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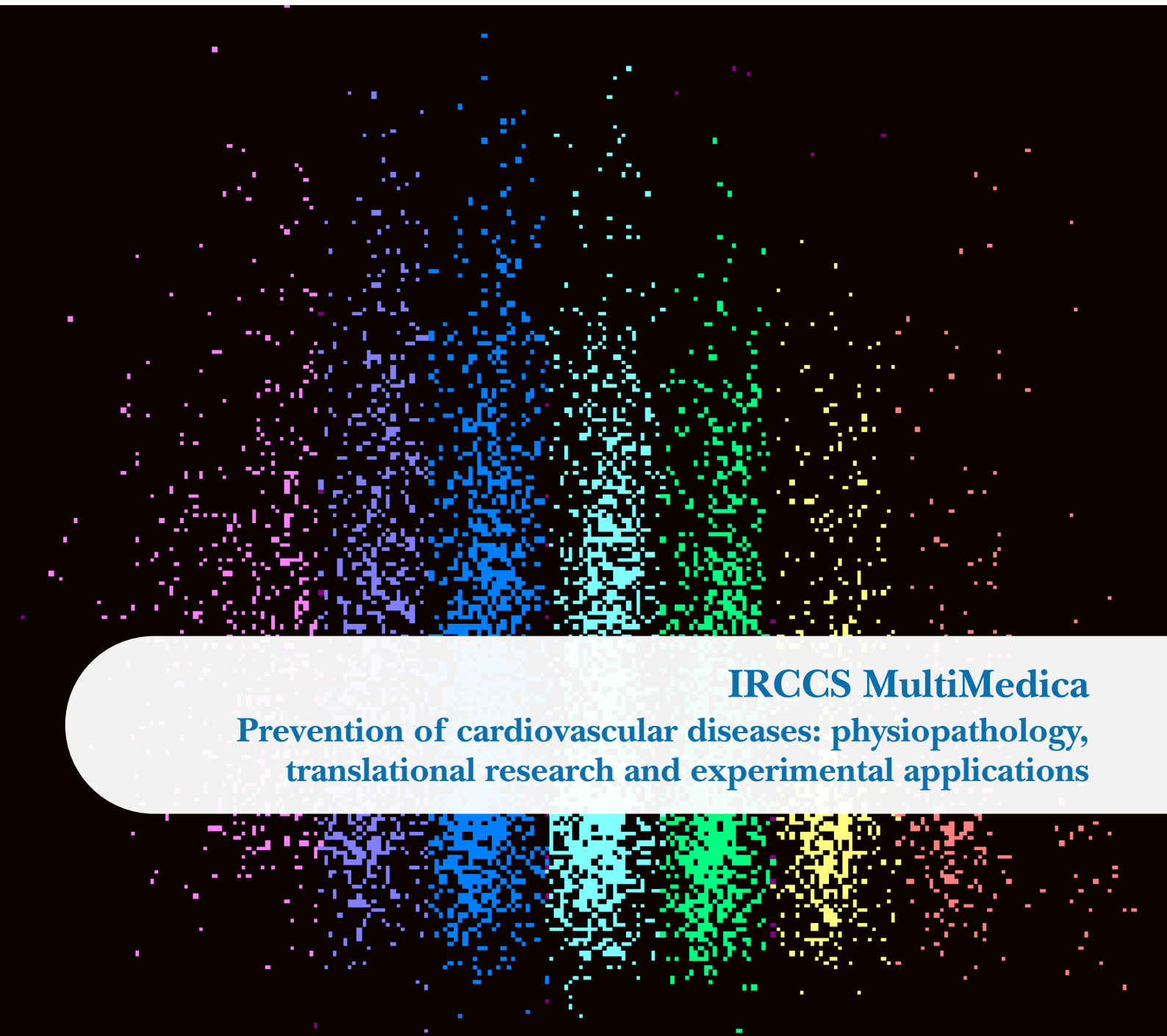

IRCCS MultiMedica

Prevention of cardiovascular diseases: physiopathology, translational research and experimental applications

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Contents

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IRCCS MultiMedica
Prevention of cardiovascular diseases: physiopathology, translational research and experimental applications

Cardiovascular research at MultiMedica focuses on understanding the molecular, cellular, and clinical mechanisms underlying atherosclerosis and cardiometabolic diseases. Our work integrates basic, translational, and clinical research, with the overarching goal of improving disease prevention, risk stratification, and therapeutic management for patients across the spectrum of cardiovascular conditions.

A central area of investigation concerns lipid metabolism and the pathways regulating LDL cholesterol, with particular attention to the molecular mechanisms that influence atherosclerotic plaque formation and progression. Studies exploring the biology of key proteins, their expression in different tissues, and their systemic functions contribute to clarifying how lipid-related processes interact with vascular inflammation and endothelial dysfunction.

Diagnostic innovation represents another major component of our research program. Advanced imaging technologies—including nuclear medicine approaches such as PET and SPECT—are employed to refine the evaluation of suspected coronary artery disease and to improve the accuracy of ischemia detection. These tools support clinical decision-making and help tailor management strategies based on individual risk profiles.

Research at MultiMedica also addresses the interplay between cardiovascular health, immunity, and ageing. Particular attention is devoted to understanding how immune cell phenotypes change with age and frailty, and how these alterations contribute to cardiovascular vulnerability. The identification of circulating biomarkers involved in immune regulation and tissue homeostasis is a growing field of interest, with potential implications for early detection of frailty and age-associated cardiometabolic risk.

In parallel, increasing efforts are devoted to RNA-based mechanisms, including the role of microRNAs in endothelial function, inflammation, vascular remodelling, and plaque stability. These small molecules are emerging as promising candidates for diagnostic and therapeutic applications, reflecting a broader shift toward precision medicine in cardiovascular research.

Across all these areas, MultiMedica's research is characterized by strong integration between laboratory science and clinical practice. Multidisciplinary collaboration – spanning cardiology, internal medicine, molecular biology, geriatrics, diabetology, and advanced imaging – ensures a comprehensive approach to cardiovascular disease. National and international partnerships further strengthen the impact of our work and enhance the translation of scientific discoveries into improved patient outcomes.

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PCSK9 in extrahepatic tissues: What can we expect from its inhibition?

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Keywords
PCSK9;
extrahepatic tissues;
monoclonal antibodies;
small interfering mRNA

ABSTRACT

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is an enzyme that belongs to the serine protease family and plays a key role in regulating low-density lipoprotein cholesterol (LDL-C) levels in the blood. PCSK9 binds to the LDL receptor (LDLR), targeting it for degradation, resulting in an increase in circulating LDL-C levels. Loss-of-function mutations in the PCSK9 gene are associated with lower LDL-C levels and lower cardiovascular risk; in contrast, gain-of-function mutations are a cause of familial hypercholesterolaemia. The identification of PCSK9 as a pharmacological target led to the development of inhibitors for the treatment of hypercholesterolaemia. To date, the monoclonal antibodies evolocumab and alirocumab (which target plasma PCSK9) and the small-interfering RNA inclisiran (which targets hepatic PCSK9 mRNA) have been approved for the treatment of hypercholesterolaemia. Although hepatic PCSK9 plays a central role in regulating plasma LDL-C levels, this protein is also expressed in other tissues, including the brain, pancreas, heart, kidney, intestine and adipose tissue. In extrahepatic tissues, the functions of PCSK9 are both dependent and independent of LDLR and not necessarily harmful. For this reason, it is essential to uncover any potentially harmful effects of therapies that inhibit PCSK9, beyond their known LDL-C-lowering and CV risk-reducing effects.

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Introduction

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is an enzyme that belongs to the serine protease family and is an important regulator of low-density lipoprotein cholesterol (LDL-C) levels (1). PCSK9 binds to the LDL receptor (LDLR) and initiates endocytosis and subsequent lysosomal degradation of the LDLR, preventing the receptor from returning to the cell surface (1). This leads to an increase in circulating LDL-C levels. The *PCSK9* gene contains several polymorphisms, including gain-of-function and loss-of-function mutations, which significantly affect normal PCSK9 signalling and cholesterol metabolism (2). PCSK9 is predominantly expressed in the liver, which produces the bulk of circulating PCSK9, which in turn regulates plasma LDL-C levels.

The role of PCSK9 in determining plasma LDL-C levels and its association with cardiovascular disease has been suggested by the observation that loss-of-function mutations in the PCSK9 gene are associated with lower LDL-C levels and lower cardiovascular risk (3-6); in contrast, gain-of-function mutations have been identified as a cause of familial hypercholesterolaemia, with elevated LDL-C levels from birth and high cardiovascular risk (2, 7-11). Once PCSK9 was identified as a pharmacological target, research focused on the development of inhibitors to control hypercholesterolaemia. To date, two monoclonal antibodies (mAbs, evolocumab and alirocumab) targeting circulating PCSK9 and a small interfering ribonucleic acid (siRNA, inclisiran) targeting hepatic PCSK9 mRNA have been approved for the treatment of hypercholesterolaemia. Both approaches have

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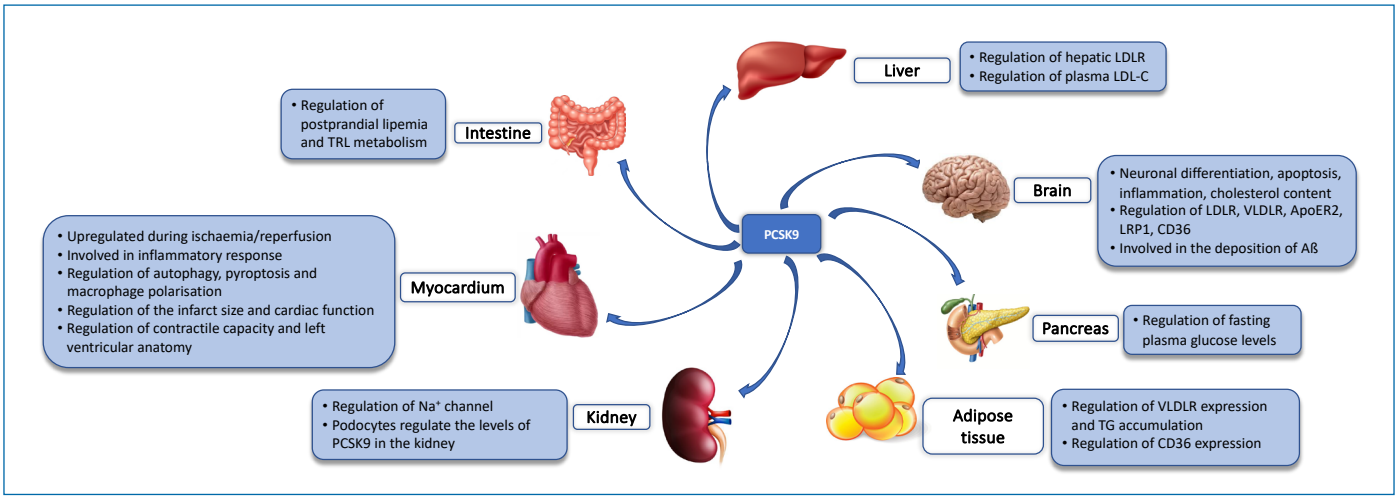

Figure 1 | Effects of PCSK9 in the liver and extra-hepatic tissues.

shown great efficacy in lowering LDL-C levels (~50-60% for mAbs and ~50% for siRNA); outcome trials have reported clinical benefit for both mAbs (12, 13); the ongoing ORION-4 is investigating the effect of inclisiran on clinical outcomes in patients with cardiovascular disease (NCT03705234).

Although PCSK9 has been studied primarily in the liver due to its important role in regulating plasma LDL-C levels, it is also expressed in other tissues, albeit to a lesser extent, such as the brain, pancreas, heart, kidneys, intestine, and adipose tissue. In these tissues, most of the effects exerted by PCSK9 are associated with metabolic pathways involving LDL-C, but relevant effects independent of LDLR metabolism have also been described (**Figure 1**). Based on this observation, questions have been raised about the possible effects of PCSK9 inhibitors in extrahepatic tissues. In this review, we aim to discuss the evidence available to date on this topic.

PCSK9 and the brain

PCSK9 was first discovered in neuronal cells undergoing apoptosis, and it appears to play a role in neuronal differentiation, cholesterol regulation, apoptosis, and inflammation in the brain (14). The brain is the most cholesterol-rich organ, but its cholesterol metabolism is uncoupled from peripheral tissues, as neither cholesterol nor PCSK9 can cross the blood-brain barrier under physiological conditions (15). In addition to the LDLR, PCSK9 regulates the levels of other receptors involved in the transport of cholesterol into neurons, including very low-density lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (ApoER2), as well as LDL receptor-related protein-1 (LRP1) and the scavenger receptor CD36 (16), which are highly expressed in the central nervous system (CNS). PCSK9 is present in cerebrospinal fluid at a low but constant level, in contrast to serum PCSK9 levels, which show large diurnal fluctuations (17), suggesting that the regulation of PCSK9 in cerebrospinal fluid may be different from that of PCSK9 in the bloodstream.

Although lowering LDL-C levels to very low levels is associated with clinical cardiovascular benefits, concerns have been raised about possible negative effects on cognitive function, as cholesterol is an essential component of myelin. This interaction between cholesterol homeostasis and cognition appears to be of particular importance in dementia.

Alzheimer's disease (AD), the most common cause of dementia, is a neurodegenerative disease characterised by continuous cognitive decline leading to poor quality of life (18). A body of evidence suggests that PCSK9 plays a role in AD, although our understanding of it is still incomplete. The accumulation of ß-amyloid (Aß) is a hallmark of AD; it arises from the amyloid precursor protein through the action of ß-site amyloid precursor protein-cleaving enzyme 1 (BACE1). Overexpression or inhibition of PCSK9 leads to decreased or increased expression of BACE1, respectively, and thus to higher or lower deposition of Aß (19). An indirect effect of PCSK9 has also been suggested, through the increase in systemic LDL-C levels, which may increase Aß deposition either by disturbing the balance of oxysterols (which can efficiently cross the blood-brain barrier) or by impairing Aß transport and degradation or by damaging the blood-brain barrier through an inflammatory process (20). Impaired Aß clearance in the brain promotes the onset and progression of AD. LRP1 and CD36 are two lipoprotein receptors that play a crucial role in Aß clearance by transporting Aß from the brain into the blood (21, 22). PCSK9 could therefore interfere with this clearance process by negatively regulating these receptors. In mice, mAbs against PCSK9 were able to reduce cerebral Aß burden, an effect that did not occur in mice lacking LRP1 (23). Another mechanism likely linking PCSK9 and AD is the induction of a pro-apoptotic effect in neuronal cells through the degradation of ApoER2 (24).

It is noteworthy that monoclonal antibodies, due to their size, cannot cross the intact blood-brain barrier under physiological conditions. However, under certain pathological conditions (such as ischaemic stroke) the permeability of the blood-brain barrier may be impaired. Short-term clinical trials with mAbs targeting PCSK9 found no association between very low LDL-C levels and cognitive impairment (13, 25-29). The EBBINGHAUS trial was specifically designed to investigate cognitive function in patients enrolled in FOURIER who received either evolocumab or placebo in addition to statins (25). No significant differences in cognitive function were observed between the two groups over a median of 19 months (25). In the open-label extension study (FOURIER-OLE), patients were followed up for a median of 5 years: there was no clear monotonic trend between a lower achieved LDL-C level and the risk of neurocognitive events (30). Genetic studies support the lack of association between PCSK9 inhibition and impaired cognitive function. PCSK9 LOF variants, which determine lifelong exposure to low LDL-C levels, were not found to be associated with neurocognitive abnormalities in blacks

participating in the REGARDS study (31). Mendelian randomisation analyses using data from a combined sample of ~740,000 participants showed no significant effects on cognition associated with genetic inhibition of PCSK9 (32), which was confirmed by another Mendelian randomisation analysis (33). In contrast, genetic HMGCR inhibition was associated with reduced cortical surface area, worsened reaction time, and impaired cognitive performance (32), which is consistent with the results of some studies on statins (34, 35). However, some studies have suggested a possible association between PCSK9 inhibition and cognitive impairment. The results of a Mendelian randomisation study have raised the possibility that exposure to PCSK9 inhibitors may predispose individuals to AD (36). A recent analysis of a large pharmacovigilance database found a disproportionality signal related to PCSK9 inhibitors (either as a class or as a single drug) and mental impairment (including memory impairment and amnesia) (37). However, it must be emphasised that this study was conducted with a database of spontaneous reports. Adequate long-term clinical trials could definitely shed more light on this topic.

PCSK9 and the pancreas

Cholesterol homeostasis appears to be essential for pancreatic ß-cell function (38). These cells express both LDLR and PCSK9, which may thus modulate LDLR expression and influence cell function. It is noteworthy that patients with familial hypercholesterolaemia who carry genetic defects leading to reduced LDLR expression/function are less likely to develop diabetes. On the other hand, statins have been shown to increase the risk of new-onset diabetes, especially in pre-diabetics or patients with established risk factors for diabetes (39), although the clinical benefit outweighs the risk. Based on its mechanism of action, PCSK9 inhibition has therefore been suspected of promoting the onset of diabetes, like statins.

PCSK9 loss-of-function variants have shown differential effects on glucose homeostasis, likely related to the genetic background of individuals and the type of the effect on PCSK9 (40). A Mendelian randomisation study showed that PCSK9 variants associated with lower LDL-C levels were also associated with higher fasting plasma glucose levels and increased risk of new-onset type 2 diabetes (41). This confirms a previous observation suggesting that exposure to LDL-C-lowering genetic variants is associated with a higher risk of type 2 diabetes (42). Variants in the *PCSK9* gene and variants in the *HMGCR* gene had approximately the same effect on diabetes risk per unit lower LDL-C level (43). However, pharmacological inhibition of PCSK9 does not appear to increase the risk of new-onset diabetes. A prespecified analysis of the FOURIER trial showed that evolocumab was effective in diabetic and non-diabetic patients and did not increase the risk of new-onset diabetes or worsen blood glucose levels during a median follow-up of 2.2 years (44). The FOURIER-OLE study showed that long-term LDL-C lowering with evolocumab was safe and well tolerated, and resulted in a further reduction in cardiovascular events compared with delayed treatment initiation (45). Interestingly, the rate of new-onset diabetes was not higher in patients who achieved very low LDL-C levels (<20 mg/dL) (30). A meta-analysis of 39 randomised clinical trials involving 66,748 patients treated with alirocumab or evolocumab showed that the use of these PCSK9 inhibitors was not associated with an increased risk of new-onset diabetes (27).

To better explore the potential impact of inhibiting PCSK9 on the development of diabetes, some studies have investigated the role of circulating versus locally produced PCSK9 in animal models. PCSK9 deficiency in mice has been shown to be associated with impaired glucose tolerance due to abnormalities in pancreatic islets, likely due to cholesterol overload of ß-cells and decreased pancreatic insulin secretion (46). However, this effect seems to be independent of circulating (liver-derived) PCSK9 but rather related to locally produced PCSK9 (46): liver-selective PCSK9 knockout mice, mimicking the condition of patients treated with a PCSK9 inhibitor, retain extrahepatic production of PCSK9 (in contrast to the condition of a loss-of-function mutation in the *PCSK9* gene, which affects all sites of production), with normal insulin production, LDLR expression and cholesterol levels in pancreatic islets (46). The ß-cell-specific knockout of PCSK9 resulted in unchanged circulating LDL-C levels with concomitant down-regulation of cholesterogenic genes, which should prevent cholesterol load and toxicity in ß-cells as well as alteration of glucose homeostasis (47). Of note, in this study PCSK9 was selectively inactivated only in mature ß-cells, resulting in residual PCSK9 expression in pancreatic islets (~30%) (47). Silencing PCSK9 expression in endocrine pancreas precursors and mature ß-cells and δ-cells resulted in a 90% reduction in PCSK9 expression in the pancreas (48). Circulating PCSK9 levels remained unchanged, but glucose intolerance was observed in mice due to defective insulin secretion (48). Increased LDLR expression and the resulting cholesterol accumulation were identified as the cause of the observed effect (48). Based on these observations, therapeutic inhibition of PCSK9 should not impair ß-cell function. Indeed, mAbs targeting PCSK9 act against circulating protein derived from the liver. Inclisiran, the siRNA approach that specifically targets PCSK9 mRNA in the liver (thanks to its structure that ensures specific recognition at the hepatic level), should not have diabetogenic properties.

PCSK9 and the heart

Adult differentiated cardiomyocytes constitutively express and release PCSK9 (49). PCSK9 expression is upregulated by inflammation and hypoxia (characteristic of an ischaemic heart) in cardiomyocytes (50, 51). Oxidised LDL, a marker of oxidative stress associated with reduced cardiac function, also increase the expression and release of PCSK9 (49), which appears to affect cardiomyocyte function in an autocrine manner, leading to reduced contraction and relaxation velocity, with the LOX-1 receptor being the most likely candidate to trigger the action of OxLDL (52). During ischaemia/reperfusion (such as acute myocardial infarction, MI), PCSK9 is also upregulated in immune cells, such as neutrophils, monocytes, and macrophages, which are immediately recruited to the ischaemic tissue. PCSK9 promotes the release of pro-inflammatory cytokines from macrophages, leading to a further reduction in cardiomyocyte viability and induction of cardiac cell apoptosis (51).

PCSK9 has been shown to be upregulated in the ischaemic heart of mice, with the zone adjacent to the infarcted areas showing the highest PCSK9 expression and intense autophagy activity, a self-degradation process activated during stress to promote cell survival and cardiac homeostasis (50). PCSK9 released by these cells contributes in determining the infarct size and cardiac function (50): mice lacking the PCSK9 gene or treated with a PCSK9 inhibitor showed better heart function and smaller infarct size (50). PCSK9 expression and release, as well as autophagy, were highest one week after the ischaemic event and then declined (50, 53). Analysis of heart sections from patients who had died of acute myocardial infarction showed PCSK9 and markers of autophagy being strongly expressed in the border zone between ischaemic and normal areas (50). It is likely that the release of PCSK9 early after the onset of myocardial ischaemia may be considered a protective response by stimulating autophagy, at least in the short term. It remains to be elucidated whether sustained PCSK9 release and autophagic response during hypoxia may have deleteri-

ous effects by inducing cell self-digestion and cell death. It is noteworthy that high expression of PCSK9 after myocardial infarction promotes pro-inflammatory M1 macrophage polarisation associated with poor myocardial repair, whereas PCSK9 deficiency promotes anti-inflammatory M2 macrophage polarisation and better protection against myocardial injury (54). Inhibition of PCSK9 has been shown to ameliorate myocardial injury after ischaemia/reperfusion by inhibiting autophagy and inflammation (55).

During acute cardiac ischaemia/reperfusion, high levels of circulating PCSK9 may trigger inflammatory and oxidative processes in ventricular cardiomyocytes, leading to cardiac dysfunction. Therefore, inhibition of PCSK9 could have a cardioprotective effect against ischaemia/reperfusion injury. Indeed, administration of a PCSK9 inhibitor prior to ischaemia resulted in a cardioprotective effect by inhibiting apoptosis, improving cardiac mitochondrial function, reducing infarct size and improving left ventricular function in rats (53, 56). When the PCSK9 inhibitor was administered during ischaemia or reperfusion, no benefits were observed (56). These results are not related to the lipid-lowering effect of the PCSK9 inhibitor, but rather to attenuated cardiac mitochondrial dysfunction and mitochondrial fission, and reduced apoptosis in the ischaemic myocardium (56). Another relevant observation is that PCSK9 regulates pyroptosis in cardiomyocytes during chronic myocardial ischaemia. Pyroptosis is a form of inflammatory programmed cell death that is closely associated with activation of the NLRP3 inflammasome (57). Both PCSK9 and pyroptosis-related proteins have been found to be highly expressed in the zone adjacent to the infarct (58). In *vitro*, PCSK9 activated the NLRP3 inflammasome and further enhanced pyroptosis in cardiomyocytes (58). Consistent with the results in mice, serum levels of PCSK9 and proteins related to pyroptosis were higher in patients with chronic myocardial ischaemia than in healthy subjects (58). Analysis of heart sections from patients who had died of acute MI showed that all these proteins were present in high concentrations in the zone adjacent to the infarcted area (58).

PCSK9 deficiency is associated with increased LDLR and CD36 expression in the heart, leading to lipid accumulation, and altered mitochondrial metabolism (59). These effects manifest as impaired cardiac function and heart failure with preserved ejection fraction (59). However, circulating PCSK9 does not affect cardiac metabolism: mice selectively lacking PCSK9 in the liver exhibit normal cardiac function (59). On the other hand, cardiomyocyte-specific deficiency of PCSK9 resulted in reduced contractile capacity, impaired cardiac function and left ventricular dilatation (60). Interestingly, individuals carrying a loss-of-function mutation in the *PCSK9* gene have increased epicardial fat accumulation and increased left ventricular mass index without alterations in the ejection fraction (59, 61). However, this finding could not be replicated in another study (62). Overall, these observations suggest that therapies targeting PCSK9 should not have negative effects on cardiac metabolism.

Accordingly, clinical trials have shown that therapy with the PCSK9 inhibitors evolocumab and alirocumab can significantly reduce the risk of myocardial infarction. The ODYSSEY OUTCOMES trial showed that alirocumab added to statin therapy reduced the overall incidence of MI, particularly the risk of type 1 (atherothrombotic, the most common form) and type 2 (myocardial oxygen supply-demand mismatch) MI (by 13% and 23%, respectively), but not type 4 (associated with percutaneous coronary intervention) MI (63). The benefit of alirocumab in reducing these types of MI was more pronounced when the increase in biomarkers (as a measure of infarct size), exceeded three times the upper normal limit (63). As an explanation for the effect on type 2 MI, the authors suggested that alirocumab may have improved myocardial oxygen supply (63). On the other hand, a prespecified analysis of the FOURIER trial showed that evolocumab significantly reduced the risk of the first MI by 27%; more specifically, type 1 MI was reduced by 32% and type 4 MI by 35%, while no effect was observed for type 2 MI (64). Evolocumab significantly reduced the risk of non-STEMI and STEMI (by 23% and 36%, respectively) (64). A meta-analysis of data from 3 clinical trials of inclisiran failed to demonstrate difference in the risk of MI between patients randomised to inclisiran or placebo (65); the ongoing outcomes trial ORION-4 will shed light on this point.

A post-hoc analysis of the ODYSSEY OUTCOMES trial showed that, among post-ACS patients, alirocumab reduced the risk of MACE in patients without a history of heart failure, but not in patients with a history of heart failure, despite comparable reductions in LDL-C levels (66). In addition, there was a significant increase in non-fatal MI (66), and no effect of alirocumab on hospitalisations for HF, either overall or in the two subgroups (66). This finding is consistent with previous observations that statins do not reduce cardiovascular events in HF patients (67). This suggests the hypothesis that the clinical course of advanced heart failure does not appear to be influenced by anti-atherosclerotic therapies, as deterioration of myocardial function drives disease progression rather than atherosclerotic cardiovascular events (67). The limitations of this subgroup analysis do not allow any conclusions to be drawn, only the need to investigate the clinical efficacy of PCSK9 inhibitors in specific trials. The ongoing EVO-HF pilot trial is investigating whether evolocumab is effective in stable HF patients with reduced ejection fraction of ischaemic origin (NCT03791593).

PCSK9 and the kidney

Lipid and lipoprotein abnormalities are common features in patients with chronic kidney disease (CKD), in whom ASCVD is an important cause of mortality and morbidity. Each type of CKD has a typical phenotype, but overall, at least in the early stages, there are increased triglycerides, decreased high-density lipoproteins, and an excess of small, dense low-density lipoprotein particles. In the kidney, PCSK9 is involved in the regulation of the epithelial Na^+ channel (ENaC) by reducing its expression on the cell surface; this regulation appears to be independent of PCSK9 protease activity (68).

Podocytes (also known as visceral epithelial cells) are highly specialised cells lining the outer surface of the glomerular capillary. Dysfunction of these cells, as seen in patients with nephrotic syndrome, is associated with hypercholesterolaemia, mainly due to increased production and decreased clearance of apoB-containing lipoproteins. Studies in patients with kidney disease have shown that circulating PCSK9 levels are significantly increased compared to healthy subjects, but decrease during remission of the disease (69-72). This finding has also been confirmed in animal models of nephrotic syndrome (71). Injection of a nephrotoxic serum into C57BL/6J mice resulted in an increase in plasma PCSK9 and hypercholesterolaemia associated with a decrease in LDLR (71); this increase in PCSK9 is due to increased expression, increased secretion and decreased clearance (71). Interestingly, PCSK9 clearance was only reduced twofold after podocyte injury, and PCSK9 mRNA was generally not increased, suggesting a post-transcriptional mechanism by which damaged podocytes trigger a signal to the liver that leads to increased PCSK9 secretion (71). On the other hand, mice lacking PCSK9 show a reduced response to the treatment with nephrotoxic serum, with hypercholesterolaemia induced to a lesser extent, suggesting that multiple mechanisms are likely involved in the dyslipidaemia associated with nephrotic syndrome (71).

It is noteworthy that CKD patients receiving haemodialysis have

lower blood LDL-C and PCSK9 levels than healthy people, but those receiving statin therapy have comparable PCSK9 levels to healthy people (73).

Statins are widely prescribed to treat hypercholesterolaemia in patients with CKD. However, it must be emphasised that the clearance of most statins is affected by renal function, leading to excess of drug-drug interactions. In addition, statin therapy is effective in patients with mild-to-moderate CKD, while patients with advanced CKD benefit less (74-76). Evolocumab and alirocumab have been tested for efficacy in CKD patients and provided consistent results in both patients with preserved and impaired renal function (77). An analysis of the efficacy of evolocumab according to the renal function in the FOURIER trial showed that the reduction in LDL-C levels and relative risk reduction were similar for both primary and secondary endpoints in all stages of CKD (including patients with preserved function, stage 2 CKD and ≥stage 3 CKD) (78). The absolute risk reduction for the composite of cardiovascular death, MI, or stroke with evolocumab was numerically greater in patients with more advanced CKD (78). In an analysis of data from ODYSSEY phase 3 trials, alirocumab was shown to lower LDL-C levels independent of the presence or absence of impaired renal function (79). A pre-specified analysis of the ODYSSEY OUTCOMES trial of alirocumab found that alirocumab was effective in reducing LDL-C levels, major cardiovascular events and death across the range of renal function evaluated in patients with recent ACS and dyslipidaemia despite intensive statin therapy (80). However, it should be emphasised that eGFR <30 mL/min/1.73 m^2 was an exclusion criterion in this trial, and eGFR <20 mL/min/1.73 m^2 was an exclusion criterion in FOURIER. Based on this last observation, it is clear that further specifically designed trials are needed to assess whether therapy with mAbs against PCSK9 can have a negative impact on kidney disease and whether it is as effective in patients with advanced kidney disease.

PCSK9 and the intestine

The gut is involved in maintaining cholesterol homeostasis in the body through balanced metabolic cross-talk with the liver (81). PCSK9 is expressed in the small intestine of mice and humans (82). In mice, PCSK9 is expressed throughout the digestive tract and in the colon at levels similar to the liver. In the human intestine, PCSK9 is localised in the cytoplasm and accumulates in the subapical and basolateral compartments of the enterocytes. The cellular distribution of PCSK9 appears to be heterogeneous depending on the intestinal tract: in the duodenum, PCSK9 is expressed at both the apical and basolateral poles, whereas in the ileum it is mainly expressed at the apical pole. This heterogeneity is probably related to the function of PCSK9: in the upper part of the small intestine, PCSK9 is expressed at both poles of the enterocyte according to the absorption process and lipoprotein secretion, whereas in the ileum, which secretes less lipoproteins, PCSK9 is expressed only at the apical side. Furthermore, PCSK9 is not secreted from mature enterocytes in vitro and does not contribute to circulating PCSK9 levels (83), although this remains to be confirmed in vivo. PCSK9 appears to play a critical role in postprandial lipaemia. Indeed, PCSK9-deficient mice are protected from postprandial lipaemia (82). However, in mice, circulating PCSK9 rather than intestinal PCSK9 regulates postprandial lipaemia: both PCSK9-deficient and wild-type mice treated with alirocumab showed reduced postprandial lipaemia (via an LDLR-dependent pathway), an effect not observed in mice specifically lacking intestinal PCSK9 (84). Accordingly, subjects carrying PCSK9 loss-of-function variants had a more favourable lipid profile on fasting and attenuated levels of postprandial TG, apoB48, and total apoB (85), suggesting a role for PCSK9 in regulating TG-rich lipoproteins (TRLs) metabolism. In addition, treatment with evolocumab significantly reduced the postprandial lipaemic response to a mixed high-fat meal in type 2 diabetics, although the production and release of chylomicrons from intestine were not affected (86). Similarly, alirocumab reduced fasting plasma levels of TG and apoB48 and postprandial plasma response of TG and apoB48 in patients with type 2 diabetes on intensive insulin treatment (87). Of note, two studies found no effect of PCSK9 inhibition on the postprandial response in healthy normolipidaemic subjects (88, 89), suggesting that inhibition of PCSK9 may be of particular importance in diabetics who have increased production and impaired clearance of TRLs.

Treatment of human enterocytes with recombinant human PCSK9 markedly increased intestinal production and secretion of apoB and apoB48 by 50%; this effect was due to both an increase in apoB mRNA and an enhanced post-transcriptional apoB protein stability (90). Considering that there is one apoB molecule per TRL particle, this increase in TRL-apoB by PCSK9 suggests a potential doubling of the amount of pro-atherogenic TRL intestinal remnant particles in the circulation after a meal. LDLR expression in enterocytes was reduced by 50%, with concomitant increases in NPC1L1 protein levels, MTP protein levels and lipid transfer activity (90). Of note, treatment of enterocytes with PCSK9 siRNA reversed all observed effects (90). Accordingly, delivery of wild-type PCSK9 or a gain-of-function mutant to epithelial cells in the basolateral medium reduced LDLR at the basolateral membrane and caused marked perturbations in cholesterol homeostasis, including increased cholesterol uptake from the apical membrane via upregulation of NPC1L1, CD36 and ACAT2 and downregulation of HMG-CoAR activity (91).

In vivo, PCSK9 expression significantly reduced LDLR levels in the small intestine, but not in the large intestine in transgenic mice expressing human PCSK9 in multiple tissues (90); MTP expression and activity were increased in the small intestine (where chylomicrons are assembled), but were not detected in the large intestine (90). Similarly, plasma PCSK9 levels correlated positively with the pool size and production rate of intestinal TG-rich lipoproteins (containing apoB48), but not the fractional catabolic rate in men with varying degrees of insulin resistance (92). In addition, intestinal expression of the *PCSK9* gene was positively associated with genes involved in de novo cholesterol synthesis (*HMGCR* and *ACAT2*) and lipoprotein uptake (*LDLR*) (92).

Altogether, these observations suggest that pharmacological inhibition of PCSK9 may have a beneficial effect on postprandial lipaemia, an effect that may be particularly relevant in diabetic patients who have excessive postprandial lipaemia.

PCSK9 and the adipose tissue

Adipose tissue plays a central role in energy balance and storage, but is also involved in the metabolism of TG-rich lipoproteins (93). Adipose tissue also contains a very large pool of free cholesterol and promotes the transfer of cholesterol to HDL (94). Previous studies have shown that circulating PCSK9 regulates VLDLR expression and TG accumulation in visceral adipose tissue: PCSK9$^{-/-}$ mice exhibited significant visceral adipose tissue accumulation compared to wild-type mice, which was associated with adipocyte hypertrophy and increased fatty acid uptake as well as greater cell surface expression of VLDLR, with a mechanism being independent of LDLR (61, 95). Similar effects were observed following specific inactivation of hepatic PCSK9 in wild-type animals (95). In addition, carriers of the PCSK9 R46L loss-of-function variant had higher body mass index and increased percentage of total and android fat mass compared to

non-carriers (61). Circulating PCSK9 is also involved in the degradation of CD36, an important receptor participating in the metabolism of fatty acids and triglycerides in the liver and visceral adipose tissue: PCSK9$^{-/-}$ mice showed high expression of CD36 in adipose tissue, whereas adipocytes treated with PCSK9 showed a strong reduction in cell surface expression of CD36 (96).

PCSK9 is also abundantly expressed in the visceral adipose tissue (97). Of note, statins upregulate PCSK9 expression (98), an effect that has also been demonstrated in adipose tissue (99). PCSK9 expression in adipose tissue is positively correlated with body mass index in humans, suggesting that obesity and adiposity promote PCSK9 expression (97). Insulin and LDL upregulated the expression of PCSK9, LDLR, SREBP-1c and SREBP2 in human adipocytes, and atrial natriuretic peptide partially reversed these effects; this latter observation should be of interest in patients with obesity and hypertension (97). An analysis in overweight/obese individuals with normal LDL-C levels showed that individuals with lower than median plasma levels of PCSK9 had higher expression of LDLR in their white adipose tissue, accompanied by increased expression of CD36, IL-1ß secretion, postprandial hypertriglyceridaemia, lower white adipose tissue function and a lower disposition index, indicating a predisposition to type 2 diabetes (100). Increased LDL uptake is thought to impair adipocyte differentiation and consequently lead to white adipose tissue dysfunction (101), with a concomitant upregulation of MCP-1 expression that promotes the cross-talk between adipocytes and macrophages (100).

Among the various body fat depots, visceral epicardial adipose tissue (EAT) is a proxy of total visceral adiposity and a reliable marker of cardiovascular risk (102, 103). Under physiological conditions, it provides mechanical protection and also functions as an energy supplier, thermoregulator, and endocrine organ. However, under pathological conditions, EAT dysfunction can be detrimental and promote CVD progression (103). EAT is a source of PCSK9; local PCSK9 levels (but not plasma PCSK9 levels) correlated with EAT thickness and local inflammation (104); conversely, PCSK9 R46L carriers have higher EAT thickness compared to non-carriers (61). Further studies are needed to better define the role of PCSK9 in this specific tissue and the potential consequences of PCSK9 inhibition.

Conclusion

Since its discovery, PCSK9 has been shown to be a major determinant of circulating LDL-C levels through its main function in the liver, but also plays a key role in other tissues and organs. The rapid development and approval of PCSK9 inhibitors for the treatment of hypercholesterolaemia has paved the way for many unanswered questions related to the potential adverse effects of inhibiting PCSK9 in tissues and organs other than the liver. Many studies have attempted to answer these questions and provide evidence that inhibition of PCSK9 has not adverse effects, but many questions remain unresolved. Although PCSK9 inhibitors have been shown to be beneficial (the results for inclisiran are awaited), particularly in the context of cardiovascular health and metabolic disorders, the use of PCSK9 inhibitors could have effects on several other organs and tissues, particularly in the context of neurocognitive disorders, ß-cell function and diabetes, cardiac metabolism, heart failure and chronic kidney disease. To date, there is no evidence that PCSK9 inhibitors have negative effects on neurocognitive disorders or ß-cell function. In addition to the proven benefit for ASCVD, PCSK9 inhibition does not appear to negatively affect cardiac metabolism or have a negative impact on the clinical course of advanced heart failure. Monoclonal antibodies targeting circulating PCSK9 have been shown to be effective in reducing the risk of ASCVD in both patients with preserved and impaired renal function, although there are no specifically designed trials to assess their efficacy in patients with advanced kidney disease.

Results from experimental models are critical to understanding the underlying molecular mechanisms, but have limited translatability to humans. Similarly, observations in individuals carrying genetic variants of PCSK9 do not always translate to clinical trials of pharmacological inhibition of PCSK9, although they are critical in defining the precise role of this protein. Notwithstanding the role that PCSK9 inhibitors play in controlling hypercholesterolaemia, specific studies are needed to understand the long-term effects of PCSK9 inhibition.

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Is there still a place for fenofibrate-statin combination therapy?

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ABSTRACT

Although low-density lipoprotein cholesterol (LDL-C) is the main target for the prevention of atherosclerotic cardiovascular disease (ASCVD), hypertriglyceridaemia (HTG), a common condition characterised by elevated blood triglyceride (TG) levels, contributes to residual cardiovascular risk independently of LDL-C levels. Elevated TG levels are a feature of atherogenic dyslipidaemia, which also includes low HDL-C levels and high levels of atherogenic small, dense LDL, together with accumulation of atherogenic remnant particles.

Treatment of HTG includes lifestyle interventions, but these are not always sufficient to significantly reduce TG levels in people at high cardiovascular risk. Current guidelines for the treatment of dyslipidaemias recommend the use of statins as the first choice in people with HTG (TG >200 mg/dL) and high CV risk, and consideration of the use of specific TG-lowering drugs, such as fenofibrate, bezafibrate or icosapent ethyl if HTG persists.

Fenofibrate acts by activating the peroxisome proliferator receptor alpha (PPARα), a nuclear receptor that plays an important role in lipid and lipoprotein metabolism, glucose homeostasis and inflammation. Several clinical trials have shown that fibrates may reduce the incidence of major cardiovascular events only in patients with high TG levels and low HDL-C levels, a finding that was also observed with fenofibrate in combination with a statin compared to statin therapy alone. The recent failure of the PROMINENT trial with pemafibrate in combination with a statin highlighted the notion that treatment with fibrates provides a clinical benefit only if they lower apoB levels.

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Introduction

Low-density lipoprotein cholesterol (LDL-C) is a causal factor for atherosclerotic cardiovascular disease (ASCVD) and is the main target for ASCVD prevention [1]. Although several drugs are available that effectively lower LDL-C levels, many patients continue to experience cardiovascular events even when their LDL-C is at goal. Many factors contribute to the residual CV risk beyond LDL-C levels, including hypertriglyceridaemia (HTG) [2].

HTG is a common condition characterised by elevated levels of triglycerides (TG) in the blood. TG are energy-storage molecules made up of glycerol and fatty acids. They are stored in adipose tissue until they are needed. In the blood, TG are transported via lipoproteins, and in particular via TG-rich lipoproteins, which include very-low-density lipoproteins (VLDL), chylomicrons and their remnants. The remnants originate from partial lipolysis mediated by lipoprotein lipase, are TG-depleted and cholesterol-enriched compared to their naïve counterparts and are highly atherogenic [2]. The most important apolipoprotein of TG-rich lipoproteins is apolipoprotein B (apoB), which present in one copy per particle.

The main causes of HTG are an unbalanced diet, being overweight or obese, metabolic syndrome, excessive alcohol consumption, taking certain medications and genetics. Elevated levels of TG are a feature of the so-called atherogenic dyslipidaemia, which is also characterized by low levels of HDL-C and high levels of small dense LDL. A common feature in atherogenic dyslipidaemia is an increase of either apoB or non-HDL-cholesterol, both parameters reflecting the global number of atherogenic lipoproteins. Atherogenic dyslip-

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idaemia is associated with an increased CV risk [3] and severe HTG (TG levels >500 mg/dL) can lead to acute pancreatitis, a potentially life-threatening condition.

Treatment for HTG includes dietary changes, weight control, increasing physical activity and reducing alcohol consumption. However, these approaches are not always sufficient to significantly reduce TG, especially in people at high CV risk, who may need specific drugs to lower TG levels and reduce CV risk. The most common drugs used to control HTG are fibrates and omega-3 polyunsaturated fatty acids. However, these drugs can be ineffective for severe HTG, which requires specific treatments to massively reduce TG levels.

Fibrates include clofibrate (the first drug developed, which is no longer available due to the increased risk of adverse effects), gemfibrozil, fenofibrate, bezafibrate, ciprofibrate and the most recent pemafibrate. These molecules work by activating the peroxisome proliferator receptor alpha (PPARα). PPARα belongs to the nuclear receptor superfamily and plays an important role in physiological processes such as lipid and lipoprotein metabolism, glucose homeostasis and inflammation [4]. Activated PPARα forms a heterodimer with another nuclear receptor, the retinoid X receptor, which binds to specific peroxisome proliferator response elements, resulting in either activation or inhibition of several genes involved in lipid metabolism. This in turn leads to a decrease in TG and an increase in HDL-C levels, with the efficiency depending on the molecule and the baseline lipid levels. Activation of PPARα leads to the stimulation of fatty acid oxidation, an increase in lipoprotein lipase (LPL) synthesis and a decrease in apoC-III expression, resulting in increased lipolysis and improved clearance of TG-rich lipoproteins. Fibrates also stimulate lipolysis in adipose tissue, releasing fatty acids into the bloodstream. Finally, fibrates reduce the hepatic synthesis of TG by inhibiting the enzymatic activity of diacylglycerol acyltransferase (DGAT), a key enzyme in TG synthesis. In addition to lowering triglycerides, fibrates can also increase levels of HDL-C. The increase in HDL-C results from the PPARα-mediated stimulation of the expression of apo A-I and apo A-II and a reduction in the activity of the cholesteryl ester transfer protein (CETP), which transfers cholesterol from HDL to VLDL in exchange for TG.

Current guidelines for the treatment of dyslipidaemias recommend the use of statins as the first choice to reduce CVD risk in HTG individuals (TG >200 mg/dL) at high CV risk [5]. In high-risk or very-high-risk patients who have high TG levels (135-499 mg/dL) despite statin treatment, icosapent ethyl in combination with a statin should be considered [5]. Fenofibrate or bezafibrate may be considered in combination with a statin in patients in primary prevention or in high-risk patients with LDL-C at goal and TG >200 mg/dL [5]. Of note, in the recently released 2023 ESC guidelines for the management of cardiovascular disease in patients with diabetes the use of fibrates is no longer considered to manage elevated TG levels in these patients due to the little benefit demonstrated in RCTs, aside from sub-group analysis including subjects with very high TG levels [6].

Fenofibrate

Fenofibrate is by far the most commonly used fibrate in clinical practice. Fenofibrate is a pro-drug that is converted in the liver to the pharmacologically active metabolite fenofibric acid. Following oral administration, fenofibrate is rapidly absorbed; the extent of absorption ranges from 30-50% when the drug is taken in a fasting state to 60-90% when administered after a meal [7]. Fenofibrate does not accumulate with repeated administration, and fenofibric acid is >99% bound to plasma albumin. It is excreted mainly as fenofibric acid and its glucuronide conjugate in the urine, with smaller amounts excreted in the faeces [8].

While gemfibrozil inhibits hepatic uptake of statins through OATP1B1 and competes for the same glucuronosyltransferases that metabolise most statins, determining a clinically relevant drug -drug interaction, fenofibrate is glucuronidated by enzymes not involved in the glucuronidation of statins. Therefore, fenofibrate-statin combinations are less likely to cause myopathy than combination therapy with gemfibrozil and statins. In fact, co-administration of fenofibrate and atorvastatin, for instance, did not result in relevant clinical-pharmacokinetic drug interactions in healthy subjects [9].

Evidence from cardiovascular endpoint trials

Clinical trials with fibrates have provided conflicting results. In the Helsinki Heart Study (HHS) primary prevention trial, 4,081 asymptomatic middle-aged men (40-55 years) with primary dyslipidaemia (non-HDL-C ≥200 mg/dL) without CVD were treated with gemfibrozil or placebo [10]. Gemfibrozil lowered total cholesterol, LDL-C, non-HDL-C and TG, while it increased HDL-C. After 5 years, a 34% reduction in the primary endpoint (fatal and non-fatal myocardial infarction (MI) and cardiac death) was observed in the gemfibrozil group compared with placebo [10]. In the subgroup of patients with TG >2.3 mmol/L and LDL-C/HDL-C ≤5 the benefit was even greater (71% risk reduction) [11]. The benefit of gemfibrozil was confirmed in a secondary prevention trial in men with low HDL-C, with a 22% reduction in the primary endpoint (non-fatal MI or coronary death) [12]. However, two subsequent trials with bezafibrate, the BIP and LEADER trials, could not confirm this positive effect on the primary endpoint in the overall population [13, 14]. Of note, the Bezafibrate Infarction Prevention (BIP) trial reported a 41.8% reduction in the primary endpoint in the subgroup of patients with high TG and low HDL-C levels [13] and reduced the incidence of myocardial infarction in patients with metabolic syndrome during long-term follow-up (6.2 years for events and 8.1 years for mortality data) [15]. In addition, a 40% reduction in the secondary endpoint of non-fatal CHD events was observed in patients aged <65 years in the Lower Extremity Arterial Disease Event Reduction (LEADER) trial testing bezafibrate in patients with peripheral artery disease (PAD) [14].

The FIELD (Fenofibrate Intervention and Event Lowering in Diabetes) was the first cardiovascular outcomes trial of fenofibrate [16]. Patients with type 2 diabetes mellitus who were not taking statins at baseline were treated with fenofibrate or placebo. Fenofibrate did not reduce the risk for the primary endpoint (first occurrence of non-fatal myocardial infarction or death from coronary heart disease), but it did reduce the risk for total CVD events (HR 0.89 [0.80-0.99], P=0.035) and coronary revascularisation (HR 0.79 [0.68-0.93], P=0.003) [16]. It should be noted that in this trial, patients in the placebo group were significantly more likely to take statins than patients in the fenofibrate group (36% vs 19%), which may have reduced the expected effect of fenofibrate. The effect of fenofibrate in the subgroup of patients with marked dyslipidaemia (TG >2.3 mmol/L and lower HDL-C) was significant (HR 0.73 [95% CI 0.58-0.91], P=0.005) [17]. The subsequent outcome trial of fenofibrate, the ACCORD (Action to Control Cardiovascular Risk in Diabetes) Lipid, investigated the effect of fenofibrate or placebo in addition to simvastatin in patients with type 2 diabetes [18]. After a mean follow-up of 4.7 years, the combination of fenofibrate and simvastatin did not reduce the rate of the primary endpoint (first occurrence of non-fatal myocardial infarction, non-fatal stroke, or death from cardiovascular causes) compared to simvastatin alone [18]. However, in the prespecified subgroup of patients with low HDL-C (≤34 mg/dL) and high TG levels (≥204 mg/dL) fenofibrate therapy resulted in a significant 31% risk reduction [19], which is consistent with the

results of post-hoc subgroup analyses in other fibrate trials [11, 13, 17]. It is worth noting that variants in the *PPARα* gene can influence the response to fenofibrate in patients with type 2 diabetes [20].

The ACCORDION study was a post-trial follow-up of the participants (90%) of the ACCORD Lipid study. The mean overall duration of follow-up was 7.7 years for the primary outcome and 9.1 years for all-cause mortality [21, 22]. This extended follow-up confirmed the neutrality of fenofibrate in the overall study cohort, but the incidence of the primary endpoint was 27% lower in patients with atherogenic dyslipidaemia, which is consistent with the results of the original ACCORD trial [21]. A secondary analysis of trial and post-trial data in patients who had atherogenic dyslipidaemia of the ACCORDION study showed that treatment with fenofibrate during the initial trial period was associated with a legacy benefit of improved survival over the post-trial follow-up, an effect that was observed despite similar achieved lipid levels during the follow-up [22]. These findings support the use of fibrates as an add-on to statins to reduce CV risk in diabetic patients with atherogenic dyslipidaemia.

Putative explanations for the different clinical outcomes between fenofibrate and pemafibrate

The clinical efficacy of a new selective PPAR-alpha modulator, pemafibrate, has been evaluated in the PROMINENT trial conducted in patients with type 2 diabetes, mild to moderate HTG and low HDL-C [23]. More than 95% of patients were on background statin therapy at baseline. Despite significant reductions in TG (26.2%), VLDL-C (25.8%) and remnant cholesterol (25.6%), the incidence of major adverse cardiovascular events (a composite of myocardial infarction, ischemic stroke, hospitalization for unstable angina warranting unplanned coronary revascularization, or death from cardiovascular causes) was similar in patients treated with pemafibrate or placebo [23]. Both LDL-C and apoB were significantly increased after pemafibrate therapy [23].

What possible explanations are there for this difference in the effect of fenofibrate and pemafibrate? Some studies contain information that could help explain this difference, particularly with regard to the different effects on atherogenic lipid parameters, including apoB, LDL-C and sd-LDL. A phase 3 study compared the efficacy and safety of pemafibrate with fenofibrate in Japanese patients with high TG and low HDL-C levels [24]. Pemafibrate 0.4 mg/day and fenofibrate 200 mg/day -the usual doses of these two drugs- produced similar reductions in TG levels and remnant cholesterol [24]. Both drugs caused an increase in LDL-C, but this was greater with pemafibrate than with fenofibrate (+19.3% versus +6.6%, p=0.001). ApoB levels were slightly increased with pemafibrate treatment while decreasing with fenofibrate (+3.2% versus -7.3%, p<0.001) [24]. It is noteworthy that diabetic patients who received the fenofibrate/simvastatin combination therapy showed no increase in LDL-C levels in the ACCORD Lipid trial [18].

The deleterious effect of pemafibrate 0.4 mg/day was confirmed in European patients with high TG and low HDL-C on statin therapy [24]: pemafibrate 0.4 mg/day (twice daily) increased LDL-C by 20.5% (p<0.001 versus placebo) and no significant effect was observed in either apoB or non-HDL-C levels [24]. So the increase in LDL-C was largely due to an increased amount of cholesterol per particle rather than an increase in LDL particle number, as demonstrated by ion mobility analyses, which showed that pemafibrate increased the concentration of large LDL particles and decreased the concentration of small dense LDL particles [25], consistent with other observations [26].

The results of a meta-analysis of three randomised clinical trials have suggested that pemafibrate is more effective than fenofibrate in reducing TG-rich lipoproteins [27]. Indeed, pemafibrate reduced more TG, VLDL-C, remnant cholesterol, apoB48 and apoC-III and increased more HDL-C and apoA-I compared with fenofibrate [27]. However, the dose of fenofibrate was only 100 mg daily in these trials. No significant difference in non-HDL-C and apoB levels was observed between the two groups, and a slight LDL-C-increasing effect was observed in the pemafibrate group, which is consistent with previous observations [27]. A more-in-depth analysis showed that LDL-C levels increased in patients with higher baseline TG levels and lower baseline LDL-C levels [26], which is likely explained by the effect of pemafibrate on TG-rich lipoprotein catabolism, leading to increased conversion of VLDL to LDL and a change in LDL composition. However, when calculating the levels of small dense LDL in the PROMINENT study, no difference was found between the pemafibrate and placebo groups [28], suggesting that the influence of TG on small LDL-C levels is attenuated when LDL-C is tightly controlled [29].

Overall, these observations suggest that the effect on apoB levels rather than the TG-lowering efficacy may be crucial for the potential beneficial effect of a fibrate-based therapy, together with the choice of the right type of patient to be treated, potentially with regard to PPAR-α gene polymorphisms modulating response to (feno)fibrate.

Meta-analyses of fibrate trials

A meta-analysis of 18 trials with 45,058 participants showed that fibrate therapy resulted in a 10% relative risk reduction for major CV events and a 13% relative risk reduction for coronary events, but had no effect on stroke, all-cause mortality, CV mortality, sudden death, or non-CV mortality [30]. Overall, fibrates lowered total cholesterol, LDL-C and TG levels and increased HDL-C levels, with gemfibrozil being the most effective [30]. Patients with higher baseline TG levels (≥2.0 mmol/L) appeared to benefit more from fibrate therapy [30]. The beneficial effect on CV risk in individuals with atherogenic dyslipidaemia was noted in the meta-analysis of data from 6 trials with more than 25,000 participants [31]. While fibrate therapy did not reduce the rate of vascular events in 9,872 subjects with neither high TG nor low HDL-C, a significant benefit was observed in 5,068 subjects with high TG and low HDL-C, with a relative risk reduction of 29% (RR 0.71, [0.62-0.82], P<0.001) [31]. It is worth noting that benefit was also observed in 7,389 subjects with high TG and in 15,303 subjects with low HDL-C (RR 0.84, 95% CI 0.77 to 0.91, P<0.001) [31]. Another meta-analysis of 5 trials of fibrates found similar results: a significant protective effect was observed in patients with high TG levels or atherogenic dyslipidaemia, in whom fibrates reduced CV risk by 28% (15% to 39%; P < 0.001) and 30% (19% to 40%, P <0.0001), respectively, but only by 6% (–2% to 13%, P=0.13) in patients without atherogenic dyslipidaemia [32].

Fenofibrate and statins combination therapy

The rationale for using a combination therapy is that it provides complementary mechanisms of action on lipid metabolism, leading to a better improvement in the lipid profile. Monotherapy with high intensity statins can lead to greater improvements not only in LDL-C but also in TG; however, this type of approach still does not correct all the lipoprotein abnormalities in patients with combined hyperlipidaemia. On the other hand, fibrates significantly reduce TG-rich lipoproteins, as well as the LDL fraction of small, dense particles. Fibrates and statins thus regulate serum lipids by different mechanisms, so that combination therapy could offer desirable advantages in patients with combined hyperlipidaemia, at least if this combination therapy produces a complementary reduction in the total

number of atherogenic lipoproteins, i.e. a reduction in apoB levels, compared with statin monotherapy.

As mentioned above, the ACCORD Lipid trial showed that the combination fenofibrate/simvastatin did not reduce the rate of major adverse cardiovascular events compared to simvastatin alone [18], although a positive effect was observed in the subgroup of patients with elevated TG levels and low HDL-C levels [18, 19]. The DIACOR (Diabetes and Combined Lipid Therapy Regimen) study investigated the effect of simvastatin/fenofibrate combination therapy on inflammatory biomarkers in patients with diabetes [33]. The combination was not superior to monotherapies in modulating inflammatory biomarkers, while the overall lipid profile was better [33]. Similar results were observed in the SAFARI trial, in which the combination fenofibrate/simvastatin 160/20 mg improved the lipid levels more than simvastatin 20 mg alone in patients with combined hyperlipidaemia, especially a 10% complementary decrease in apoB levels [34]. Two doses of the fixed dose combination (FDC) fenofibrate/simvastatin were compared for efficacy and safety with the monotherapies in patients at high CV risk and with mixed dyslipidaemia [35]. After 12 weeks, both FDC doses significantly reduced TG and increased HDL-C levels compared with simvastatin; LDL-C levels were not increased as instead observed with fenofibrate alone; non-HDL-C and apoB decreased with both FDC doses [35].

The effect of a FDC of fenofibrate 100 mg and atorvastatin 40 mg has been investigated in adults with mixed dyslipidaemia [36]. The FDC was more effective in lowering TG and non-HDL-C (-49.1% and -44.8%, respectively) than monotherapies with atorvastatin 40 mg (-28.9% and -40.2%, respectively) or fenofibrate 145 mg (-27.8% and -16.1%, respectively) [36]. As expected, the decrease in LDL-C was significantly greater in the FDC group than in the fenofibrate 145 mg monotherapy group (–42.3% versus –13.9%; $P < 0.001$) but was not significantly different from the decrease in the atorvastatin monotherapy group (–43.1%; n.s.). However, the decrease in apoB levels was significantly greater with the FDC than with atorvastatin 40 mg monotherapy (-40.5% versus -35.7%, respectively, p=0.046) [36]. This treatment was generally well tolerated and argued for the use of the combination to better control the lipid profile.

The co-administration of rosuvastatin 10 mg or 20 mg with fenofibric acid was more effective in reducing TG levels and increasing HDL-C levels compared to rosuvastatin monotherapy in patients with mixed dyslipidaemia, while LDL-C lowering was comparable [37]. Combination therapy with rosuvastatin 10 mg led to a greater reduction in non-HDL-C and apoB than rosuvastatin alone (non-HDL-C: -44.7% versus -39.8%, p<0.001; apoB: -39.2% versus -34.1%, p<0.001). However, no differences were observed for the same parameters between combination therapy with rosuvastatin 20mg and rosuvastatin 20 mg monotherapy groups [37]. The fixed-dose combination of rosuvastatin and fenofibric acid (20 mg/135 mg, 10 mg/135 mg, and 5 mg/135 mg) was compared with simvastatin 40 mg in 474 patients with high levels of LDL-C and TG [38]. A greater reduction in LDL-C levels was observed in patients treated with all doses of the rosuvastatin/fenofibric acid combination than with simvastatin alone [38]. All other biochemical parameters (including non-HDL-C, apoB, TG, HDL-C and hs-CRP) were improved more by the combination [38], and side effects were comparable between groups.

A study comparing the non-lipid effects of rosuvastatin-fenofibrate (160 mg/10 mg) combination with rosuvastatin monotherapy (10 mg) in high-risk Asian patients with mixed hyperlipidaemia showed that the incidence of muscle or liver enzyme elevations were similar in the two groups (2.8% and 3.9% in the combination and rosuvastatin groups, respectively, p = 1.00) over a 24-week treatment period [39]. Overall, the proportion of patients experiencing adverse events was comparable in both groups [39]. Higher elevations of homocysteine, blood urea nitrogen, and serum creatinine and a greater reduction in leukocyte and haemoglobin levels were observed in the combination group [39], which may indicate cautious use in individuals with renal dysfunction.

A fixed-dose combination of fenofibrate and pravastatin (160 mg and 40 mg) was given to high-risk patients with mixed hyperlipidaemia for 12 weeks. Compared to pravastatin alone, greater reductions in non-HDL-C, LDL-C, TG and apoB were observed, with comparable incidences of adverse events [40]. This FDC therapy was shown to be effective and safe over a 52-week period and resulted in greater reductions in lipid levels than pravastatin 40 mg in a group of high-risk hyperlipidaemic patients [41].

Altogether, the results of clinical trials suggest that the combination of fenofibrate with a statin is effective in improving atherogenic dyslipidaemia, especially in terms of complementary decrease in apoB levels, and may provide clinical benefit in patients with elevated TG levels and low HDL-C levels. The presence of a statin in the combination ensures the reduction in LDL-C essential to reduce the CV risk. Of note, the effect is similar for all statins (class effect), and thus similar benefits can be expected regardless of which statin is used in combination with fenofibrate. Since fenofibrate appears to provide significant microvascular benefits in patients with type 2 diabetes, specifically a reduction of the progression of diabetic retinopathy [42, 43], the combination of fenofibrate with a statin may be a valuable tool for these patients; despite this consideration, fibrates are no longer recommended in the recently released 2023 ESC guidelines for the management of CVD in diabetic patients [6].

Conclusions

Fibrates have been in use for many decades and have proven effective and safe treatments of atherogenic dyslipidaemia. Their current position in the management lies primarily in combination with a statin. Most data documenting efficacy and safety of statin-fibrate combinations come from fenofibrate/fenofibric acid. Beneficial anti-atherogenic effects of the combination regimens are linked with ApoB reductions [44] that have been achieved in a number of trials of fenofibrate and statin combinations. Pharmacological differences between fenofibrate and pemafibrate, the latter associated with ApoB increase in the PROMINENT trial, might explain the observed lack of clinical benefits in contrast to fenofibrate.

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Competing interests

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Role of nuclear medicine assessing patients with suspected coronary artery disease

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ABSTRACT

Nuclear medicine is a critical component in the field of cardiology as it provides diagnostic and prognostic insights that are essential for the effective management of heart disease.

Both single photon emission computed tomography (SPECT) and positron emission tomography (PET) play a significant role in assessing the likelihood of ischemic heart disease based on pre-test probabilities. Both SPECT and PET should be integrated into the clinical pathway according to the patient's individual risk profile, symptoms, and initial test results. The guidelines recommend using these imaging modalities to refine risk stratification, particularly in intermediate-risk patients, and to guide further invasive diagnostic or therapeutic procedures based on the imaging findings.

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Keywords
Coronary artery disease; single photon emission computed tomography; positron emission tomography; non-invasive tests

Initial evaluation of patients with suspected ischemic heart disease

According to the 2019 European Society of Cardiology (ESC) guidelines on chronic coronary syndromes, a patient with suspected ischemic heart disease, commonly referred to as coronary artery disease (CAD), is typically identified based on risk factors, clinical presentation, and initial non-invasive evaluation [1].

The presence of risk factors such as hypertension, dyslipidemia, diabetes, smoking, a family history of early coronary artery disease, and obesity increases the likelihood of coronary artery disease. Symptoms can be summarized as dyspnea, typical angina pectoris characterized by chest pain or discomfort that occurs with exertion or emotional stress and is relieved by rest or nitroglycerin, atypical angina and non-anginal chest pain when not all the criteria for typical angina are met.

A detailed initial assessment, including medical history, physical examination, and diagnostic tests like an electrocardiogram (ECG), is used to define the pre-test probability of CAD, based on age, sex, and the nature of chest symptoms.

Subsequent management can range from lifestyle modifications and medical treatment for low-risk patients to more aggressive interventions such as revascularization for those at high risk.

Noninvasive diagnostic evaluation

Non-invasive tests to assess ischemia include several techniques, such as exercise testing, stress echocardiography, myocardial perfusion imaging by nuclear imaging with Single Photon Emission Computed Tomography (SPECT) or Positron Emission Tomography (PET), and cardiac magnetic resonance (CMR). These tests help to detect myocardial ischemia and evaluate the need for further invasive investigations such as coronary angiography.

Each non-invasive diagnostic test has a particular range of clinical likelihood of obstructive CAD where the usefulness of its application

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is maximal [1]. Given the clinical likelihood of obstructive CAD and the likelihood ratio of a particular test, one can assess the post-test probability of obstructive CAD after performing such a test [1]. Using this approach, one can estimate the optimal ranges of clinical likelihood for each test, in which they can reclassify patients from intermediate to either low or high post-test probability of CAD [2].

Patients can be categorized as having low, intermediate, and high pre-test probability of having ischemic heart disease and the choice of diagnostic tests is guided by these categories [1]:

- Low Pre-test Probability (<15%): For these patients, non-invasive testing might often be unnecessary, and routine testing is not recommended as it could lead to false positives and unnecessary further invasive procedures.
- Intermediate Pre-test Probability (15-85%): This group benefits the most from non-invasive imaging tests like stress echocardiography, SPECT and PET. SPECT is commonly used due to its availability and efficacy in detecting areas of reduced myocardial perfusion indicative of CAD. PET, while less commonly available, provides higher accuracy and better quantification of myocardial blood flow, and may be particularly useful in certain complex cases.
- High Pre-test Probability (>85%): In these patients, direct invasive strategies such as coronary angiography are often considered appropriate due to the high likelihood of significant coronary artery disease. However, PET can be used in specific scenarios to assess myocardial viability, especially when considering revascularization options.

Coronary Computed Tomography Angiography (CTA) is the preferred test in patients with a lower range of clinical likelihood of CAD, no previous diagnosis of CAD, and characteristics associated with a high likelihood of good image quality. It detects subclinical coronary atherosclerosis but can also accurately rule out both anatomically and functionally significant CAD. It has higher accuracy values when low clinical likelihood populations are subjected to examination [3]. Trials evaluating outcomes after coronary CTA to date have mostly included patients with a low clinical likelihood [4, 5].

The non-invasive functional tests for ischemia typically have better rule-in power. In outcome trials, functional imaging tests have been associated with fewer referrals for downstream coronary angiography compared with a strategy relying on anatomical imaging [6-8].

The clinical significance of high-risk ischemic patterns

Before revascularization decisions can be made, functional evaluation of ischemia (either non-invasive or invasive) is required in most patients. Therefore, functional non-invasive testing may be preferred in patients at the higher end of the range of clinical likelihood if revascularization is likely or if the patient has previously been diagnosed with CAD.

When severe myocardial ischemia, indicative of substantial coronary artery obstruction, is identified, it represents a key determinant in the decision-making process for proceeding with interventional procedures. Patients displaying severe ischemia are often recommended for coronary angiography, which can lead to interventions such as percutaneous coronary intervention (PCI) or coronary artery bypass grafting (CABG). These procedures aim to restore adequate blood flow to the ischemic areas, thereby improving symptoms, cardiac function, and overall prognosis [9, 10].

The ISCHEMIA (International Study of Comparative Health Effectiveness with Medical and Invasive Approaches) trial provided significant insights into the impact of the extent of myocardial ischemia on therapeutic decision-making in patients with stable CAD.

This trial explored the outcomes of patients with moderate to severe ischemia who were treated either with conservative medical therapy alone or with an initial invasive strategy involving angiography and possible revascularization [11].

The trial demonstrated that the initial invasive strategy did not significantly reduce the risk of major cardiovascular events compared to medical therapy alone in the overall cohort. However, subgroup analyses suggested that patients with more extensive ischemia might benefit more from revascularization in terms of symptom relief and quality of life improvements [11].

These findings emphasize the importance of personalized treatment strategies based on the extent of ischemia. While the results challenge the necessity of routine invasive procedures for all patients with moderate to severe ischemia, they highlight the need for a tailored approach, considering the individual patient's ischemic burden and symptomatic status.

Clinicians are required to carefully assess the extent of myocardial ischemia using non-invasive imaging techniques in stable CAD patients [1].

The ESC Guidelines summarize the definitions of high event risk for the different test modalities in patients with established chronic coronary syndromes [1, 12-14]:

- Exercise ECG: cardiovascular mortality >3% per year according to Duke Treadmill.
- Score SPECT or PET perfusion imaging: area of ischemia ≥10% of the left ventricle myocardium.
- Stress echocardiography: ≥3 of 16 segments with stress-induced hypokinesia or akinesia.
- CMR: ≥2 of 16 segments with stress perfusion defects or ≥ 3 dobutamine-induced dysfunctional segments.

The role of nuclear medicine in patients with suspected CAD

Nuclear medicine is a critical component in the field of cardiology, offering diagnostic and prognostic insights that are essential for the effective management of heart diseases. This branch of medicine utilizes radioactive substances, known as radiotracers, to create images of the heart and study its function and structure in detail.

Both SPECT and PET play significant roles in assessing the likelihood of ischemic heart disease based on pre-test probabilities [1]. Guidelines outline specific scenarios in which SPECT and PET are particularly valuable, emphasizing their utility in refining diagnostic accuracy and guiding clinical decision-making [1].

Myocardial perfusion imaging with SPECT has been generally regarded as the reference standard for the evaluation of myocardial perfusion [1]. SPECT imaging is a robust tool for diagnosing CAD by evaluating myocardial perfusion deficits during stress testing. It is particularly useful for assessing the severity and extent of ischemia, helping to guide decisions about the necessity for angiography or revascularization [1].

Otherwise, PET offers several advantages over SPECT, including higher spatial resolution, the ability to quantitatively assess myocardial blood flow, and reduced radiation exposure to the patient [1]. PET is highly effective in evaluating myocardial viability and differentiating between scarred and hibernating myocardium, which is crucial for planning revascularization in patients with severe ischemia or complex coronary anatomy [1].

Both SPECT and PET should be integrated into the clinical pathway according to the patient's individual risk profile, symptoms, and initial test results. The guidelines recommend using these imaging modalities to refine risk stratification, particularly in intermediate-risk

patients, and to guide further invasive diagnostic or therapeutic procedures based on imaging findings.

A patient-centric approach, using the best available diagnostic tools to inform treatment strategies, thereby optimizing care for patients with suspected or confirmed CAD, is advocated to apply the best cost-effectiveness approach to an increasing disease.

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BPIFB4 protein and monocyte phenotyping: a preclinical asset for marking the frailty condition

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Keywords
Frailty; longevity; monocytes; biomarker

ABSTRACT

Frailty is a state of increased vulnerability to stressors arising from the systemic decline in physiological reserve mechanisms with aging. Advanced age impacts on frequency and phenotype of immune cells such as monocytes and macrophages. BPIFB4, a host defense protein with immunomodulatory activity, is protective in healthy long-living individuals in whom monocytes and macrophages have a favorable redistribution and phenotype. Although we reported an inverse correlation of the homozygous LAV-BPIFB4 haplotype with frailty in elderly subjects, the role of the circulating BPIFB4 levels as a frailty biomarker has not yet been characterized. In this study we investigated the correlation between BPIFB4 levels and both the frailty assessment/health status and monocytic profile in frail subjects.

Participants (40 frail individuals and 20 age-matched healthy volunteers) were subjected to standardized questionnaires to assess frailty risk, routine clinical examinations and blood tests; monocytes were analyzed by flow cytometry.

Overall, 70% of the frailty cohort had mild frailty, 25.5% had moderate frailty, and 5% had severe frailty. Compared to healthy controls, frail subjects showed lower levels of circulating BPIFB4 that inversely correlated with the relative risk index for hypertension and cardiovascular disease. The total circulating monocyte frequency is reduced in frail subjects compared to healthy controls. CD14++CD16– classical monocytes and CD14+CD16++ non-classical monocytes were significantly increased in frail people compared to healthy controls, whereas intermediate CD14++CD16+ monocytes were reduced. The M2/M1 monocytic balance was also altered in frailty condition. No relationship between BPIFB4 plasma levels and monocytes' subsets was found.

Our findings highlight that BPIFB4 protein has a potential prognostic value for marking the frailty condition.

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Introduction

People worldwide can expect to live longer. By 2030, about 20% world's population will be aged 60 years or over, which is why frailty is expected to reach epidemic proportions in the coming decades. Frailty represents an age-related dysregulation of the physiological functions and reserve mechanisms associated with adverse health outcomes. A state of vulnerability to stressors and dysfunctional homeostasis persists in frail subjects [1]. Frailty is recognized by clinicians, but its definition requires a complex systemic approach that takes into account biological and psychosocial correlates, and single symptoms are not sufficient to highlight it [2, 3]. Precisely because of its syndromic nature, the research area lacks an operational assessment tool for frailty that meets international consensus [4-6]. Among the various frailty assessment tools, we particularly highlight Fried et al. [7] frailty phenotype and Rockwood's [8] cumulative deficit model, which have achieved an international reputation. The frailty phenotype ranges from not-frail to pre-fail and frail, [7], and the different frail states are gradually strongly associated with a higher risk of

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developing adverse geriatric outcomes. In agreement with Wleklik et al., frailty develops in 25% to 62% of patients with cardiovascular diseases and, meanwhile, the presence of CVDs implies the increased risk of developing frailty in older people [9]. Furthermore, multimorbidities (such as hypertension, diabetes, and COPD) which are common in old and frailty, pose a detrimental predictor of health outcomes in older patients [10]. In the aging context, long-living individuals constitute a model of exceptional healthy aging, considering their ability in overcoming and coping better with age-related diseases and frailty, despite their biological age. Previous work from our group identified a longevity-associated variant (LAV) of BPIFB4 associated in homozygosity with exceptional longevity in different independent populations [11, 12]. The bactericidal/permeability-increasing fold-containing family-B-member-4 (BPIFB4) is a secreted protein highly abundant in respiratory secretions, in the upper airways and proximal trachea. Besides the longevity-associated variant (LAV), which is constituted by the minor allele of rs2070325 that is part of a four SNPs haplotype, the gene BPIFB4 presents other two isoforms: the wild type (WT)-BPIFB4, which is constituted by major alleles of the four SNPs, and the rare-variant (RV)-BPIFB4, found to be a biomarker of vascular dysfunction and hypertension [11, 13]. Compared to the other two isoforms, LAV-BPIFB4 gene transfer was found to exert advantages by reducing atherosclerosis progression and inflammation in ApoE$^{-/-}$ mice [14], by contrasting immunosenescence and aorta senescence in a murine model of advanced age, [15], by restoring the heart function in a model of diabetic cardiopathy [16]. Furthermore, the serum of long-living individuals is enriched in BPIFB4 compared to controls and frail people, thus classifying their health status [17]. Moreover, Malavolta et al identified LAV-BPIFB4 haplotype was significantly under-represented in frail subjects and the LAV-BPIFB4 gene therapy in old mice clinically attenuated the progression of frailty [18]. LAV-BPIFB4 also showed an interesting involvement with regard to the immune compartment. Indeed, the longevity-associated variant of BPIFB4 showed the ability in driving both dendritic cells toward a regulatory phenotype [19] and the macrophage-skewing toward a pro-resolving M2 phenotype in atherosclerotic subjects [14]. Considering the age-related changes in innate immune cells and that circulating BPIFB4 levels were found to associate with the abundance of pro-resolving monocytes and macrophages in long-living individuals [20], here we evaluate the profile of monocytes and macrophages in recruited frail subjects compared to healthy volunteers and the potential correlation with BPIFB4 circulating levels. Indeed, more efforts are needed to find optimal biomarkers associated with frailty capable of being valuable for the early diagnosis or prognosis of frailty in older people. From a translational point of view, the main purpose of this work was to evaluate the possible usefulness of BPIFB4 as a prognostic tool for marking frailty.

Materials and methods

Study design and sample characteristics

The study is a single-center, cross-sectional survey conducted between January 2016 and January 2017 among a group of older patients recruited from a random sample stratified by age and gender, at the Department of Medicine, Surgery and Dentistry "Scuola Medica Salernitana", University of Salerno and the University Hospital "San Giovanni di Dio e Ruggi d'Aragona", Salerno Italy. The primary objective was to assess, through a validated questionnaire and clinical examinations, the health status and frailty index of the young old and the old/great old, respectively. The secondary objective was to understand, through blood tests, whether the immunophenotype and genotypic characterization had a possible correlation with frailty. It is important to use screening and assessment tools to investigate the different dimensions of health and identify the frailty condition earlier to help patients recover function and prevent adverse outcomes.

The study was performed on a group of 67 individuals, n=47 frail patients and n=20 aged-matched healthy volunteers free from risk factors for, and clinical evidence of clinical signs and symptoms of relevant communicable disease agents and chronic diseases, and treatments related to medical conditions.

For each patient, venous blood (10 mL) was withdrawn for analyses and detailed anamnesis was collected. All participants signed an informed consent for the management of personal anamnestic data and blood samples. The study was approved by the Campania Sud ethical committee and conducted in accordance with the ethical principles deriving from the Declaration of Helsinki (N.78 _r.p.s.o. del 04/07/2018." *Studio per la valutazione della correlazione tra le isoforme del gene BPIFB4 e il rischio di fragilità umana*").

Of the forty-seven frail patients recruited, 40 were selected and completed the study, as the eligibility criterion was that the patients' phenotype fell within the threshold value of frailty [7], that the patients were aged 65 to 90 years or older and that there were no obvious disabilities; seven fell within the exclusion criteria as they did not have the above characteristics and belonged to the robust subjects [1]. The patients met the criteria outlined in international clinical practice guidelines for the identification and management of frailty in older adults [21].

Data collection for baseline evaluation

Standardized questionnaires ascertained self-rated health status, health habits, weight loss, and self-reported medical diagnoses of cardiovascular events (hypertension, angina pectoris, chronic heart failure, stroke), diabetes, chronic pulmonary disease, and cancer.

The multidimensional procedure "Comprehensive Geriatric Assessment" was used to assess the functional ability [22, 23], physical, cognitive, and mental health [24, 25], and socio-environmental status [26, 27] of older patients. Functional status was ascertained by asking old patients whether they had difficulty performing 12 tasks of daily living, tasks included in instrumental activities of daily living (IADLs) and activities of daily living (ADLs) [28]. Physical function was assessed with several questions from the Physical Activity Scale for Elderly (PASE) [29], which includes standardized performance-based measures of physical function, such as time (seconds) taken to walk 4 meters [25] and grip strength (kilograms) of the dominant hand (2 measures on mean), using a Smedley handheld dynamometer. Cognitive and mental health was assessed with the Mini-Mental State Examination (MMSE) [30] and the Geriatric Depression Scale (GDS) [24]. The Social Support Assessment (SSA) was used to assess whether older patients had social relationships and, if so, whether the level of support was high, fair or low [27]. Through standardized clinical examinations, such as electrocardiogram, echocardiography, and pressure report, and subsequent evaluation of the data by physicians, cardiovascular diseases (hypertension, angina pectoris, chronic heart failure, stroke) were validated [31].

Further examinations ascertained: body weight (kg) and height (cm) to calculate body mass index (BMI); blood test to determine fasting glucose level; and M1/M2 immunophenotypic analysis and genotype characterization.

Rockwood frailty index data

Rockwood's Frailty Index was calculated using information collected during various routine health assessments of older adults,

specifically 38 variables were considered to have an accurate index [32, 33]. Issues related to functional difficulties, such as difficulty in washing, dressing, sitting or getting up from a chair, walking, eating, taking care of the house, using the toilet, climbing or descending stairs, grocery shopping, household chores, preparing meals, taking medication, managing money, staying in bed at least half the day due the health, and reducing habitual activity, were coded as binary variables, using the convention that "0" indicates no deficit and "1" indicates the presence of a deficit. For the self-rated health question "How do you rate your health?" a six-point Likert scale was used for responses, where the endpoints are labeled 0 = excellent, 0.25 =very good, 0.5 = good, 0.75 = poor, and 1 = poor. For the question "Has your health changed in the last year", the response includes 0 = better/same, 1 = worse.

Standardized measures, to define physical health, including time taken to walk 4 meters, the cutoff of which was coded as a binary variable, where 1 ≥10 frailty index criterion and 0 ≤10 non-frailty index criterion; and grip strength, which was stratified into quartiles based on gender and body mass index (BMI) [7] and then recoded into binary as follows:

1 male = presence of grip strength if BMI ≤24 and kg ≤29; BMI 24.1- 26 and kg ≤30; BMI 26.1- 28 and kg ≤30; BMI >28 and kg ≤32.

0 male = absence of grip strength if BMI ≤24 and kg >29; BMI 24.1-26 and kg >30; BMI 26.1-28 and kg >30; BMI >28 and kg >32.

1 female = presence of grip strength if BMI ≤23 and kg ≤17; BMI 23.1- 26 and kg ≤17.3; BMI 26.1 -29 and kg ≤18; BMI >29 and kg ≤21.

0 female = absence of grip strength if BMI ≤23 and kg >17; BMI 23.1-26 and kg >17; BMI 26.1- 29 and kg >18; BMI >29 and kg >21.

Variables related to cognitive health (GDS), such as "feeling that everything is an effort", "feeling depressed" and "feeling happy", were coded through a three-point Likert scale, 0 = rarely, 0.5 = sometimes, 1 = most of the time. Regarding the Mini-Mental State Examination (continuous variable), recoding was done according to the severity of impairment [31], assigning 1 for scores <10 defined as "severe dementia", 0.75 for scores ≥10 and ≤17 classified as "moderate dementia", 0.5 for scores ≥ 18 and ≤20 defined as "mild dementia", 0.25 for scores >20 and <24 "mild cognitive impairment" (MCI), and 0 for scores ≥24 "no cognitive impairment" [33].

The comorbidities were assessed both as a cumulative total and as a single disease, such as hypertension, diabetes, chronic obstructive pulmonary disease (COPD), chronic heart failure, angina pectoris, stroke, and cancer, labeled with a three-point Likert scale with endpoints such as 0 = no disease, 0.5 = suspected presence, 1 = presence of disease.

By dividing weight (kg) by height squared (m^2), body mass index (BMI) was calculated in old patients. The BMI (variable continuous) was recoded, according to the criteria established by the WHO, considering 0 = normal weight, 0.5 = overweight, and 1 = obese. The mini-nutritional assessment (MNA) was coded into three-point Likert scales labeled as 0 if the score is between 24 and 30 and shows "normal nutritional status", 0.5 if the score is between 17 and 23.5 "risk of malnutrition", and 1 if the score is < 17 "malnutrition" [17].

Social Support Assessment (SSA) has been coded to 0 "low social support" if the range is 0 to 2.9; 1 "moderate support" if the range is 3 to 5.9; and 2 "high support" if the range is 6 to 10 [27].

The frailty index was calculated based on the score of the deficits present in the patients in relation to the total number of deficits considered. Based on severity, the frailty index was divided into "mild" if the score was between 0 to 13.9, "moderate" if it was between 14 to 24.9, and "severe" if it was between 25 to 38.

Flow cytometry and immune phenotypical analysis

Peripheral blood mononuclear cells (PBMC) were extracted from whole blood by density gradient (Ficoll). After separation, PBMC were collected and washed for the subsequent experiments.

Conjugated monoclonal antibodies against CD14, CD16, CD86, and CD163 were purchased from BD Biosciences. After 20 minutes of incubation at room temperature in the dark, cells were washed with staining buffer and resuspended for the FACS analysis. For each test, cells were analyzed using a FACS Verse Flow Cytometer (BD Biosciences).

ELISA assay

Plasma levels of BPIFB4 were measured using an ELISA Kit (Cusabio CSB-YP003694HU) following the manufacturer's protocol. Concentration values were subjected to statistical analysis by using GraphPad Prism 6.0 software for Windows (GraphPad software).

Genotyping

Genetic analysis for the SNP rs2070235 (p.Ile229Val) on BPIFB4 was assessed in all subjects. From all samples collected, leucocytes were used to extract their genomic DNA (DNeasy kit, Qiagenâ). Then, the DNA was quantified to normalize concentrations run on quantitative polymerase chain reaction (PCR)-Taqman-based method.

Statistical analysis

Descriptive statistics were used to summarize patients' characteristics considering perception of current and last-year health status, diseases at baseline, and frailty indexes; responses to all items were shown with absolute and relative frequency values for categorical variables and mean and standard deviation for continuous variables. Multivariate analysis plots were used to show changes in values of the multidimensional comprehensive geriatric assessment (for better understanding, CGA data were recoded into 5-point Likert with endpoints labeled as -2 worst health status and 2 best health status). Poisson regression analysis was performed to calculate significant predictors of BPIFB4 protein and Rockwood frailty index on the measured variable. The incidence ratios (IRRs) and their 95% confidence intervals (CIs) were used in the Poisson regression models to measure the independent associations between the different variables and the outcomes of interest. Pairwise correlation analysis was performed between BPIFB4 protein and patrolling. For all analyses, values of 0.05 or less were considered statistically significant. Data analyses were conducted using STATA software (Release 16.1, StataCorp LLG, College Station, TX, USA, 2019).

Results

Demographic characteristics, perceived health status, and frailty indexes

Forty people, 35% female and 65% male, with a mean age of 73.5±5.4 (range 65-86 years), with different health conditions and frailty were evaluated (**Table 1**). Overall, 70% of the cohort had mild frailty, 25.5% had moderate frailty, and 5% had severe frailty. 77.5% of patients rated perceived health as fair to good, while perceived health in the last year was rated worse for 65% of the old.

Among chronic diseases, the highest rates are evident for hypertension, cardiovascular heart failure, and diabetes (**Table 1**).

Comprehensive geriatric assessment of patients

Figure 1 shows the results of the comprehensive geriatric assessment. Physical health, assessed through standardized measures of

Table 1 | Baseline patient and disease characteristics of the 40 analyzed patients and 20 healthy controls enrolled in the study.

Patients characteristics (Tot sample = 40)	N	%	Healthy controls (Tot sample = 20)	%
Gender				
Female	14	35	7	35
Male	26	65	13	65
Age	73.5 ± 5.4 (65-86)		70 ± 4.9 (65-75)	
Perception of current health status				
Poor	2	5	–	–
Fair	11	27.5	–	–
Good	20	50	12	60
Very good	4	10	7	30
Excellent	3	7.5	1	10
Perceived health status in the last year				
Worse	26	65	N/A	N/A
Better	14	35	N/A	N/A
Prevalent Disease at Baseline				
Hypertension	32	82.5		
Diabetes	15	38.5		
COPD	14	35.9	N/A	N/A
CHF	28	71.8		
Angina pectoris	10	25.6		
Stroke	4	10.2		
Cancer	1	2.6		
Rockwood frailty index (RFI)				
Mild	28	70		
Moderate	10	25.5	N/A	N/A
Severe	2	5		

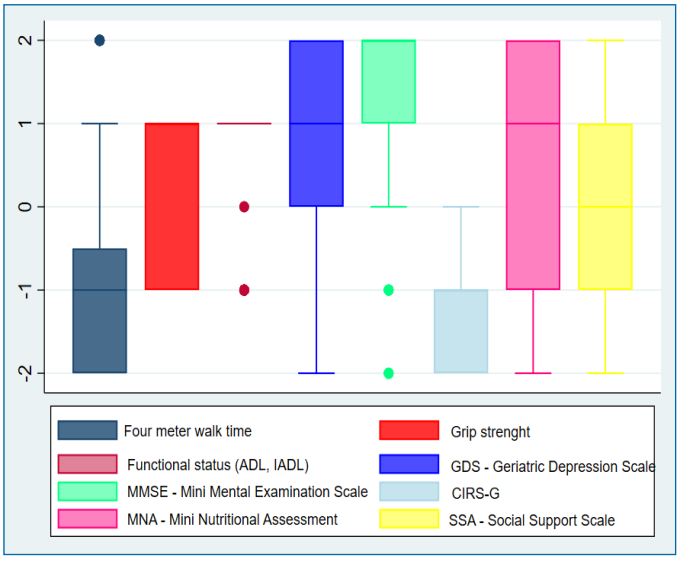

Figure 1 | Comprehensive geriatric assessment of patients. *Note: CIRS-G Cumulative Illness Rating Scale in Geriatrics; ADL Activity of Daily Living; IADL Instrument Activity Daily Living.*

some performance, including time taken to walk 4 meters, showed that 75% of the patients took longer than the established standard; while for grip strength, 2/4 of the older people presented grip ability. Regarding functional status, delineated through activities of daily living (ADL, IADL), it was inferred that 1/4 of the cohort had functional deficits.

Cognitive and mental health was good, 2/3 of the old did not suffer from depression, and only 17.5% had moderate or mild dementia. As for comorbidities, they were particularly evident in each patient. Regarding the screening of nutritional status, 1/3 of the patients had a risk of malnutrition. Finally, the social support need assessment showed that patients were equally divided between those who had high support and those who had low support.

Analysis of BPIFB4 blood levels in frail patients

The bactericidal/permeability-increasing fold containing family B member 4 (BPIFB4), characterized as both a longevity-associated and a host defense protein with a proven immunomodulatory activity (19, 20, 34), displayed prognostic relevance and inversely correlated with disease severity in COVID-19 and atherosclerosis. Furthermore, circulating levels of BPIFB4 are increased in healthy Long Living Individuals LLIs as compared to old controls [20] as a putative biomarker of life-long expectancy.

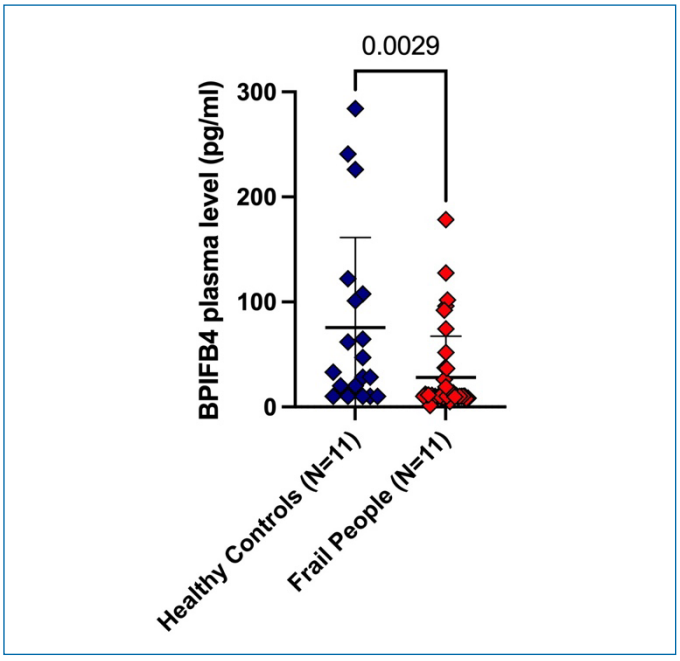

Figure 2 | ELISA quantification of BPIFB4 plasma levels in Healthy control and Frail People (non parametric Mann-Whitney U test).

We examined the plasma BPIFB4 levels in N=40 frail people and N=20 healthy controls, for comparison (**Figure 2**). Notably, BPIFB4 values were significantly lower in frail individuals as compared with old controls pointing to BPIFB4 as a bona fide biomarker inversely related to frailty condition.

Significant predictors of BPIFB4 protein, genotype, and Rockwood frailty index

The Poisson regression model constructed to study the relationship between BPIFB4 protein and patients' comorbidities showed a significant protective effect for hypertension (IRR = 0.32; 95% CI 0.12-0.844; p = 0.02) and cardiovascular disease (IRR = 0.50; 95% CI 0.26-0.97; p = 0.04), while there were no significant relationships with diabetes, COPD and stroke (Model 1 **Table 2**). Furthermore, Poisson regression results revealed that statistically significant predictors of RFI were homo/hetero genotype (IRR = 0.78; 95% CI 0.64-0.95; p = 0.01) (Model 2 **Table 2**).

Characterization of monocytic dynamics in frail elderly

The frequency and phenotype of different immune cell populations are severely affected by the advanced age and its related comorbidities. Our data demonstrate that total circulating monocyte frequency is significantly reduced in N=11 immunophenotyped frail subjects as compared with healthy controls (**Figure 3**).

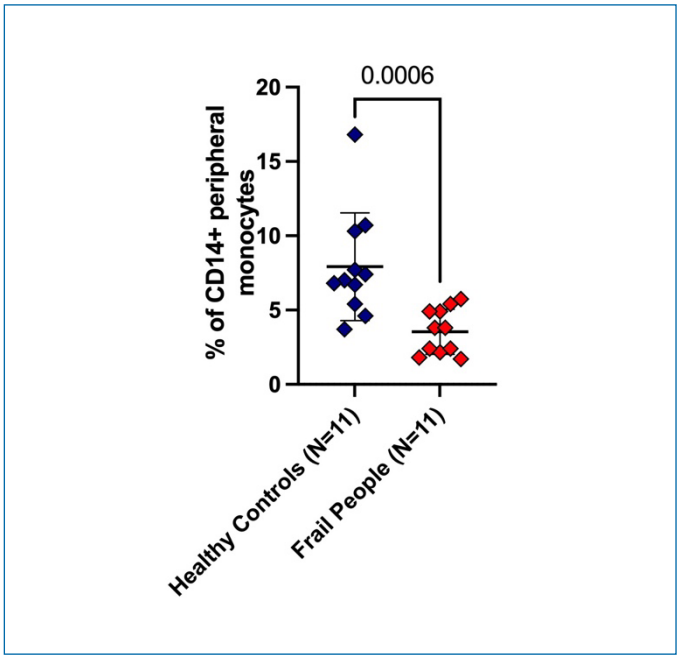

Figure 3 | Frequency of monocytes in Healthy Controls (N=11) and Frail People (N=11) expressed by percentage of total CD14+ positive cells in cytofluorimetric analysis. (non parametric Mann-Whitney U test).

Table 2 | Correlation analysis between BPIFB4 protein and patients' comorbidities.

Model 1. BPIFB4 (sample size = 39) Log likelihood = -50.03, x^2 = 15.42 (6 df), p = 0.017	**IRR**	**95% CI**	**p**
Hypertension	0.32	0.12 - 0.84	0.02
Diabetes	1.03	0.55 - 1.94	0.91
COPD	0.79	0.41 - 1.53	0.49
CHF	0.50	0.26 - 0.97	0.04
Angina pectoris	0.87	0.36 - 2.12	0.77
Stroke	1.11	0.23 - 17.22	0.89
Model 2. RFI (sample size = 39) Log likelihood = -116.11, x^2 = 6.28 (2 df), p = 0.043			
BPIFB4	0.99	0.99 - 1.00	0.50
Genotype			
WT	1	1	1
homo/hetero	0.78	0.64 - 0.95	0.01

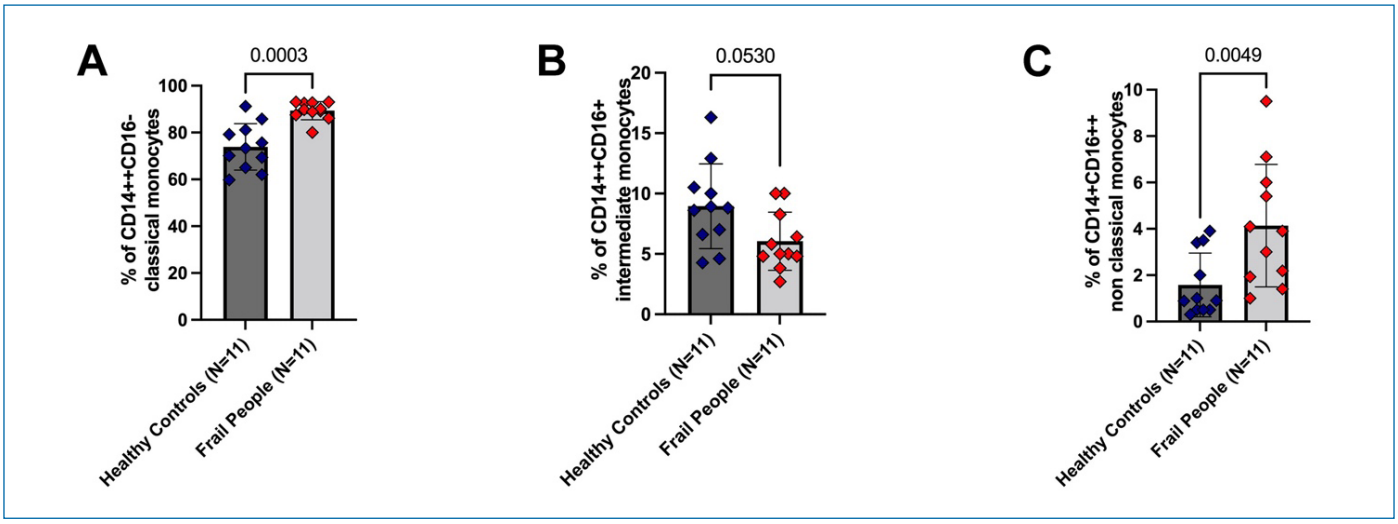

Figure 4 | FACS analysis of monocyte subpopulations in the healthy control group (N=11) and frail people group (N=11). In (A) is expressed the % of CD14++CD16- Classical monocytes, which appears to be increased in Frail People versus Healthy controls). In (B) it is expressed the % of CD14++CD16+ Intermediate monocytes that result decreased in Frail People compared to Healthy controls while in (C) it can be appreciated the difference in % of CD14+CD16++ Non-Classical monocytes subgroup that is increased in Frail People (non parametric Mann-Whitney U test).

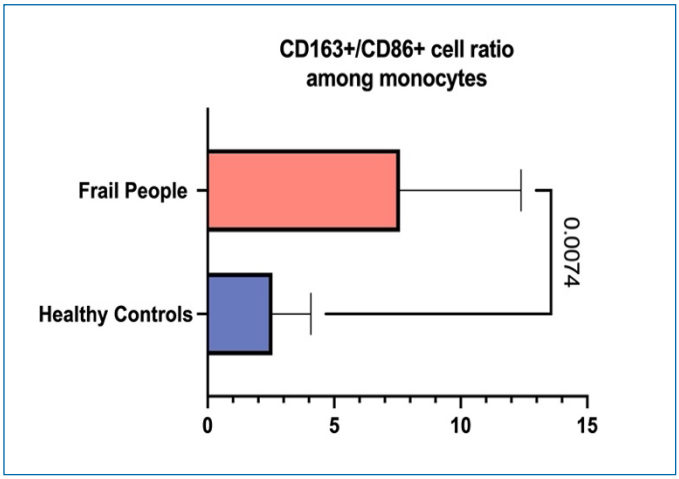

Figure 5 | CD163+/CD86+ ratio in CD14+ cells in Frail People (N=11) and Healthy Controls (N=11) (non parametric Mann-Whitney U test). In Frail People there is a higher ratio, meaning that CD14+CD163+ M2 monocytes are more abundant compared to CD14+CD86+ M1 monocytes.

Looking for differences in subsets of monocytes, CD14++CD16– classical monocytes and non-classical CD14+CD16++ monocytes were significantly increased in frail people compared to old controls, whereas intermediate CD14++CD16+ monocytes were reduced (**Figure 4**).

Moreover, when we profiled total CD14+ monocytes according to their surface levels of

CD86, a classical M1 pro-inflammatory marker, and CD163, a canonical M2 pro-resolutive marker, we described an altered balance of M2/M1 in frailty conditions compared to old volunteers (**Figure 5**).

As levels of CD163 are strongly regulated by mediators in the inflammatory response [35], its enhanced expression on monocytes from frail elderly may be a potential biomarker reflecting efforts by the immune system to resolve immune activation and inflammation (typically referred to as *inflammaging*).

Correlation analysis between BPIFB4 and circulating monocyte subsets.

As in LLIs BPIFB4 levels are associated with a favorable redistribution of monocyte compartment and macrophage polarization *in vitro,* we asked if the reduced BPIFB4 levels in frail people may dictate or contribute to the altered monocyte frequency and phenotype (**Table 3**).

Table 3 | Correlation analysis between BPIFB4 protein and patients' monocyte pool.

	BPIFB4	Non-classical monocytes	Intermediate monocytes	Classical monocytes	CD14 monocytes frequency
BPIFB4	1.000				
Non-classical monocytes	0.159	1.000			
Intermediate monocytes	0.021	0.179	1.000		
Classical monocytes	-0.100	-0.742*	-0.784*	1.000	
CD14 monocytes frequency	0.209	0.199	-0.184	0.019	1.000

Note: * significant correlation with p value ≤0.05.

Correlation analysis between the BPIFB4 protein and all the monocytes' subsets showed no relationship. Statistical significance was shown only between classical monocytes and non-classical monocytes ($p = 0.008$) and between classical monocytes and intermediate monocytes ($p = 0.004$).

Discussion

The main objectives of this study were to assess the health status and frailty index of a group of young old and old/great old, respectively, and to present associative clinical evidence between frailty and both frailty-specific protein biomarkers and immunophenotypically peculiar assets. Frailty is considered a complex and multidimensional syndrome influenced by both clinical features and social and environmental determinants of health. Frail people have a multisystemic reduction in normal physiological functions leading to increased vulnerability to stressful events and a reduced ability to restore homeostasis [4, 36]. The old population is normally characterized by a progressive loss of physiological reserves, but in frailty, this mechanism is even more evident [37]. Frailty is known to be associated with increased adverse sequelae [38, 39], depression [40], reduced self-sufficiency [41], fractures [42], cognitive impairment [43], hospitalization [44, 45], the need for long-term care interventions [41], reduced levels of quality of life [46, 47], and premature death [48, 49]. Therefore, in our study, using Rockwood and Mitnitski's model [33], a frailty index was constructed that provides a holistic view of different dimensions of health to identify this condition early, helping patients to prevent associated adverse outcomes. The older one gets the greater the risk of developing chronic diseases and multimorbidity (presence of two or more chronic diseases) situations [50, 51]. Accordingly, our results showed that comorbidities were particularly evident in each patient. Although the presence of multiple chronic conditions is associated with the development of frailty [52], frailty is not necessarily the result of chronic disease. On the other hand, it is also true that intensive or overtreatment of chronic diseases can increase adverse health outcomes in frail people [52, 53] as clarified by Elliot et al. [54] who claimed that frailty can hinder adherence to both pharmacological and rehabilitative therapies. Several studies have shown that a cardinal manifestation of frailty is loss of physical function [55]. Poor physical function, muscle atrophy, and dyspnea are shared conditions between the phenotypic model of frailty and sarcopenia. The approach to the diagnosis of sarcopenia involves the search for symptoms such as falls, weakness, slowness, self-reported muscle atrophy, or difficulty performing activities of daily living [56, 57]. According to the European Working Group on Sarcopenia in Older People (EWGSOP) (56], the approach to the diagnosis of sarcopenia should be stepwise and begins with the measurement of muscle strength, usually grip strength, following which sarcopenia may be suspected. In our research, the results of standardized measures of some performances, including time taken to walk 4 meters, grip strength, and activities of daily living (ADL, IADL), showed that half of the cohort analyzed had suspected sarcopenia. In agreement with Coelho-Junior et al. [58], the risk is that a physically inactive lifestyle may lead to the progression of both conditions. In support, Landi et al. [59] evaluating this scenario, proposed sarcopenia as the biological substrate of frailty, while Marzetti et al. [60] combined the two conditions into a new clinical entity called physical frailty-and-sarcopenia. On the other hand, the pathophysiology of frailty and sarcopenia may have a multifactorial etiology involving many of the biological features of aging (e.g., genomic and epigenetic instability, loss of proteostasis, mitochondrial shortening, telomere shortening, stem cell depletion, cellular senescence) [61, 62].

In agreement with Solfrizzi et al. [63] who studied cognitive and mental components, they deduced a correlation between physical frailty and cognitive impairment, defined as cognitive frailty. The cognitive and mental health of the patients in our study was good, most of the old people examined did not suffer from depression, and only a small percentage (1/4) had moderate or mild dementia. It is important to remember, however, that cognitive frailty, like physical frailty, can be delayed at least in the early stages and that its presence can lead to an increased risk of events that negatively impact health [64], such as worsening quality of life [65], increased hospitalizations and mortality [66].

It is well known that health is a reflection of several factors in addition to biomedical factors, such as social [67] and environmental factors [68, 69]. Social determinants of health, such as work, social networks, eating habits, and internal and external living environment, can indeed decrease an individual's intrinsic and extrinsic abilities, making him or her frail. The results of our study showed that patients were equally divided between those with high support and those with low support. In agreement with Aliberti et al. [26], the old person needs the support of family and third parties, as well as cultural activities and recognition can have a significant impact in terms of personal well-being. Azzopardi et al. [67] in the context of frailty, point out that social aspects and especially environmental and personal (e.g., relational) factors of an individual are not sufficiently considered by social and health professionals.

Collectively, the results of our study showed that two-thirds of patients had mild frailty, so it was possible to intervene to help patients regain function. As changes in the proteome and the degree of peripheral immune response are also related to the progression into frailty [70], we propose protein biomarkers and immune traits related to the frailty condition and its progression. Indeed, the diagnosis of frailty is usually clinical and based on selected criteria, which are sometimes inconsistent. Therefore, there is an urgent need to identify and validate novel biomarkers.

While most popular circulating markers are those related to the inflammatory response (eg, C-reactive protein [CRP], IL6, and tumor necrosis factor-alpha [TNFα]) or oxidative stress and/or hormones (insulin-like growth factor-1 [IGF1], testosterone) [71] here for the first time the peculiar asset of monocytes and BPIFB4 circulating factor may constitute new disease biomarkers and therapeutic opportunities in the complexity of the frailty condition. BPIFB4 has already been shown to serve as a biomarker of healthy aging [14 , 20, 72] and display prognostic significance in vascular pathology and COVID-19 [73] mainly influencing mono-macrophage skewing. Here BPIFB4 plasma levels are inversely correlated with frailty condition even though no significant correlations were found between BPIFB4 and different monocyte subsets characterizing frail elderly. Noteworthy, we corroborated a decline in monocyte frequency in frail people compared to the non-frail group. The peripheral reduction of monocytes may suggest their robust recruitment in the damaged tissue as also suggested by the higher levels of monocyte chemoattractant protein-1 (MCP-1) characterizing frail vs non frail-group [74]. The functional changes in peripheral monocyte response deserve much attention as they reveal an expected inflammatory arm (CD14++CD16- Classical monocytes *high*) but counterbalanced by a reparative polarized activity (CD14+CD16++ Non-Classical monocytes *high* and CD14+CD163+ monocytes *high*) of monocytes. Indeed, as levels of CD163 are strongly regulated by mediators in the inflammatory response [35], its enhanced expression on monocytes from frail elderly may be a potential biomarker reflecting efforts by the immune system to resolve immune activation and inflammation (typically referred to as *inflammaging*). On

the other hand, the prevalence of CD14+CD16++ Non-Classical monocytes and CD14+CD163+ monocytes failing to be activated may reflect an exhausted state of the frail vs non-frail group. This scenario also emerged from the transcriptome on the single-cell level in human-aged immune cells from frail (n = 5; age, 88.0 ± 5.8 years) individuals compared to healthy old individuals (n = 6; age, 85.8 ± 11.1 years) [70].

Even though not investigated in our report, probably a different monocyte balance in the dynamic course of the frailty (pre-frail/frail) may be useful to dissect the pathophysiological process and its clinical progression. The lack of association with BPIFB4 levels can be in part explained by the sample size, which is an obvious limitation of our study, or probably because high BPIFB4 levels correlate with pro-resolutive M2 response only when resulted protective and useful to blunt inflammatory tone [14, 15, 75, 76], while in this context the CD163+/CD86+ balanced ratio contributed to the impaired nature of frail monocytes. Likewise, monocyte variations in some frail individuals can reflect a plethora of comorbidities-associated states for some of which BPIFB4 levels do not guarantee a proper degree of protection. Indeed, here BPIFB4 levels resulted protective for hypertension and cardiovascular disease, while there were no significant relationships with diabetes, COPD, and stroke (Model 1 **Table 2**). This is consistent with the cardiovascular benefits of carrying the LAV isoform of the BPIFB4 gene associated with healthy aging and a high degree of protection from hypertension, ischemia, and atherosclerosis. Furthermore, the association of the LAV haplotype with lower frailty in elderly subjects and the reduced frailty observed in mice treated with LAV-BPIFB4 gene therapy are in perfect agreement [18]. Here we confirmed a protective incidence relationship between frailty and homo/hetero BPIFB4 genotype (Model 2 **Table 2**). This genetic association may be strengthening the diagnosis of frailty which can take advantage of biomarkers, genetic and proteomic research, and incorporation of sociodemographic variables associated with frailty.

Conclusions

The epidemiological transition has led to a longer life expectancy with an increase in chronic diseases compared to acute diseases [77]. This may predispose to a progressive, whole-organism process of decompensated homeostasis with a substantial contribution from the impaired immune responses.

However, frailty is often reversible [78] in the early stages, before the onset of functional impairment. Therefore, early identification, through protein biomarkers and immunophenotypically at the pre-fragility or mild frailty stage, is important to help patients regain function and prevent adverse outcomes associated with the syndrome [79]. Our findings highlight that BPIFB4 protein has a potential prognostic value for marking the frailty condition deserving much attention in the near future.

Author contributions

E.C. designed and conducted the study, coordinated the research team, and wrote the manuscript. and S.M.A. performed statistical analysis and data interpretation and wrote the manuscript. F.M., V.L., C.B., A.C. and P.D.P., A.M. performed laboratory activities. M.C.C. and C.V. cared for the subjects of the study and evaluation of their health status and reviewed critically the paper. M.C. reviewed critically the paper. A.A.P. performed data interpretation and reviewed the manuscript. A.A.P. supervised the project in its entirety and provided financial support. All authors approved the final version to be published.

Competing interests

All authors declare no financial or competing interests that are directly relevant to the content of this manuscript.

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Declarations

Ethics approval and consent to participate.

The study was approved by the Campania Sud ethical committee and conducted in accordance with the ethical principles deriving from the Declaration of Helsinki (N.78 _r.p.s.o. del 04/07/2018." *Studio per la valutazione della correlazione tra le isoforme del gene BPIFB4 e il rischio di fragilità umana*"). All participants signed an informed consent for the management of personal anamnestic data and blood samples.

Consent for publication

Not applicable

Availability of data and materials

Data, materials, and protocols will be available on request by emails to the corresponding authors due to privacy/ethical restrictions.

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Effect of anti-PCSK9 drugs on the association of PCSK9 to LDL

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Keywords
Atherosclerotic cardiovascular disease; lipid-lowering therapy; monoclonal antibodies; small interfering RNA; LDL-cholesterol; PCSK9

ABSTRACT

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a protein known to interact with the LDL receptor, thereby promoting its degradation and blunting the uptake of LDL from the circulation. In this context, anti-PCSK9 monoclonal antibodies (mAbs) and siRNAs have been approved for the treatment of hypercholesterolaemia. Previous studies have shown that a significant proportion of circulating PCSK9 is associated with LDL. The aim of our research is to investigate the effect of mAbs and siRNA on the association of PCSK9 protein with LDL. In this study, 10 statin-intolerant patients received treatment with anti-PCSK9 mAbs or siRNA, in addition to therapy with a low-dose statin and ezetimibe. Their plasma samples were analysed before and after 1, 3, and 6/9 months of treatment. The results showed that both the monoclonal antibodies and inclisiran reduced LDL-C levels by 50% to 60%. LDL-C levels decreased from 92±28 mg/dL to 44±26 mg/dL after siRNA treatment and reached 97±9, 27±10, 32±14, and 23±10 mg/dL after mAbs therapy. The circulating PCSK9 level decreased by 70% after the first siRNA injection, while it increased 10-fold after mAbs therapy. Regardless of treatment, the percentage of PCSK9 bound to LDL did not vary from baseline and remained constant during the treatment period. Whether this is of physiological relevance remains to be addressed.

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Background

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a protein that plays a crucial role in the regulation of plasma low-density lipoprotein cholesterol (LDL-C) levels [1]. It is primarily produced in the liver [2]. The PCSK9 protein binds to LDL receptors (LDLR) on the cell surface, leading to internalisation of the PCSK9-LDLR complex and targeting LDLR for lysosomal degradation, thus reducing the cell's ability to remove LDL-C from the bloodstream [3]. This leads to higher levels of LDL-C in the circulation and contributes to the development of atherosclerosis and cardiovascular disease [4, 5]. Lowering LDL-C levels is a key focus in the prevention and treatment of cardiovascular disease [6]. Lifestyle changes, including a healthy diet, regular exercise and avoiding tobacco use, can help to lower LDL-C levels. In addition, therapies such as statins are commonly prescribed to reduce LDL-C and lower the risk of cardiovascular events (7). The central role of PCSK9 in modulating LDL-C levels has driven the development of several approaches to inhibit this protein [8].

Evolocumab and alirocumab are fully humanised monoclonal antibodies (mAbs) that target circulating PCSK9 and have been investigated in several clinical trials, including the two outcome trials FOURIER and ODYSSEY OUTCOMES [9, 10]. Inclisiran is a small interfering RNA (siRNA) that specifically inhibits the hepatic synthesis of PCSK9 [11]. Both treatments lead to increased expression of LDLR in the liver, enhancing the removal of LDL-C from the blood [12, 13]. PCSK9 inhibitors are used as a therapeutic option to lower LDL-C levels in individuals with hypercholesterolaemia and a high risk of cardiovascular events [14]. Several studies are investigating the possible effects of PCSK9 inhibition beyond LDL-C levels [15]. In this study, we investigated the effect of mAbs and siRNA on the association of PCSK9 protein with LDL.

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Methods

Subjects and samples

For this study, we have selected 10 patients who were treated with a statin plus ezetimibe and experienced statin intolerance. Of these, 9 were classified as medium risk and 1 was classified as high risk for intolerance. Subsequently, the patients were divided into three groups, of which 5 subjects received a monoclonal antibody anti-PCSK9, 4 others received siRNA treatment, in addition to therapy with a low-dose statin and ezetimibe, and 1 patient replaced the statin with siRNA. Plasma samples were collected from each patient at baseline and after 1, 3 and 6/9 months of therapy. All blood samples were subjected to low-speed centrifugation (3000 rpm, 12 min) to obtain plasma to which the protease inhibitor (Halt™ Protease Inhibitor Cocktail, Thermo Fisher, Italy) was added. Each participant gave written informed consent for the study. This study was conducted in accordance with the Declaration of Helsinki.

Iodixanol density gradient ultracentrifugation

Lipoproteins were isolated from plasma using three layer-density of OptiPrep™ solvent as previously described [16].

Statistics

Statistical analyses were performed with GraphPad Prism 9.0. Data were analysed using the unpaired t-test and the one-way ANOVA test.

Results

The study cohort consisted of n=10 subjects, with 9 subjects being treated with alirocumab, evolocumab or inclisiran in addition to their existing therapy, while the remaining 1 subject was treated only with inclisiran. Plasma samples were collected before the therapies and at 1, 3, and 6/9 months (T1, T2 and T3, respectively) after the first injection. All samples were analysed for PCSK9 and lipoprotein distribution. Baseline levels of total cholesterol, LDL-C, HDL-C and TG of the subjects are reported in **Table 1**.

In patients receiving monoclonal antibody therapy, approximately 12% of the total PCSK9 was bound to LDL at baseline (T0). After one month of treatment with an anti-PCSK9 monoclonal antibody (T1), a 70% reduction in LDL-C levels (from 97±9 mg/dL to 27±10 mg/dL) was observed. Conversely, plasma PCSK9 levels increased 10-fold, from 562±156 ng/mL to 4,925±1400 ng/mL. Interestingly, the percentage of circulating PCSK9 bound to LDL remained unchanged throughout the therapy duration, despite the marked changes in LDL-C and PCSK9 levels. The data observed at T1 was confirmed at T2 and T3, where LDL-C levels were 27±10, 32±14 and 23±10 mg/dL, respectively; while PCSK9 concentration reached 4,925±1400, 5,360±755 and 5,843±920 ng/mL, respectively (**Figure 1**). The percentage of PCSK9 bound to LDL remained consistent with the level observed at T1, reaching values of 15% at both T2 and T3 (**Figure 1**).

Compared to anti-PCSK9 mAb therapy, patients treated with inclisiran showed a decrease in both LDL-C and PCSK9 plasma levels (from 92±28 mg/dL to 44±26 mg/dL and from 691±187 ng/mL to 212±63 ng/mL, respectively) one month after the first inclisiran injection (T1) (n=5; **Figure 2**). The calculated percentage of association between PCSK9 and LDL at T1 was comparable to that at T0. After three and nine months of inclisiran treatment (T2 and T3, respectively), the percentage of PCSK9 bound to LDL remained unchanged, with no further variations in LDL-C and PCSK9 levels.

Discussion

Several studies have shown that PCSK9 is associated with LDL in plasma [5, 17, 18]. To date, the nature and the physiologic role of the PCSK9 associated with LDL and other lipoproteins, such as Lp(a) [18], remains unclear. Some observations suggest that LDL-bound PCSK9 is the more functional form of this protein, as the interaction with an LDL particle protects PCSK9 from cleavage by furin and the protein remains bound to the particle in its active form [19]. On the other hand, in vitro studies showed that the addition of recombinant PCSK9 to LDL reduces the affinity of PCSK9 for LDLR, suggesting that LDL-bound PCSK9 is a less functional form of plasma PCSK9 [5, 20].

PCSK9 inhibitors (monoclonal antibodies and siRNA) have been

Table 1 | Report of total cholesterol (TC), HDL-C, LDL-C and TG in plasma patients before treatments.

Subjects	TC (mg/dL)	HDL-C (mg/dL)	LDL-C (mg/dL)	TG (mg/dL)
Patients with mAbs (N=5)	165±15	40±7	97±9	141±48
Patients with siRNA (N=5)	163±35	46±11	92±28	124±44

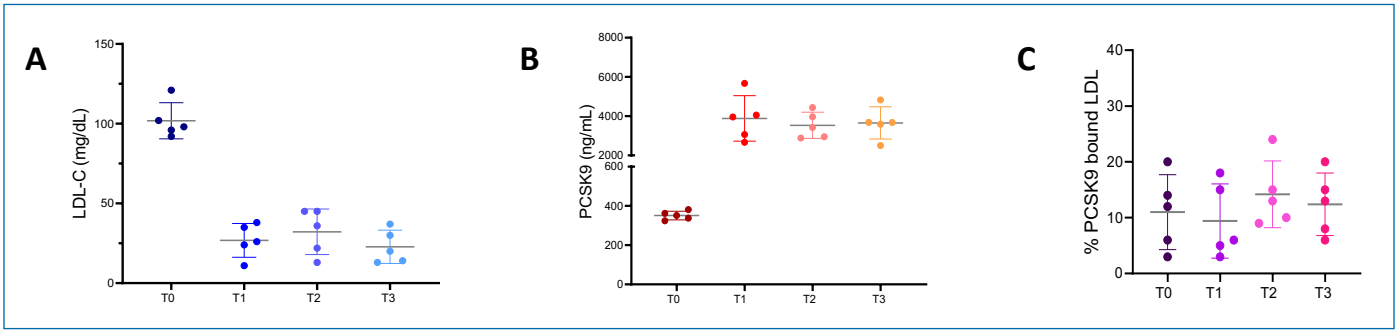

Figure 1 | Comparison among (A) LDL-C and (B) PCSK9 levels before (T0) and after (T1, T2, T3) anti-PCSK9 mAbs administration (n=5 for each group; p<0.005). (C) The percentage of circulating PCSK9 bound to LDL during the therapy (values are means±standard errors).

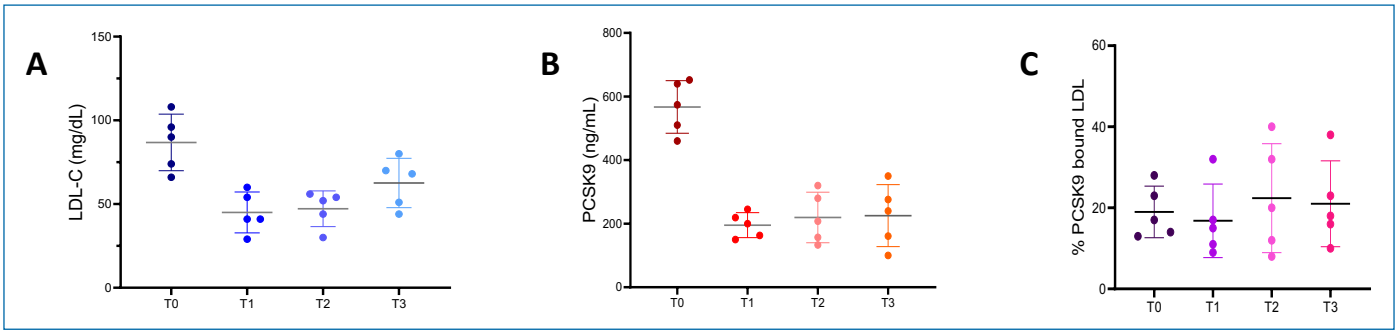

Figure 2 | Comparison among (A) LDL-C and (B) PCSK9 levels before (T0) and after (T1, T2, T3) inclisiran administration (n=5 for each group; p<0.005). (C) The percentage of circulating PCSK9 bound to LDL during the therapy (values are means±standard errors).

developed and approved for the treatment of hypercholesterolemia and are used in patients who need substantial reductions in their LDL-C levels to lower cardiovascular risk. In the present study, we aimed to investigate the association between PCSK9 and LDL particles in patients treated with two different anti-PCSK9 approaches.

According to the results from clinical trials [9, 10], we have demonstrated that both monoclonal antibodies and inclisiran reduce LDL-C levels by 50 to 60%. On the other hand, we clearly show that plasma PCSK9 levels differ significantly between the two therapies. In fact, mAbs increase plasma PCSK9 levels by up to 10-fold, which is due to the large amount of PCSK9 bound to the antibodies, whereas inclisiran reduces plasma PCSK9 protein levels by about 70%.

We found that while LDL-C levels were significantly reduced with both treatments, circulating PCSK9 levels behaved as expected (increased with the mAbs and decreased with inclisiran), while the percentage of PCSK9 bound to LDL did not vary from baseline and remained constant during the treatment period. Nevertheless, a relatively large amount of PCSK9 remains bound to LDL, especially after treatment with monoclonal antibodies. Whether this is of physiological relevance remains to be addressed. The nature of this association is currently being analysed to determine its status and whether the bound PCSK9 is structurally different from the unbound form. Preliminary observations from our ongoing study suggest that it is not the monoclonal antibody-bound form of PCSK9 that binds to lipoproteins, but the free form. Furthermore, we found that the lipoprotein-bound PCSK9 is not in the cleaved form (unpublished data). The mature form of PCSK9 (62 kDa) is believed to be more effective than the furin-cleaved form (55 kDa) in degrading the LDLR [21]; through western blotting analysis, we observed that the mature specifically associates with the LDL subfraction. This observation supports a previous finding that the PCSK9 species associated with LDL is primarily the intact heterodimer form, whereas the free PCSK9 (non-LDL-bound) is primarily in the furin-cleaved conformation [22]. It is tempting to speculate that the PCSK9-LDL-bound form can explain the biological function of LDLR, but further investigations are needed. It is worth noting that all subjects had previously been treated with statin therapy; therefore, the concentration of PCSK9 protein was elevated. In addition, the distribution of PCSK9 and its binding to lipoproteins may be influenced by prior therapy. Further studies on subjects before therapy are underway.

Our previous studies have investigated the interaction between PCSK9 and LDL [16], showing that high salt concentrations disrupt this binding, suggesting a non-covalent interaction. Moreover, our studies have explored the type of lipoproteins bound to PCSK9, uncovering a preference for a specific LDL subfraction. This particular subfraction, resembling remnant lipoproteins, exhibits increased buoyancy compared to mature LDL, characterised by enriched levels of apoE and apoCs, alongside elevated triglyceride content (unpublished data). This observation led us to hypothesise that PCSK9 may enter the bloodstream in association with VLDL, which are then metabolized to IDL, which could explain our finding. This possibility is currently being investigated in a specifically designed study. Overall, the association of lipoproteins with PCSK9 is thought to influence the PCSK9 activity towards the LDL receptor [23], which calls for further investigation into the potential biological significance of this LDL subfraction.

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Authorship and author contribution statement

SM wrote the MS and critically revised the data; VP and FME performed the laboratory work and collected all data; AP critically revised the data and wrote the MS; LG critically revised the data and read the MS; ALC designed the study, critically revised the data and wrote the MS.

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Conflict of interest

AL Catapano reports consulting fees/lecturing fees from Akcea, Amgen, Amryt, Sanofi, Esperion, Kowa, Novartis, Ionis Pharmaceuticals, Mylan, Menarini, Merck, Recordati, Regeneron Daiichi Sankyo, Genzyme, Aegerion, and Sandoz. The remaining authors have nothing to disclose.

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

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Keywords
miRNAs; atherosclerosis; theranostics, LPS; smooth muscle cells; inflammasome

ABSTRACT

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

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Introduction

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

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

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

The stages of atherosclerosis

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

miRNAs in atheroma development

LDL binding to the intima and oxidation

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

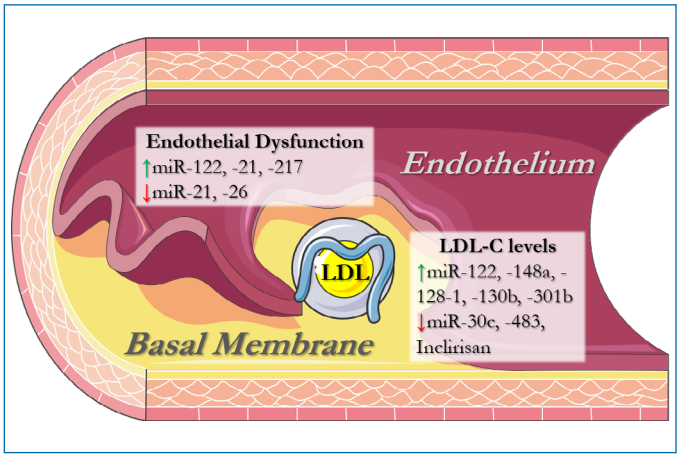

Figure 1 | microRNAs in lipoprotein adhesion to the basal membrane. *Schematic overview of the miRNAs involved in the regulation of endothelial dysfunction, LDL synthesis and uptake. Green arrow: endothelial dysfunction/LDL-C enhancement; red arrows: endothelial dysfunction/LDL-C modulation. Created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

miRNAs regulating LDL synthesis

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

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

miRNAs modulating LDL and vLDL uptake

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

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

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

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

miRNAs regulating endothelial dysfunction in atherosclerosis

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

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

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

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

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

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

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

miRNAs involved in cholesterol processing by macrophages

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

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

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

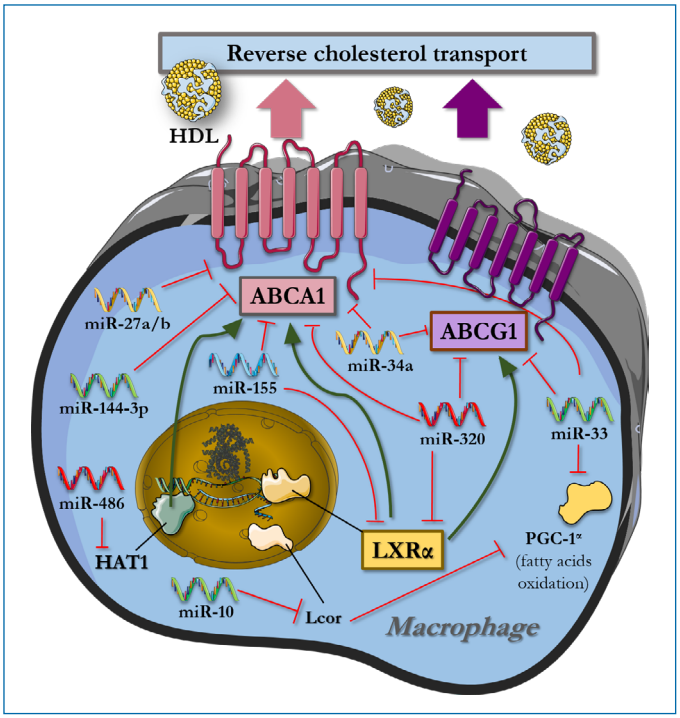

Figure 2 | Role of microRNAs in reverse cholesterol transport regulation within the macrophage. *Schematic representation of the miRNAs known to affect HDL nucleation and foam cell formation within plaque resident macrophages. Green arrows: positive transcriptional regulation; red connectors: negative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

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

The role of miRNAs in neointima expansion

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

Macrophages-T cells crosstalk and inflammation

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

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

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

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

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

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

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

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

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

miRNAs in SMC recruitment and differentiation

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

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

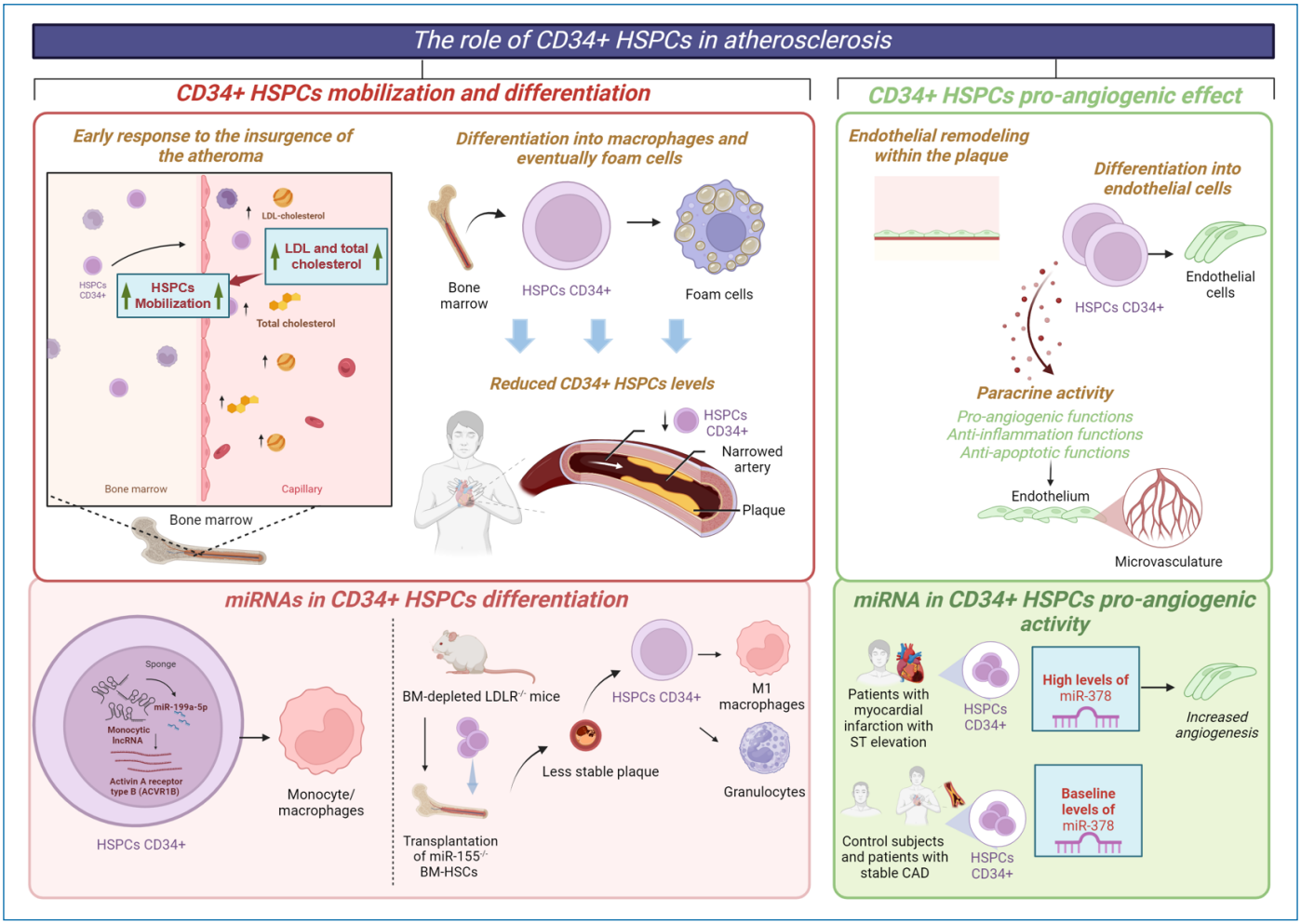

Figure 3 | miRNAs and the role of CD34+ HSPCs in atherosclerosis. *The role of miRNAs in the mobilization, differentiation (left panel), and function (right panel) of CD34+ HSPCs in the development of atherosclerosis. HSPCs: hematopoietic stem and progenitor cells; LDL: low-density lipoprotein; BM-HSCs: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

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

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

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

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

The role of miRNAs in determining plaque stability

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

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

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

miRNAs as biomarkers for cardiovascular risk in atherosclerotic patients

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

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

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

Conclusion and future perspectives for miRNA-based theranostics in atherosclerosis

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

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

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

Conflict of interests

The authors declare no conflict of interest exists.

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Author contribution

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

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Atherosclerosis and cholesterol: What we should know

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ABSTRACT

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.

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

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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.

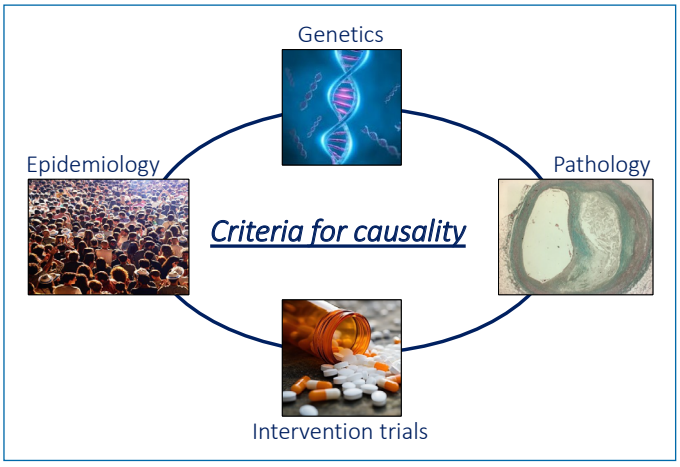

Figure 1 | LDL and atherosclerosis: Criteria for causality.

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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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Atherosclerotic cardiovascular disease and measurement of lipoprotein(a) levels in Italy

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ABSTRACT

Background: Lipoprotein(a) [Lp(a)] is a relatively new but underutilized biomarker in the context of atherosclerotic cardiovascular disease (ASCVD).
Objectives: To explore the clinical implementation of Lp(a) measurement and current practices in hospital and specialised settings in Italy.
Methods: An anonymous online questionnaire was distributed to Italian physicians to examine the habits of Italian clinicians regarding Lp(a) measurement. The survey covered three topics: 1) information on the clinical setting of the physicians, 2) questions for physicians who reported not measuring Lp(a), to understand the reasons for not requesting the test, and 3) questions for physicians who measure Lp(a), to investigate its use in patient management.
Results: A total of 978 responses were received. Overall, 63.1% of physicians reported working in a hospital; 12.2% reported being a territorial specialist. Regular Lp(a) measurement was reported by 32.1% of clinicians. Among those who do not measure Lp(a), the main barriers to implementation include high cost and limited availability of the test. The threshold value for defining elevated Lp(a) levels varies significantly among professionals, with 36.7% considering levels above 30 mg/dL to be elevated and 32.7% considering levels above 50 mg/dL to be elevated. Clinical management of patients with elevated Lp(a) primarily includes intensification of lipid-lowering therapy (69.2%), management of cardiovascular risk factors (48.7%), and lifestyle recommendations (37.4%).
Conclusions: The survey highlights the heterogeneity in the approach to managing elevated Lp(a) levels among Italian clinicians, underscoring the importance of clear guidelines and greater accessibility to the test to optimize cardiovascular risk stratification and improve clinical outcomes.

Keywords
Lipoprotein(a); atherosclerotic cardiovascular disease; clinicians; cardiovascular risk management; patient communication simulation

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Introduction

Atherosclerotic cardiovascular disease (ASCVD) represents one of the main global health challenges within the landscape of chronic diseases. Characterized by the formation and progression of atherosclerotic plaques in the arteries, ASCVD is a multifactorial condition mostly involving the deposition of lipids and inflammatory cells within the arterial wall [1]. This pathological process can impede blood flow, compromise arterial distensibility, and, in severe cases, lead to complications such as myocardial infarction, stroke, and other cardiovascular conditions.

Understanding the risk factors, prevention methods, and effective management of ASCVD is crucial for promoting cardiovascular health and reducing related morbidity and mortality.

The role of lipoprotein(a) [Lp(a)] in cardiovascular risk assessment has been and continues to be a topic of debate [2]. Lp(a) is a particle similar to low-density lipoprotein (LDL), differing only by the presence of a glycoprotein called apo(a) (which has a high homology with plasminogen) covalently linked to apoB [3]. Unlike other lipoproteins that have a clear biological function as lipid transport molecules in plasma, the function of Lp(a), after more than 50 years of research, remains practically unknown [4].

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On the other hand, the role of Lp(a) as a cardiovascular risk factor is well established. Numerous scientific studies indicate that elevated levels of Lp(a) are associated with an increased risk of adverse cardiovascular events, including stroke and myocardial infarction [5]. Therefore, measuring Lp(a) in clinical practice is becoming increasingly important for cardiovascular risk assessment, enabling targeted and personalized preventive interventions.

Plasma levels of Lp(a) in the general population vary widely, ranging from very low, almost undetectable levels (<0.2 mg/dL) to very high levels (>200 mg/dL). This variability primarily depends on the isoforms of apo(a), which differ in the size of the molecule determined by the number of repeat structures, the kringles, particularly the number of kringle 4 type 2 repeats [6].

The polymorphism of the gene encoding apo(a) size is the major predictor of plasma Lp(a) concentration and accounts for 40-70% of the variation in Lp(a) plasma levels [7]. The strong genetic influence on Lp(a) levels results in its asymmetric distribution in the population, unlike other analytes, complicating the role of Lp(a) in cardiovascular risk assessment.

Some peculiar characteristics of Lp(a), such as its significant heterogeneity, the absence of a clear physiological function, and the current difficulty in measuring it reliably and in a standardised manner, limit its use in routine clinical practice [8].

Therefore, the SISA Foundation has promoted a survey on the themes related to 'Atherosclerotic Cardiovascular Disease and the Importance of Measuring Lp(a) Levels,' targeting physicians operating in Italy.

The project's objective is to gather useful information to improve diagnostic and prognostic approaches in the near future. This includes understanding how often Lp(a) is evaluated in daily practice, the criteria used to decide whether or not to test for Lp(a), and the practical factors considered in the decision to perform the test.

This effort aims to collect essential information to determine the resource, process, infrastructure, and funding requirements needed to make Lp(a) evaluation a common practice.

Methods

The questionnaire, consisting of 23 questions, primarily included multiple-choice responses and was structured into three main areas of investigation:

- Information regarding the background and clinical setting of the physicians;
- Specific questions directed at physicians who reported not regularly measuring Lp(a) in clinical practice, to understand the motivations or practical barriers preventing them from requesting the test;
- Specific questions directed at physicians who reported regularly measuring Lp(a) in clinical practice, to delve into their approach in managing patients at high cardiovascular risk.

Participation in the questionnaire was voluntary. Consent was implied with the return of the completed questionnaire.

All responses were managed anonymously. The results were summarized using frequencies and percentages. Statistical analyses were performed using the Statistical Analysis System software (version 9.4; SAS Institute, Cary, NC, USA).

Results

A total of 978 clinicians from various settings responded to the survey: the majority reported working primarily in territorial or university hospitals (24.4% and 22.3%, respectively), and 39.2% and 24.7% of them were cardiologists or internal medicine physicians (**Table 1**). The geographical origin of the clinicians participating in the survey is illustrated in **Figure 1**.

Based on the personal experiences of the participants, the

Table 1 | Clinical settings and specializations of the physicians who participated in the survey.

Number of Clinicians	**978**
Practice Setting, %:	
University Hospital	22.29%
Institute for Treatment and Research (IRCCS)	6.44%
Territorial Hospital	24.44%
Territorial Specialist	12.17%
Specialized Lipidology Center	2.97%
Specialized Diabetology Center	2.56%
Specialized Cardiology Center	5.42%
Other	23.72%
Specialization, %:	
Cardiology (Clinical Cardiology, Hemodynamics, Electrophysiology, Interventional Cardiology)	39.16%
Diabetology	4.19%
Endocrinology	6.24%
Lipidology	1.53%
Internal Medicine	24.74%
Other	24.13%

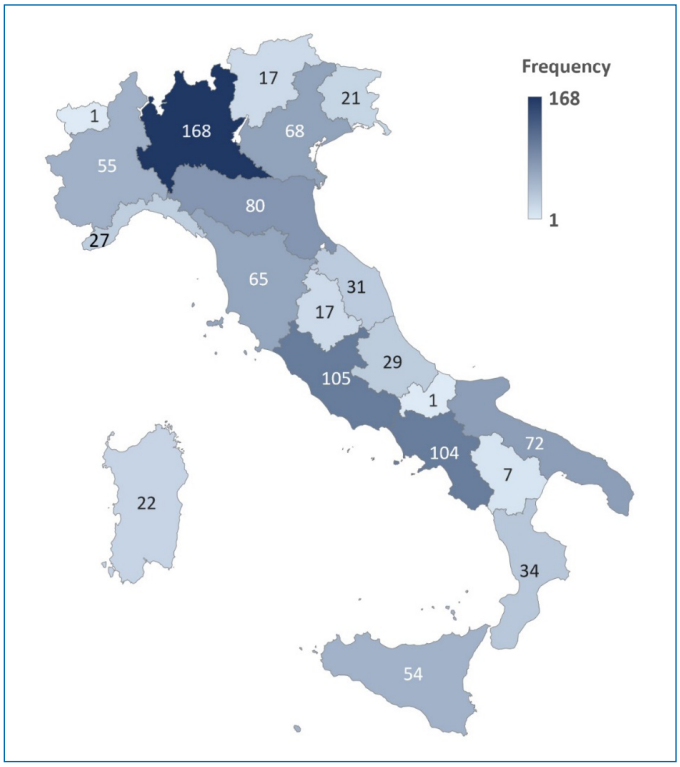

Figure 1 | Geographical distribution of the clinicians participating in the survey.

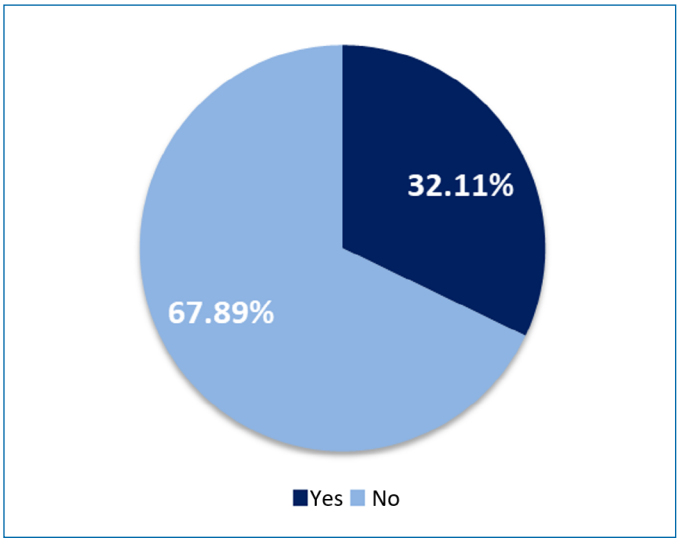

Figure 2 | Proportion of clinicians who regularly measure Lp(a) in clinical practice.

proportion of clinicians who regularly measure Lp(a) in clinical practice was found to be 32.1% (**Figure 2**).

Among the clinicians who do not measure Lp(a) in their clinical practice (N = 664), the most common reasons for not requesting the Lp(a) test were lack of reimbursement by the National Health Service, lack of treatment options for elevated Lp(a) levels, unavailability of the Lp(a) test, and the high cost of the laboratory test (**Figure 3A**). Among these physicians, the availability of specific therapies for the treatment of elevated Lp(a) levels, the availability of the measurement test, and specific recommendations in the guidelines would encourage the inclusion of Lp(a) measurement in their clinical practice (**Figure 3B**).

Among those who regularly measure Lp(a) (N = 314), a high percentage reported requesting the measurement for better cardiovascular risk stratification (**Figure 4**).

The survey shows significant variability among clinicians in the threshold considered for defining high levels of Lp(a) in relation to ASCVD risk (**Figure 5**). Most clinicians (36.7%) consider a value above 30 mg/dL (63 nmol/L) as high, while 32.7% consider a value above 50 mg/dL (105 nmol/L) as high. Only a minority consider higher values as thresholds, with 17.64% indicating 70 mg/dL (150 nmol/L), 8.94% indicating 100 mg/dL (215 nmol/L), and 4.10% considering a value of Lp(a) above 150 mg/dL (325 nmol/L) as high. **Figure 6**, on the other hand, demonstrates that the majority of clinicians adjust their therapeutic approach when Lp(a) levels exceed 50 mg/dL.

Among the categories of patients that clinicians consider eligible for Lp(a) measurement, the majority (67.7%) indicated patients with recurrent cardiovascular events despite LDL cholesterol reduction is important, followed by 64.2% who assess Lp(a) levels in patients with a family history of early cardiovascular events. A significant number of clinicians (48.6%) consider measuring Lp(a) levels important in patients with a history of myocardial infarction, and 45.2% in those with familial hypercholesterolemia. Overall, only 44.2% of clinicians find it useful to measure Lp(a) at least once in the life of every adult patient.

Faced with elevated Lp(a) levels in patients with ASCVD, most clinicians (69.2%) stated that they intensify dyslipidaemia treatment, while 48.7% actively manage other risk factors. Lifestyle

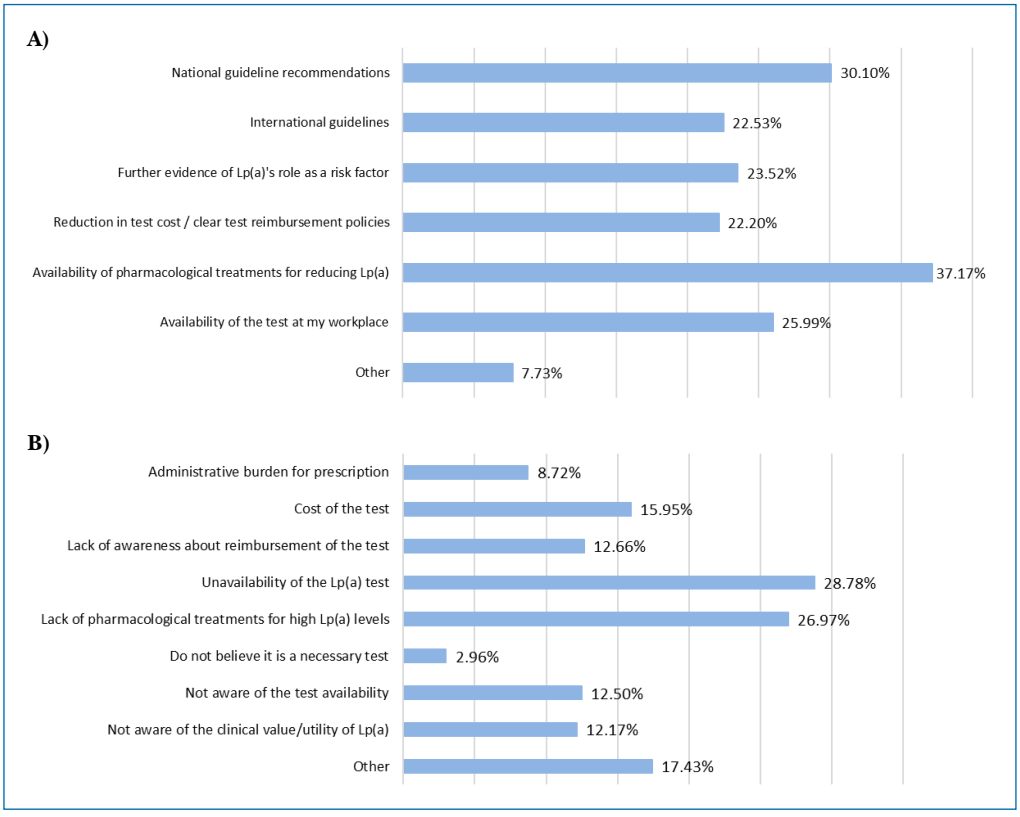

Figure 3 | Among clinicians who do not measure Lp(a) in their clinical practice, panel A shows the reasons for not requesting the Lp(a) test, while panel B shows what clinicians think would be necessary to start testing Lp(a) in clinical practice. Clinicians were allowed to provide multiple answers.

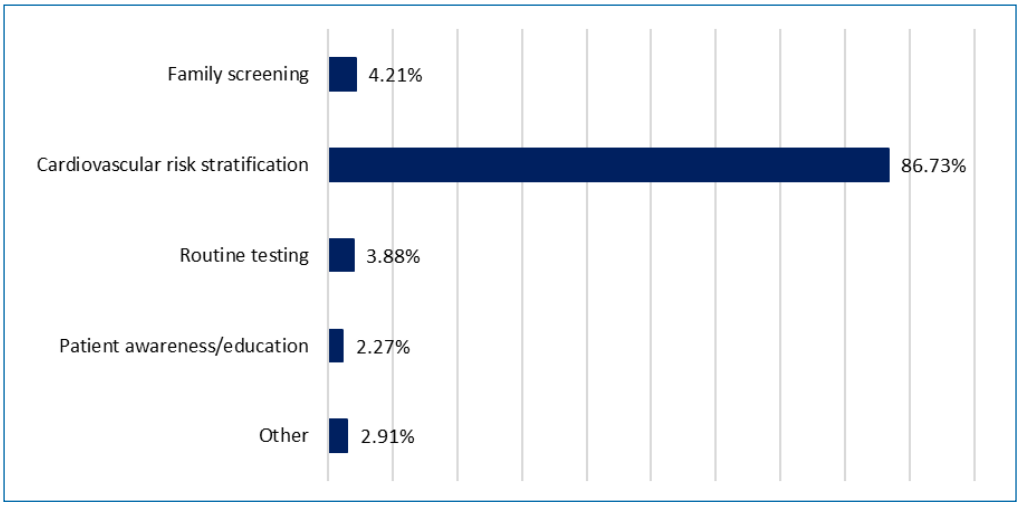

Figure 4 | Additional information for clinicians who regularly measure Lp(a) in their clinical practice. Reasons for requesting an Lp(a) test.

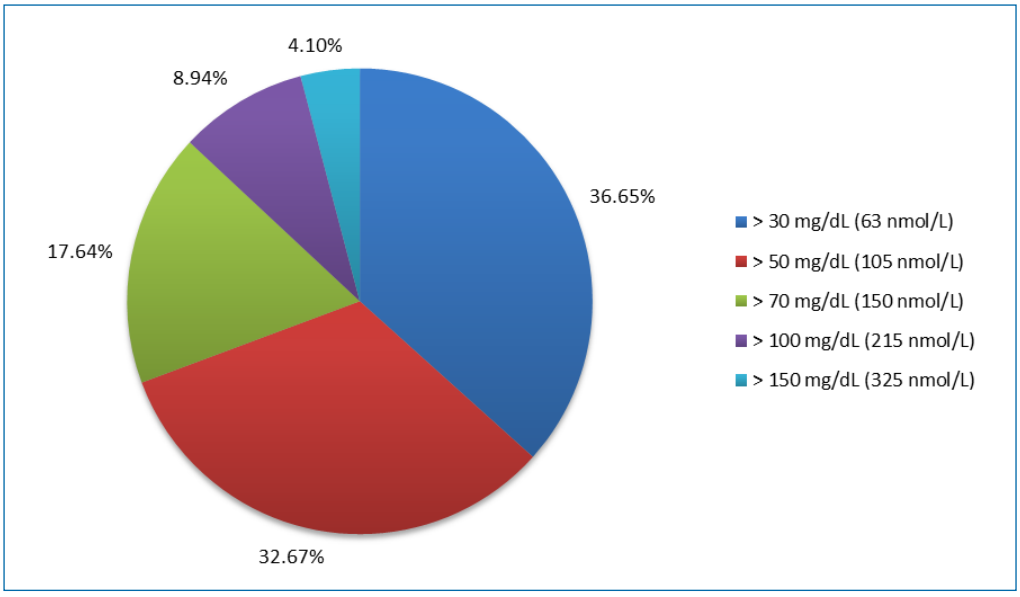

Figure 5 | Proportions of clinicians considering different levels of Lp(a) as high in relation to atherosclerotic cardiovascular disease.

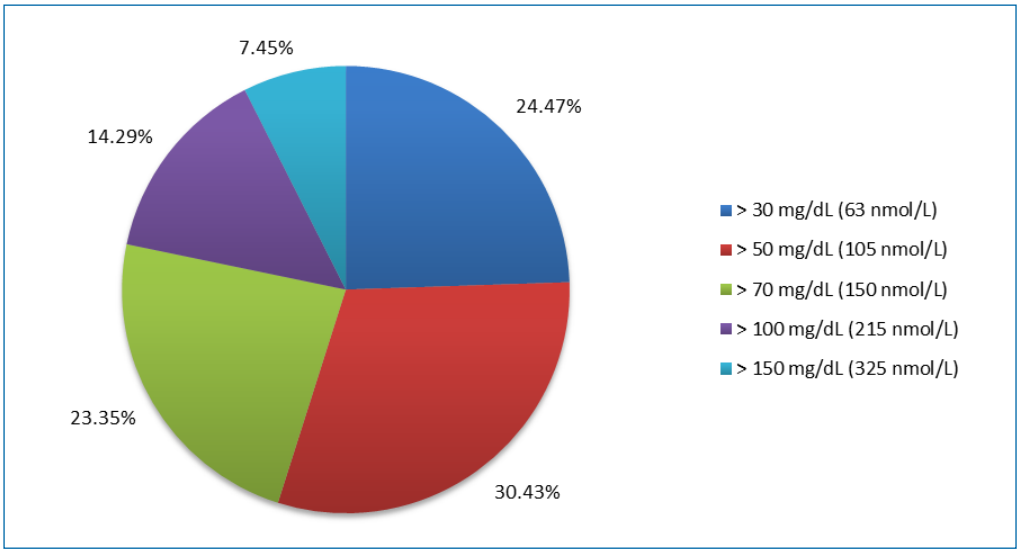

Figure 6 | Proportions of clinicians modifying therapeutic approach based on different levels of Lp(a) in atherosclerotic cardiovascular disease.

recommendations are provided by 37.4% of clinicians. Approximately 20.3% of clinicians refer patients to a colleague specializing in lipid management, and a minority (4.8%) take no action.

Finally, the majority of clinicians (76.4%) reported discussing the Lp(a) test results with the patient, explaining the clinical implications of elevated Lp(a) levels; of these, about 61% also recommend Lp(a) testing for family members. Among clinicians who choose not to discuss these results with patients, one of the most cited reasons is the lack of specific treatment to reduce elevated Lp(a) levels. This concern was highlighted by 40.4% of clinicians, underscoring a significant challenge in managing this biomarker.

Discussion

This survey depicts a detailed overview of clinicians' practices and opinions regarding Lp(a) in the management of ASCVD. The survey involved a broad sample of clinicians from various centres, predominantly those working in territorial and university hospitals. The most common specialization among the participants is cardiology, followed by internal medicine. This reflects a diverse representation of professionals managing patients with cardiovascular diseases, contributing to a comprehensive view of clinical experiences and practices.

One of the key aspects highlighted by the study is the variety of approaches in the measurement and management of Lp(a) among clinicians. While 32.1% of the participants indicated that they regularly measure Lp(a), a significant percentage cited obstacles such as the high cost of tests and the lack of reimbursement as reasons for not regularly performing this test. This evidence confirmed the results of a similar survey on European lipid clinics (12), and underscores the need to improve the accessibility and availability of the Lp(a) test in various clinical settings, especially considering the potential impact of elevated Lp(a) levels on the development of cardiovascular diseases.

Additionally, the variability in thresholds used to define elevated Lp(a) levels among clinicians reflects the lack of clear consensus in clinical guidelines (13). Most of the physicians who participated in this survey tend to consider lower levels (above 30 mg/dL or 50 mg/dL) as indicative of high risk, reflecting greater caution in identifying patients at risk of atherosclerotic cardiovascular disease. This may suggest a growing awareness of the importance of monitoring relatively low levels of Lp(a) as part of cardiovascular risk management and presents an opportunity to develop standardized criteria that can guide a more uniform and evidence-based management of patients with elevated Lp(a) levels.

The survey results on clinicians' management of elevated Lp(a) levels are particularly interesting. These data indicate that the prevailing strategy among clinicians to manage elevated Lp(a) levels involves intensifying lipid-lowering therapy and overall management of cardiovascular risk factors. Lifestyle recommendations are also considered an important component of management. However, a significant portion of clinicians feels the need to consult experts, suggesting that there may be a need for further knowledge or specialist support in this area. The reduced percentage of clinicians who do not take any action suggests a widespread awareness of the importance of addressing elevated Lp(a) levels in the management of cardiovascular diseases (14).

In contemporary medical practice, effective communication of Lp(a) test results plays a crucial role in providing personalized and rational care (15). However, it is interesting to note that some clinicians report not discussing these results with patients primarily due to the lack of a specific treatment to reduce elevated Lp(a) levels. These clinicians likely find it challenging to inform a patient about a risk factor without being able to provide a way to counteract it. This evidence underscores the critical need for medical staff education and updates regarding the currently available alternatives to counteract the increased cardiovascular risk associated with elevated Lp(a) levels. Intensifying the control of other known risk factors is currently the only strategy, and promoting this approach must become an urgent priority for scientific societies to produce and disseminate shared guidelines.

Going forward, integrating Lp(a) measurement into clinical practice is crucial for improving cardiovascular risk stratification and optimizing preventive therapies (16). This study highlights the importance of an integrated and multidisciplinary approach in the management of Lp(a), emphasizing the need for innovations in diagnostic and therapeutic practices to improve the clinical outcomes of patients with cardiovascular diseases. Continuous evolution in research and clinical practice will be essential to effectively address this critical component of cardiovascular pathology.

Authors contributions

EO and ALC were responsible for the study concept and design. SX was responsible for study management and data collection. EO and SX provided methodological knowledge and performed the analysis. MC and ALC contributed to the interpretation of the results. EO and SX wrote the article. ALC and MC critically revised for important intellectual content and approved the final article.

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Conflict of interest disclosures

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MC received honoraria for consultancy or speaker bureau from Sobi and Ultragenyx.

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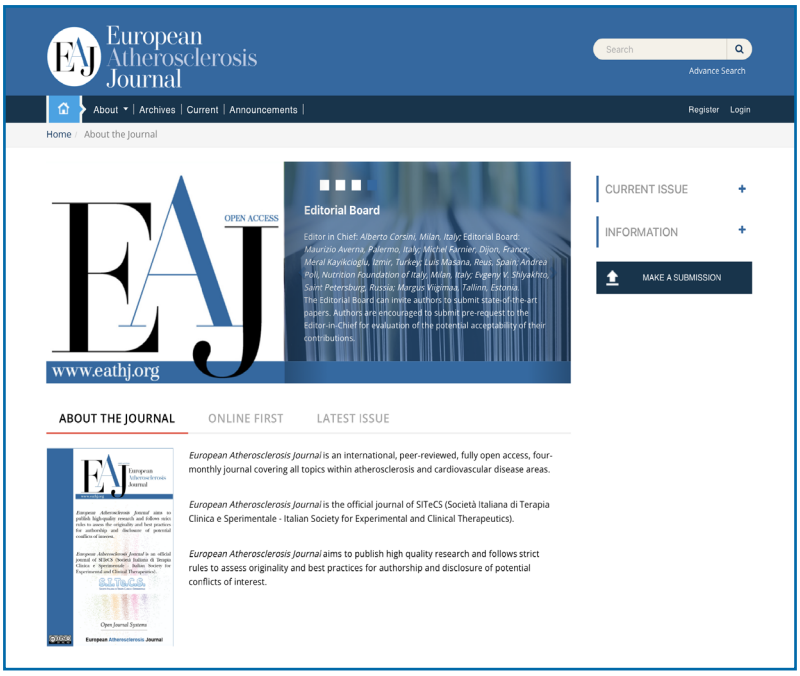

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