportional (relative) relationship between ischemic heart disease (IHD) mortality and cholesterol levels. However, cholesterol level is a strong positive risk factor for IHD mortality not only in early middle age but also in old age. Although the proportional differences in risk decrease with age, the absolute impact of cholesterol levels on annual mortality from IHD is much greater at older ages than at younger ages [4].

In summary, extensive research confirms that cholesterol is a determinant of cardiovascular risk that is consistently observed in different populations and age groups. This understanding is crucial for the development of public health strategies and individualised treatment plans.

Cholesterol is essential for cell function, as it is an essential component of all cell membranes. It co-operates with fatty acids and phospholipids to regulate membrane fluidity. Cholesterol clusters in the membranes are crucial for the localisation of receptors, including

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the LDL receptor (LDLR), in specific regions (coated pits). These areas of the cell surface are crucial for the recruitment of receptors, their ability to interact and cellular responses. Cholesterol is also crucial for the function of internal membranes, such as those of mitochondria, endosomes, and lysosomes. The body's need for cholesterol is emphasised by its ability to acquire it either from the outside, via the LDLR on hepatocytes and enterocytes, or from the inside via the mevalonate pathway. These pathways are interconnected; increased dietary cholesterol intake reduces endogenous synthesis, and vice versa. Contrary to popular belief, lowering plasma cholesterol to very low levels does not pose a biological risk, as the body can synthesise sufficient cholesterol for cell division and brain development.

When referring to plasma cholesterol, we are talking about the lipoproteins that transport cholesterol mainly in esterified form and not free cholesterol molecules. Lipoproteins carrying cholesterol, especially apoB-containing lipoproteins, are atherogenic (5). Their ability to penetrate and become trapped within the arterial wall initiates a cascade of atherosclerotic processes. Lowering LDL-C levels decreases the number of these lipoproteins and thus lowers the risk of plaque formation and progression. Remnants of lipoproteins, including very low-density lipoproteins (VLDL) and chylomicrons, also play a role in cholesterol transport and metabolism, with VLDL remnants eventually transforming into LDL.

The formation of foam cells by accumulation of excess cholesteryl esters is a key process in the initial stage of lesion development, particularly related to vascular permeability [6]. This early stage does not necessarily lead to immediate progression of the lesion. Studies conducted on young American soldiers who died in Vietnam showed numerous fatty streaks that do not always correspond to later plaque development sites. This suggests a dynamic process in the early stages, where plaques do not necessarily form at the sites of initial lipid deposition, allowing for possible damage reversal. Lowering LDL-C levels has been shown to induce plaque regression, a process in which there are significant changes in plaque composition, including a marked decrease in lipid content and an increase in the thickness of the fibrous cap (which is considered inert with respect to inflammatory activity).

LDL is a causal factor in atherosclerosis, not cholesterol itself [7]. This distinction is crucial because the role of LDL in transporting cholesterol to the arterial walls is what initiates the damage. In contrast, HDL (high-density lipoprotein), which also transports cholesterol, is not causal. Conversely, a low level of HDL-C is associated with a higher risk of cardiovascular events. However, the causal relationship between HDL-C and cardiovascular risk is more complex and less well understood than that for LDL-C. Genetic studies and clinical trials have challenged the notion that simply increasing HDL-C levels pharmacologically reduces cardiovascular risk, suggesting that HDL functionality may be more important than HDL-C levels alone [8]. Surprisingly, extremely high HDL-C levels have been associated with higher cardiovascular risk [8] casting several doubts on the antiatherogenic role of HDL and determined by the measurement of HDL cholesterol or apo A-I.

Genetic studies, including Mendelian randomisation analyses, have provided compelling evidence for the causal role of LDL-C in atherosclerosis and cardiovascular disease [7]. Individuals with genetic mutations that result in lower lifelong LDL-C levels, such as those affecting the *PCSK9* or *HMGCR* genes, have a significantly lower risk of CAD, supporting the concept that LDL-C is a causal factor in the development of atherosclerotic disease. Different genetic scores predicting a 10 mg/dL reduction in LDL-C show consistent lifelong benefits [9]. This suggests that the mechanism of LDL-C lowering, whether by statins or PCSK9 inhibitors, leads to similar outcomes. Therefore, it is the lowering of LDL-C levels that is crucial, regardless of the method used. These findings are confirmed by clinical trials of LDL-C-lowering therapies, which consistently show that reducing LDL-C levels reduces the incidence of cardiovascular events. To date, clinical trials have shown that lowering LDL-C to very low levels is associated with a further CV risk reduction with no association with excess adverse events [10].

Genetic studies, Mendelian randomisation, and clinical trials involving patients with familial hypercholesterolemia (FH) have demonstrated that cholesterol trajectories can be altered [11]. In a typical population, average cholesterol levels eventually reach a threshold where clinical disease manifests. Not surprisingly, in individuals with heterozygous FH, higher cholesterol levels from birth accelerate the progression of the disease. Early intervention to reduce LDL-C can significantly alter this trajectory, suggesting that early and sustained LDL-C reduction has a profound impact on delaying disease onset. This concept is clearly illustrated in homozygous FH, where lowering LDL-C can extend life expectancy by approximately 25 years [11]. Randomised clinical trials, observational studies, and Mendelian randomisation studies all support the notion that prolonged exposure to lower LDL-C levels accrues greater cardiovascular benefits. For instance, a lifelong LDL-C reduction of 0.3 mmol/L (10-12 mg/dL) can achieve the same cardiovascular risk reduction seen in five years of statin therapy, and this can be obtained through moderate lifestyle changes.

Triglycerides (TG) have been identified as an independent risk factor for cardiovascular disease. TG are transported by lipoproteins, mainly chylomicrons and very low-density lipoproteins (VLDL) as well as their remnants. Remnant lipoproteins are considered atherogenic, functioning similarly to LDL in terms of their pathological impact [12]. The distribution of the so-called "remnant cholesterol" is closely linked to TG levels, which makes its use as an independent marker difficult.

Mendelian randomisation studies support the causal role of remnant cholesterol in cardiovascular disease [13]. However, intervention studies specifically targeting triglycerides are limited. Lowering TG through LPL-targeted pathways, including ANGPTL3, APOC2, APOC3, and APOE, however has shown potential in observational and genetic studies. Despite numerous trials with fibrates (drugs that reduce mainly plasma TG) showing overall negative results, subgroups with high TG and low HDL-C had benefits, suggesting that targeting this subgroup may be effective. The debate on whether apoB is a more meaningful marker than TG continues. In a study that assessed the impact of genetic scores for LPL and LDL, the association of different genetic variants with apoB concentrations resulted in a log-linear relationship with the risk of coronary heart disease, establishing apoB as a reliable indicator that includes the contributions of both LDL-C and TG [9]. This suggests that the number of particles is the most accurate proxy for measuring disease causation.

This hypothesis is supported by the PROMINENT trial of pemafibrate in an ideal population (high TG, low HDL, diabetes, cardiovascular disease) [14]. Despite reductions in remnant cholesterol and TG, there was no change in apoB levels, suggesting that apoB is the primary driver of the clinical benefit. This highlights the importance of lowering apoB as opposed to simply lowering other lipid parameters. This concept is further reinforced by comparing the results of the STRENGTH and REDUCE-IT trials with omega-3 fatty acids [15, 16]. Although both trials showed a decrease in TG, only the REDUCE-IT trial showed a reduction in apoB, suggesting that the therapeutic benefit is related to apoB reduction.

In summary, apoB-containing lipoproteins fulfil the criteria for causal involvement in atherosclerosis. Lowering apoB levels is critical even with delayed intervention, although the effects may not be fully reversible.

Conclusion

The relationship between LDL-C and cardiovascular risk is well-established and supported by a wealth of epidemiological, genetic, and clinical trial data (**Figure 1**). Elevated LDL-C is a major causal factor in the development of atherosclerosis and cardiovascular disease, and interventions that lower LDL-C levels consistently reduce the risk of cardiovascular events. While the role of HDL-C in cardiovascular risk remains less clear, lowering LDL-C levels remains a cornerstone of cardiovascular disease prevention and treatment. As research advances, further insights into cholesterol metabolism and its impact on cardiovascular health may lead to new strategies for reducing the burden of cardiovascular diseases globally.

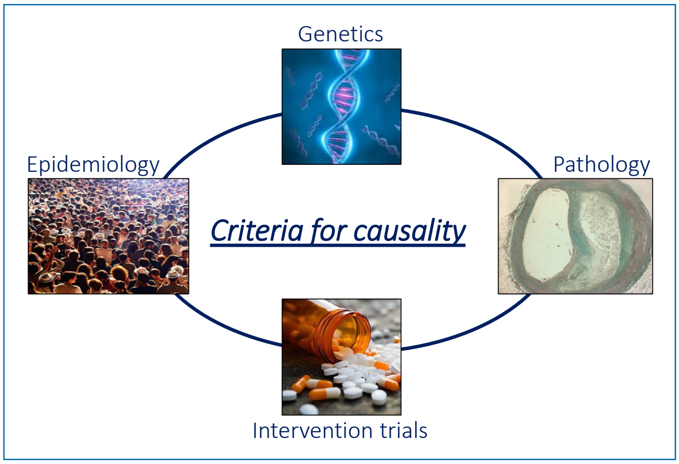

Figure 1 | LDL and atherosclerosis: Criteria for causality.

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Conflicts of interest

The author declares that he has no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. ALC has received honoraria, lecture fees or research grants from Aegerion, Amarin, Amgen, Amryt Pharma, AstraZeneca, Daiichi Sankyo, Esperion, Ionis Pharmaceutical, Medscape Education, Menarini, MSD, New Amsterdam Pharma, Novartis, Novo Nordisk, PeerVoice, Pfizer, Recordati, Regeneron, Sanofi, The Corpus, Ultragenyx, Viatris, outside the submitted work.

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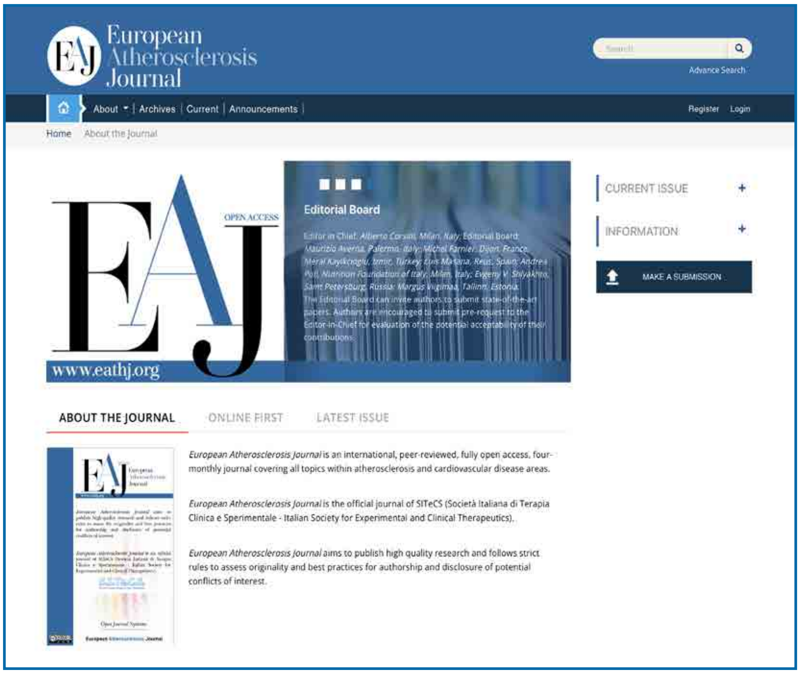

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