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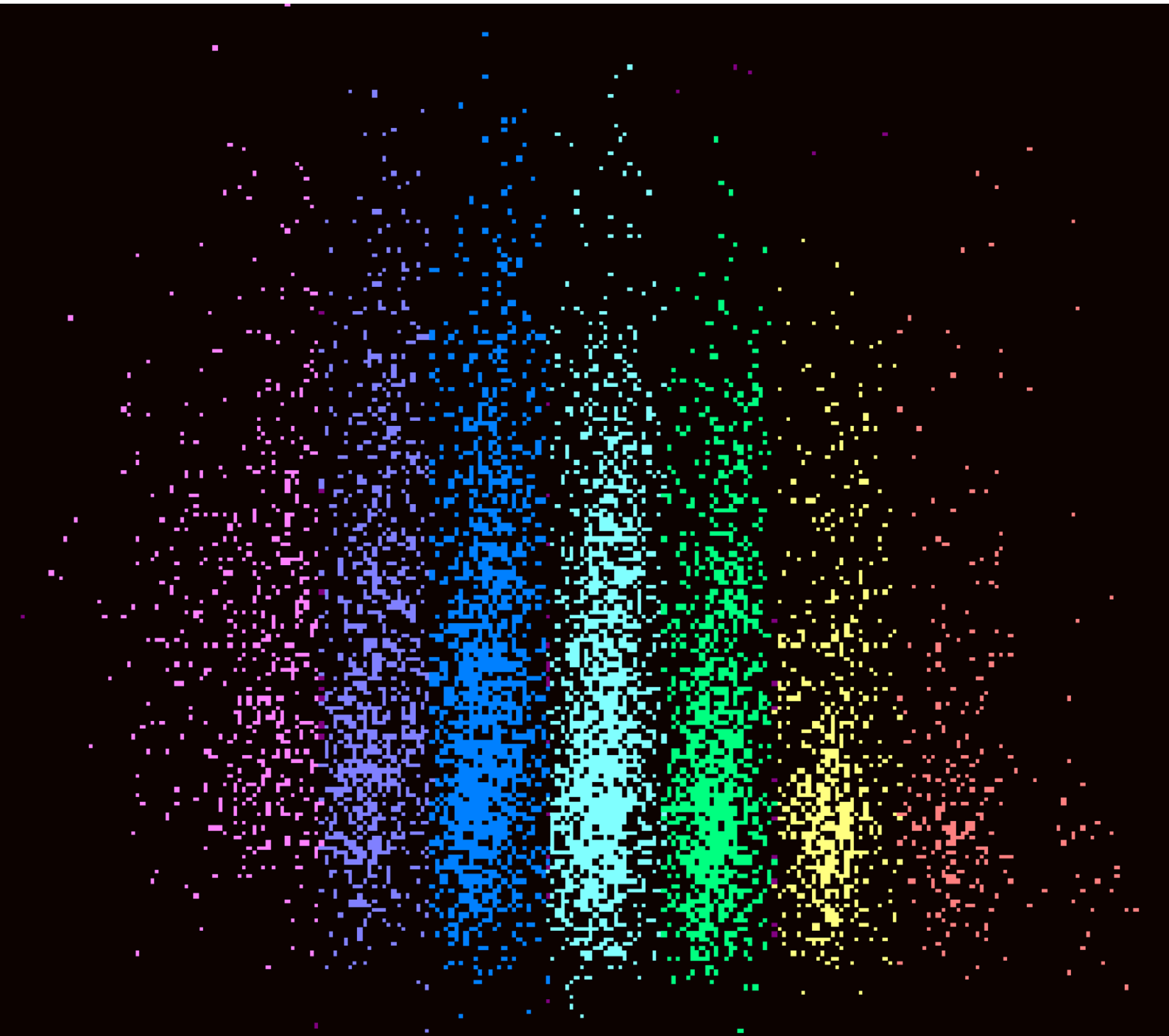

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Contents

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Postprandial implications in cardiovascular disease and potential markers to develop new therapies

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Keywords
Postprandial dyslipidemia; Triglyceride-rich lipoproteins (TRL); Endothelial dysfunction; Postprandial inflammation; Apolipoprotein B; Cardiovascular Diseases

ABSTRACT

An unbalanced diet significantly raises the risk of various chronic diseases and cancers, contributing to increased morbidity and mortality globally. Today, the link between metabolic status and cardiovascular disease is well established. Disruptions in glucose and lipid homeostasis, particularly postprandial hyperglycemia and hyperlipidemia are key risk factors for cardiovascular conditions. These postprandial metabolic disturbances promote atherosclerosis and cardiovascular injury, primarily by triggering endothelial dysfunction.

Lifestyle interventions play a pivotal role, and pharmacological treatments aimed at controlling lipid and glucose levels generally lead to improvements in both fasting and postprandial states. However, further research is necessary to establish reference values for biomarkers of postprandial dysmetabolism and to evaluate their clinical relevance. Individuals who exhibit a mismatch between fasting and postprandial levels of glucose and triglycerides, namely, those with normal or mildly elevated fasting levels but exaggerated postprandial responses, may represent a subgroup at heightened and potentially modifiable risk for both microvascular and macrovascular complications. Validating biomarkers of postprandial dysmetabolism could offer valuable clinical tools for improved risk assessment and personalized therapeutic strategies. This review summarises the unique physiology of triglyceride-rich lipoprotein metabolism after meals and the disruptions that can foster cardiovascular complications. Given the scarcity of targeted therapies, it also discusses emerging treatment candidates and their underlying mechanisms.

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Introduction

Western diets that are very rich in calories, combined with over-nutrition and physical inactivity, promote chronic low-grade inflammation that plays a pivotal role in the development of prevalent non-communicable diseases [1]. Is important to understand how this lifestyle activates immune responses for developing strategies to prevent and treat these conditions. Non-communicable diseases account for over 80% of deaths in Western societies, primarily affecting older adults and reducing healthy lifespan. These chronic conditions impose a growing socioeconomic concern, and so far their mechanisms remain poorly understood [1, 2].

The nutrient profile of a meal significantly impacts postprandial metabolism, with high-fat or high-sugar meals inducing a stronger postprandial response [3]. Assessment of the postprandial lipid profile offers a more sensitive and dynamic measure of the lipid metabolic capacity of the patients compared to fasting lipid levels. It reflects the ability of the organism to efficiently clear and process dietary lipids following a meal, thereby serving as an important indicator of general metabolic efficiency and cardiovascular risk. The postprandial state, lasting 6-12 hours, often extends to 16 hours after a meal. This state involves nutrient absorption and is marked by increased blood sugar, lipids, and low-grade systemic inflammation [4]. Postprandial inflammation can lead to endothelial dysfunction, which is a risk factor for heart disease, insulin resistance, obesity, metabolic syndrome and cardiovascular disease [5].

Recent experimental and clinical studies indicate that metabolic syndrome and cardiovascular disease may result from a systemic in-

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flammatory process [6]. This inflammation is marked by elevated levels of acute-phase proteins and pro-inflammatory cytokines, such as C-reactive protein (CRP), tumour necrosis factor-alpha (TNF-α), and interleukins (IL-1, IL-6, and IL-17), along with increased infiltration of immune cells like macrophages and T lymphocytes into insulin-sensitive tissues [7]. Both low-grade systemic inflammation and dyslipidaemia have been recognized as key contributors to atherogenesis and the associated vascular risks [8].

This review aims to summarize the differences in the metabolism of triglyceride-rich lipoproteins in the postprandial phase, as well as the alterations that can disrupt their normal metabolism and the associated complications. Given the current lack of effective therapies to manage these metabolic disturbances, it is important to explore new therapeutic targets. In this review, we have dedicated a section to summarizing several potential therapeutic targets and their mechanisms of action.

Metabolism of the postprandial lipids

Following food uptake, dietary triglycerides (TG) are absorbed in the small intestine and assemble with apolipoprotein B-48 (apoB-48) into chylomicrons by enterocyte (**Figure 1A**). They are secreted into the lymphatic circulation and subsequently enter in the bloodstream. Following their secretion into the circulation, the TG content of chylomicrons is hydrolyzed by lipoprotein lipase (LPL) to chylomicron remnants [9, 10]. These remnants are then cleared by the liver via receptor-mediated endocytosis for further metabolism [10, 11]. The low-density lipoprotein receptor (LDLR) and the LDL receptor–related protein (LRP) are fundamental hepatic receptors that mediate the endocytosis of remnant particles primarily by apolipoprotein E (apoE) binding [12-14]. The VLDL receptor (VLDLR), predominantly expressed in peripheral tissues like muscle and adipose tissue, facilitates the uptake of VLDL particles at these sites. ApoE plays a central role as a ligand, enabling TRL to interact with these receptors for effective clearance from the bloodstream [14].

VLDL is synthesized in hepatocytes from TG, cholesterol, apoB-100 and microsomal TG transfer protein (MTP). VLDL consists of about 90% lipids and 10% proteins. Within its lipid component, TG account for 70% of the total mass, while the remaining 30% is made up of cholesterol and phospholipids [15]. In circulation VLDL receive ApoCII and ApoE from high-density lipoprotein (HDL), for its maturation into fully functional VLDL . In mature form, VLDL primarily transports endogenous TG from the liver to peripheral tissues [15, 16]. VLDL is considered a pro-atherogenic factor because, in circulation, it is metabolized into IDL and subsequently into LDL, lipoprotein forms that play a central role in the development of atherosclerosis. In particular, the accumulation of LDL, especially small dense LDL particles, is associated with an increased risk of endothelial infiltration, oxidation, and atherosclerotic plaque formation. Thus, by contributing to the generation of these atherogenic particles, VLDL plays a key role in the pathogenesis of cardiovascular disease [9, 11]. The chylomicrons (**Figure 1A**) and VLDL (**Figure 1B**) follow the same metabolic pathway, where LPL hydrolyzes TG into glycerol and fatty acids. The formation and secretion of both TG-rich

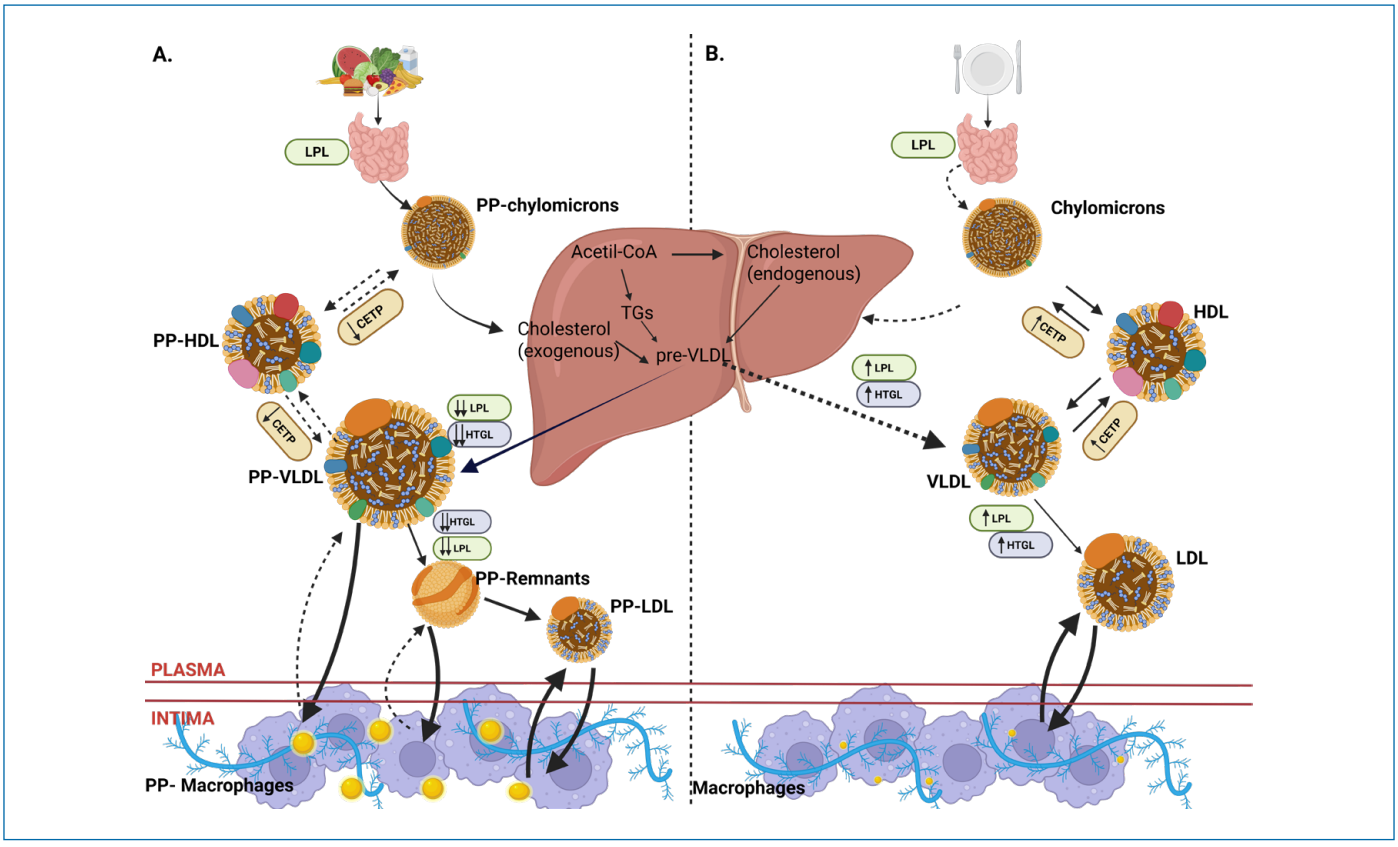

Figure 1 | **Metabolic pathways for TRL metabolism.** Comparative scheme of postprandial (A) and fasting (B) triglyceride-rich lipoprotein and remnant metabolism and its implications. LPL-lipoprotein lipase, HTGL- hepatic (TG) lipase, PP-postprandial, HDL- high density lipoproteins, LDL- low density lipoproteins, VLDL- very low density lipoprotein, CETP- Cholesteryl ester transfer protein. Created in BioRender.

lipoproteins (TRL) require MTP and apoB, without either, the synthesis does not take place [11]. Although chylomicrons and their remnants account for ~80% of the rise in postprandial TG, most particles (~80%) are liver-derived VLDL and their remnants. The concentration and synthesis rate of apoB100 significantly exceed those of apoB48, demonstrating its dominant role in the circulation of atherogenic lipoproteins. Since chylomicrons are preferentially cleared via LPL, the main atherogenic effect may arise from disrupting apoB100 catabolism [17, 18].

Modifications in TRL metabolism and clinical implications

Mutations affecting TRL metabolism can disrupt the synthesis, processing, or clearance of these lipoproteins, leading to altered lipid profiles and increased risk of metabolic and cardiovascular diseases. Such mutations may impair enzymes, receptors, or proteins involved in TRL pathways, resulting in hypertriglyceridemia, pancreatitis, and atherosclerosis. Clinically, these genetic defects can manifest as familial chylomicronemia syndrome, familial combined hyperlipidemia, or other dyslipidemias, necessitating targeted therapeutic approaches to manage elevated triglyceride levels and reduce cardiovascular risk [16].

Familial hyperchylomicronemia syndrome (FCS) is a rare autosomal recessive metabolic disorder primarily caused by mutations in the lipoprotein lipase (LPL) gene. Approximately 80% of FCS cases are due to inherited mutations in both alleles of the LPL gene. The remaining 20% result from mutations in other genes involved in LPL function, including apolipoprotein C-II (APOC2), apolipoprotein A-V (APOA5), glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 (GPIHBP1), and lipase maturation factor 1 (LMF1). Additional, as yet unidentified, genetic mutations may also contribute to the condition [19]. All of these mutations result in impaired function of the enzyme lipoprotein lipase. Familial hyperchylomicronemia syndrome is a rare and often underdiagnosed condition due to its nonspecific symptoms, making it difficult to determine its exact prevalence. Current estimates suggest it affects between 3,000 and 5,000 individuals worldwide, with a frequency of approximately 1 to 10 cases per million people [20].

Familial combined hyperlipidemia (FCH) is a common and widespread hereditary lipid disorder. It is characterized by a variable expression of elevated plasma cholesterol and triglyceride levels, typically affecting at least two members within the same family [21]. In 1973 Goldstein et al. [22] and Rose et al. [23] were initially described FCH as an autosomal dominant hereditary lipid disorder. However, it was later discovered that FCH precede a multigenic pattern with a complex mode of inheritance [24, 25]. FCH is characterized by elevated serum cholesterol (hypercholesterolemia) and/or triglyceride levels (hypertriglyceridemia). In some cases, it may also present as isolated elevations in apolipoprotein B (apoB), even when the standard lipid profile appears within normal limits [26]. The estimated prevalence of FCH ranges from 0.5% to 4% [27]. The genetic basis of FCH remains incompletely understood. Metabolic abnormalities associated with the condition include increased production of very low-density lipoprotein (VLDL) and delayed clearance of low-density lipoproteins (LDL) and triglyceride-rich lipoproteins [28].

Familial hypobetalipoproteinemia (FHBL) is an autosomal codominant disorder caused by apoB gene mutations that reduce apoB48 and apoB100 secretion. These mutations often lead to truncated proteins from premature stop codons, with over 60 identified variants. Less commonly, FHBL results from PCSK9 loss-of-function mutations. The symptoms can differ between patients; heterozygous formes are mostly asymptomatic or have moderate liver steatosis, while 5-10% may develop severe steatohepatitis that can progress to cirrhosis [29]. Most evidence suggests FHBL may protect against cardiovascular disease, but the realy mechanism is unclear. A meta-analysis of 12 studies with 57.973 subjects showed apoB truncations reduce coronary heart disease risk by 72% [30].

Familial defective apoB100 is an autosomal codominant disorder causing high cholesterol and early atherosclerosis. This results from the substitution of glutamine with arginine at amino acid position 3500 (R3500Q) in the gene for apoB100, reducing its binding to LDL receptors, which decreases LDL clearance and increases plasma LDL levels [31]. The second most frequent mutation in the same position is the substitution of tryptophan for arginine. The R3500Q mutation has been associated with a 60–70 mg/dL increase in serum LDL-C levels [32]. Both mutations (R3500Q and R3500W) of the APOB gene contribute to about 12% of familial hypercholesterolemia (FH) cases. However, mutations in APOB are generally less severe than those in the LDLR, which are the main cause of FH, resulting in moderate increases in serum LDL-C levels [33].

Moreover, overproduction of VLDL-ApoB100 may result from altered gene/protein expression and inflammatory insulin signaling. The ApoB100 rs693 polymorphism is linked to metabolic syndrome, obesity, and elevated TC, LDL-C, TG, and glucose levels [34]. A recent study by Taskinen et al. examined how intestinal triglyceride-rich lipoproteins affect residual cardiovascular risk in overweight/obese diabetic patients on statins. While apoB100 kinetics in VLDL and LDL showed no significant changes, postrandial apoB48 levels were about twice higher, especially in the VLDL1 and VLDL2 density ranges [35].

Also ***epigenetic modifications*** of genes involved in TRL metabolism occur. Epigenetic processes are natural and essential for the normal functioning of organisms. These changes modify gene expression and activity without altering the DNA sequence [36, 37]. Among various epigenetic mechanisms, DNA methylation is one of the most significant. It involves the covalent addition of a methyl group to cytosine-guanine dinucleotides (CpG), a reaction catalyzed by DNA methyltransferases [38]. Since CpG sites are often located in gene regulatory regions, their methylation can significantly influence gene expression [39]. Several studies have investigated the association between DNA methylation at various gene loci and serum TG levels, showing inconsistent results. While some studies found no significant correlation between methylation status and TG concentrations [38, 39], others reported a positive association [36, 40]. There are different epigenetic changes that might affect TG levels; some genes including: ATP Binding Cassette Subfamily G Member 1 (ABCG1), sterol regulatory element-binding protein 1 (SREBP-1) and Carnitine Palmitoyltransferase 1A (CPT1A) have been explored more than others. These genes are required for glucose metabolism and fatty acid and lipid production.

A meta-analysis explored the relationship between DNA methylation at various CpG sites within the these genes and serum TG levels [41]. It found that increased methylation of ABCG1 and SREBP-1, along with decreased methylation of CPT1A, was significantly associated with elevated serum TG levels. These results may help explain the considerable variability in hypertriglyceridemia prevalence observed across different populations. Consequently, investigating these genes may play a crucial role in the prevention of cardio-meta-

bolic risk factors, including total cholesterol and insulin levels, as well as measures of general and abdominal obesity [42-44].

Implication of TRL in cardiovascular diseases

In the postprandial state, chylomicron remnants and VLDL interact with circulating leukocytes and endothelial cells, inducing acute cellular activation characterized by integrin expression, oxidative stress, cytokines release, and complement system activation [45].

Interestingly, although postprandial inflammatory responses may occur multiple times daily following meals, there is limited evidence regarding the specific contributions of nutrients to these processes [46]. It has been demonstrated that elevated TG and glucose levels lead to an increase in neutrophil number, followed by enhanced production of pro-inflammatory cytokines and oxidative stress, which may contribute to endothelial dysfunction [46]. Moreover, TG and glucose have been shown to induce leukocyte activation, both *in vitro* [47, 48] and *in vivo* in hypertriglyceridemic patients [49]. Postprandially, TG and leukocyte number begin to rise after 1–2 hours, indicating the probable initiation of leukocyte activation. Notably, leukocyte activation was observed not only at 6 hours but persisted up to 10 hours postprandially, with no significant differences in activation levels between the 6-hour and 10-hour after oral fat ingestion. The presence of apoB on neutrophils and monocytes supports the binding of TRL to leukocytes [50]. Leukocytes from cardiovascular disease patients showing increased lipid content compared to healthy controls, likely due to the uptake of chylomicrons [51]. Postprandial lipemia has been linked to increased expression of leukocyte activation markers CD11b and CD66b in both healthy patients and those with premature atherosclerosis. CD11b aids early leukocyte-endothelium adhesion, while CD66b is a neutrophil-specific degranulation marker [52]. Elevated levels of these markers have also been observed in fasting leukocytes from patients with cardiovascular disease and diabetes [52].

Additionally, *in vitro* studies have shown that primary human monocytes can internalize remnant lipoproteins [48]. TRL are taken up by macrophages through different receptor- and non-receptor-mediated pathways [14]. Primary human monocytes activated by remnant chylomicron particles exhibited a rapid and sustained production of reactive oxygen species (ROS). Even in the absence of these particles, the monocytes secreted the pro-inflammatory chemokines monocytes chemoattractant protein 1 (MCP-1) and interleukin-8 (IL-8) [48]. Upon treatment with remnant chylomicron particles, IL-8 secretion showed a transient increase and remained detectable even in the presence of pharmacological inhibitors of IL-8 synthesis. Interestingly, monocytes pre-treated with remnant chylomicron particles demonstrated enhanced chemotactic response to MCP-1, an effect that was reversed by the addition of exogenous MCP-1 [48]. Also, neutrophil levels rise after meals in healthy patients, as well as in patients with cardiovascular disease and T2D. This postprandial neutrophil increase is correlated with the synthesis of proinflammatory cytokines and oxidative stress [50].

Some studies have uncovered a novel mechanistic connection between TRL and vascular disease, involving the formation of lipid droplets (LD) within endothelial cells. These LD can suppress nitric oxide production, impairing endothelial-dependent vasodilation

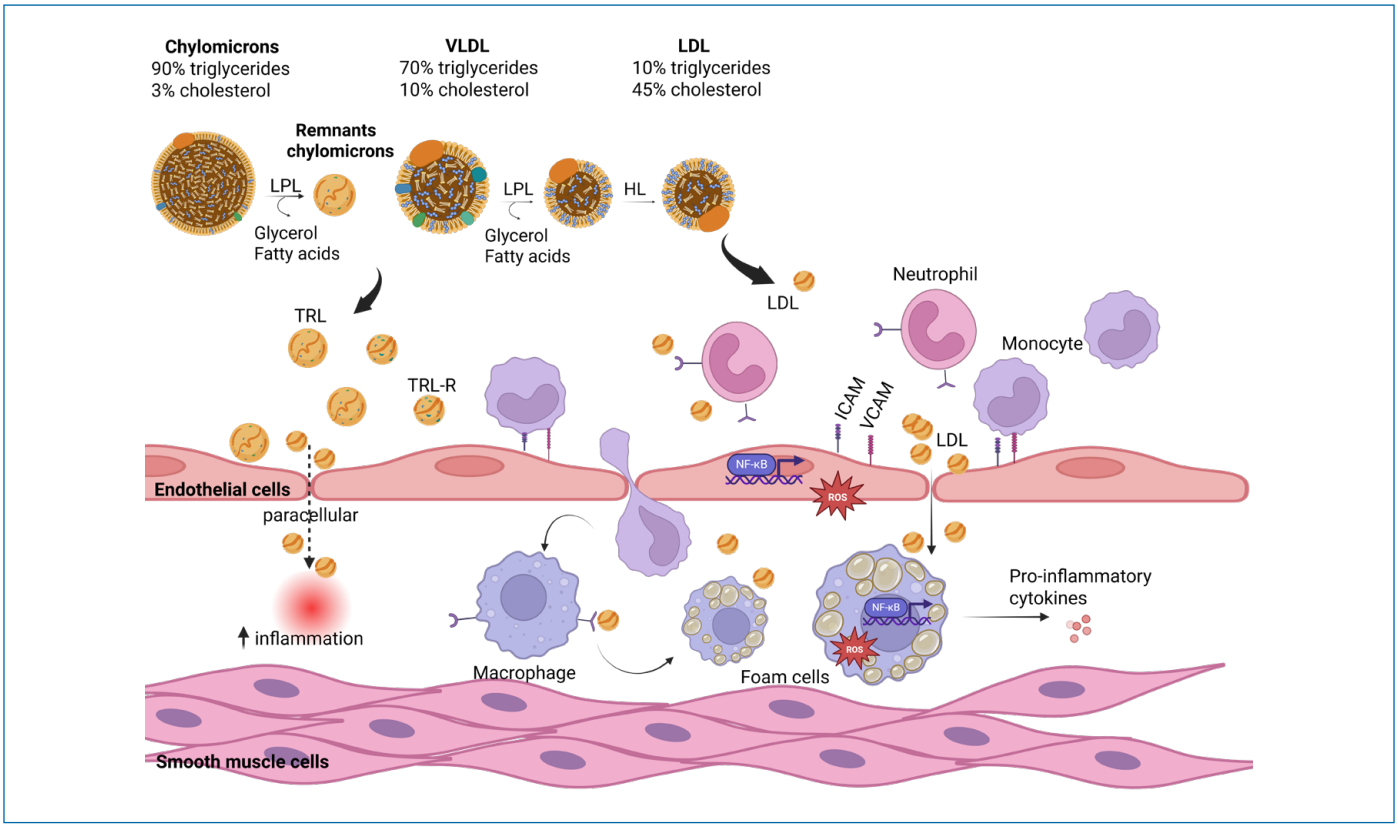

Figure 2 | **Postprandial inflammatory responses and atherosclerosis initiation.**
TRL-tryglycerides-rich lipoproteins, TRL-R-TRL remnants, LPL-lipoprotein lipase, HL- hepatic lipase, ICAM- Intercellular Adhesion Molecule, VCAM- Vascular cell adhesion molecule. Created in BioRender.

and promoting vasoconstriction, which contributes to the development of hypertension [53]. Furthermore, endothelial LD can activate the NF-κB signaling pathway, resulting in increased expression of vascular cell adhesion molecule-1 (VCAM-1). This upregulation enhances leukocyte adhesion to the endothelium, thereby promoting atherogenesis and inflammation within vascular plaques [18, 53].

Transport across the endothelium is highly efficient and does not primarily determine the concentration of atherogenic lipoproteins within the arterial wall. Instead, it is the selective retention of these lipoproteins by the extracellular matrix that governs their accumulation at sites prone to lesion development.*8* This retention occurs through ionic interactions between positively charged amino acid residues in apoB and apoE on the lipoprotein surface and negatively charged proteoglycans secreted by arterial wall cells. Similar to LDL, TRL contain a single apoB molecule per particle, but they also carry multiple apoE molecules, which enhance the binding affinity of TRL remnants for extracellular proteoglycans [17].

Consequently (**Figure 2**), postprandial inflammatory responses may include leukocyte activation through the uptake of TRL, promoting their adhesion to and activation of endothelial cells. This, may enhance the migration of inflammatory cells and lipoproteins into the sub-endothelial space, thereby accelerating the development of atherosclerosis.

Non-traditional postprandial risk factors for atherosclerosis: Telomere shortening and CHIP

Recent study have highlighted the role of telomere shortening and clonal hematopoiesis of indeterminate potential (CHIP) as non-traditional risk factors in cardiovascular disease. Telomere shorting is a hallmark of cellular aging and contributes to genomic instability. It can trigger cell-cycle arrest, senescence, and apoptosis in vascular smooth muscle cells [54], reduce the regenerative capacity of vascular endothelium [55], and can also facilitate necrotic cores formations in atherosclerotic plaques. Telomeres progressively become shorter with age and this process is accelerated by oxidative stress and chronic low-grade inflammation, both involved in the postprandial state stess.

Following an high-fat meals, a transient low grade inflammation state can be triggered, characterized by the increases in circulating TRL, oxidative stress, and inflammatory cytokines. This postprandial inflammatory response contributes to endothelial dysfunction and may amplify and influence telomere attrition in circulating leukocytes and vascular progenitor cells [56]. Repeated episodes of postprandial state stress may result in cumulative damage to telomere integrity and accelerate vascular aging.

Telomeres play an important role in the aging of hematopoietic stem cells (HSC) and in the gradual weakening of the immune system. In rare diseases like dyskeratosis congenita, where the system that protects telomeres does not work properly, bone marrow fails because HSC can no longer renew themselves [57]. But even in people without blood disorders, having short telomeres in white blood cells has been linked to a higher risk of anemia, infections, and heart disease [58, 59]. This may be because short telomeres reduce the ability of HSC to produce new blood cells and endothelial progenitor cells (EPC), which help repair blood vessels.

EPC are important for keeping blood vessels integrity, especially by helping to repair damage. However, their number and function decrease with age and in people with CVD. Studies show that EPC age faster when telomeres become too short or when there is a lot of oxidative stress [60, 61]. Interestingly, some research suggests that statin commonly used for lowering cholesterol level, may help EPC stay younger by protecting their telomeres and boosting telomerase activity [62].

Telomere shortening is also implicated in CHIP, a particular condition characterized by clonal expansion of HSC with some somatic mutations in genes like DNMT3A, TET2, or ASXL1, in patients without hematologic malignancy. CHIP affects over 10% of individuals older than 70 years old and is associated with a two-fold increased risk of coronary artery disease, independent of classical risk factors [63]. The pathogenicity of CHIP appears to be driven by chronic inflammation, mostly mutated clonal myeloid cell in particular macrophages showing an increase of IL-1β and activation of NLRP3 inflammasome, which are common influenced by postprandial state.

However, *in vivo* studies, on mice models support the "telomer-CHIP-atherosclerosis axis". TET2-KO mice develop larger atherosclerotic plaques and show elevated IL-1β expression through NLRP3 activation [56]. In humans, CHIP is associated with increased CRP levels and a pro-inflammatory gene expression profile in monocytes. Mouse models lacking telomerase components like TERC or TERT show a progressive telomere shortening, cardiac dysfunction, impaired cardiomyocyte proliferation and early death, with an increase of expression and activation of p53 [64].

All together, these data support a "telomere–CHIP–atherosclerosis axis" in which telomere attrition and clonal hematopoiesis converge to promote inflammation and vascular aging, particularly under repeated postprandial stress.

Potential postprandial lipid biomarkers

Measuring postprandial secreted markers involved in intestinal lipoprotein metabolism may facilitate the early detection of postprandial dyslipidemia and associated cardiovascular disease risk [65]. Postprandial lipid responses have been studied in research for more then 40 years and could be considered potential tools for the risk of cardiovascular disease. While the ***traditional fasting lipid profile (TG, TC, HDL-C, LDL-C)*** is commonly used to assess cardiovascular diseases risk, evidence does not support its advantage over postprandial measurements. Postprandial lipid testing provides practical benefits, such as a more accurate representation of average daily lipid levels, easier sample collection, and improved patient compliance [66]. Although fasting dyslipidemia is a well-established risk factor for cardiovascular disease, only 47.5% of patients with acute coronary syndrome exhibit abnormal lipid levels in the fasting state. Emerging evidence suggests that postprandial lipid parameters may offer superior predictive value for cardiovascular disease risk compared to traditional fasting measurements [67, 68].

The atherogenic potential of TG and TRL in the postprandial state was first proposed by Zilversmit in 1979 [69] and has been supported by numerous prospective studies. Evidence indicates that *postprandial TG levels* are more strongly associated with cardiovascular events than fasting levels [65]. Remnant lipoproteins, particularly chylomicron remnants, smaller than 70 nm, are small enough to pass the vascular endothelium and accumulate in the subendothelial space [70]. Unlike larger lipoprotein particles, these remnants can pass through endothelial junctions via paracellular transport, a process facilitated by increased endothelial permeability induced by TRL lipolysis products [71]. Once within the arterial wall, remnant lipoproteins (containing up to 40 times more cholesterol than LDL particles [72, 73] are preferentially retained in the intima, contributing substantially to atherosclerotic lesion formation. Additionally, they trigger endothelial inflammation, apoptosis, and the expression of pro-inflammatory and pro-atherogenic proteins such as MCP-1 [74] and PAI-1 [75].

LDL-C has been regarded as standard for assessing atherosclerotic cardiovascular disease risk and evaluating the efficacy of lipid-lowering therapies [16]. However, apoB provides a more broad measure of atherogenic lipoprotein particles, while VLDL, IDL, LDL, and lipoprotein(a) particles contains exactly one apoB molecule. Therefore, *apoB* may provide a more precise assessment of atherosclerotic cardiovascular disease risk, especially in patients who maintain the cardiovascular risk despite receiving optimal lipid-lowering therapy [76]. Reflecting this, current international guidelines now recognize apoB as a feasible alternative to LDL-C measurement, especially in individuals with very low LDL-C levels or those with metabolic syndrome [76]. Furthermore, apoB100 concentrations have shown a positive correlation with both systolic and diastolic blood pressure, and the apoB100/apoAI ratio has demonstrated a stronger association with cardiovascular risk than the traditional LDL/HDL ratio [77].

Antibodies targeting apoB100 (anti-apoB100) could be considered a new biomarker for identifying vulnerable atheromatous plaques. Evidence from both animal and human studies indicates that circulating autoantibodies against apoB100 are associated with the stage and severity of cardiovascular disease. These autoantibodies appear to reflect the immune response of the body to atherogenic lipoproteins and have been linked to features of plaque instability, such as inflammation and immune cell infiltration. This relationship suggests that anti-apoB100 could serve not only as markers of overall cardiovascular burden but also as specific indicators of vulnerable atheromatous plaques, those liable to rupture and trigger acute events like myocardial infarction or stroke [16]. In particular, Marchini et al. reported elevated plasma levels of pro-inflammatory anti-apoB IgG in patients with high cardiometabolic risk, including those with arterial hypertension, obesity, and metabolic syndrome. In contrast, levels of anti-apoB IgM, which are considered to have anti-inflammatory properties, were significantly reduced in these patients [16, 78].

The ***oral fat tolerance test (OFTT)***, measure lipid metabolism after a high-fat meal, but is not routinely performed in clinics due to the absence of standardized methods and reference values [79], like the oral glucose tolerance test (OGTT) which is a widely used clinical tool for evaluating glucose intolerance in pre-diabetic or diabetic conditions [80]. Several studies, using the oral glucose tolerance test (OGTT), have demonstrated significant reductions in plasma antioxidant levels and increases in markers of endothelial activation (ICAM, VCAM and E-selectin) and damage [81, 82]. These effects are different based on carbohydrate uptake, the measurement method, and individual metabolic responses [82, 83]. However, not all studies report consistent outcomes; some have shown no significant oxidative or endothelial changes after OGTT in healthy subjects [84, 85]. These difference emphasize the need for standardized methodologies and further investigation into individual susceptibility and the specific properties of different carbohydrate sources [82]. Importantly, studies show that these adverse outcomes can be relieved by the co-administration of antioxidants such as vitamins C and E, glutathione, α-lipoic acid, or statins with antioxidant properties. These interventions did not affect glucose levels, highlight that oxidative stress, not hyperglycemia itself, is the primary link to postprandial endothelial dysfunction. These findings emphasize the critical role of oxidative mechanisms in vascular health and suggest potential therapeutic strategies for protecting endothelial function during periods of acute glucose elevation [82]. In a study using the OFTT, patients with T2D (both normo- and hyperinsulinemic) and healthy individuals received 17 g fat/m² body surface area, with blood samples taken at fasting, 2 and 4 hours post-meal [86]. While TG levels were similar across groups, remnant lipoproteins rich in TG and cholesterol were elevated only in hyperinsulinemic T2D patients. This indicates that hyperinsulinemia, rather than T2D itself, may drive postprandial dyslipidemia and associated cardiovascular risk [5].

Postprandial ***glucagon like peptide 1 (GLP-1)*** has been proposed as an early biomarker of metabolic dysfunction in obesity, with some studies showing reduced GLP-1 levels in T2D patients after mixed meals [87, 88], while others report no differences in patients with metabolic syndrome [89]. Overall, findings on GLP-1 changes in obesity and insulin resistance are inconsistent, and further research is needed to explore its relationship with postprandial dyslipidemia [65].

Another recently identified biomarker of systemic inflammation, which has demonstrated strong clinical relevance and is linked to metabolic disorders like diabetes and obesity is *glycoprotein acetylation (GlycA)* [90]. GlycA responses were closely associated with postprandial TG levels, particularly with peak TG concentrations [91]. Visceral fat mass and fasting TG levels have been identified as primary determinants of both fasting and postprandial GlycA concentrations. Notably, visceral adiposity exerts a causal influence on GlycA levels, an effect that is partially mediated by circulating TG. Important, patients showing elevated GlycA responses also demonstrated higher predicted cardiovascular disease risk, highlighting GlycA as a potential marker of cardiometabolic health and systemic inflammation [91]. However, the fact that make GlycA as a promising marker for assessing inflammation in metabolic states is its consistent responsiveness to dietary intake [91], a characteristic not shared by many conventional inflammatory markers, such as CRP [4].

In a study of 30,000 pacients from the Copenhagen General Population Study, higher plasma ***glycerol and β-hydroxybutyrate*** which were considered markers of triglyceride metabolism, were linked to increased risk of cardiovascular, cancer, and other mortality, independent of plasma triglyceride levels and BMI [92]. Patients with the highest plasma glycerol levels (>80 μmol/L) had significantly higher risks of cardiovascular, cancer, and other mortality compared to those in the lowest amount (<52 μmol/L). Similarly, those with the highest plasma β-hydroxybutyrate (>154 μmol/L) had increased risks of cardiovascular, cancer, and other mortality compared with the lowest levels (<91 μmol/L). No significant interaction was found between the two markers across mortality outcomes [92].

From a biochemical perspective, linking plasma glycerol and β-hydroxybutyrate to triglyceride metabolism may seem simplistic. However, since free glycerol mainly comes from triglyceride breakdown and is trapped intracellularly by glycerol kinase (active mostly in the liver), plasma glycerol is widely accepted as a reliable marker of whole-body triglyceride breakdown. In contrast, free fatty acids from triglyceride breakdown are widely used for energy, lipogenesis, or ketogenesis. They can also be produced *de novo* from glucose or fructose in tissues like the liver, muscle, and adipose tissue [93, 94].

Ketone bodies include acetoacetate and β-hydroxybutyrate, with β-hydroxybutyrate being the most stable in plasma and thus the preferred biomarker. Ketogenesis is driven by triglyceride breakdown, not *de novo* synthesis, and since the liver cannot use ketones for energy, all produced ketone bodies are released into the bloodstream. Therefore, β-hydroxybutyrate serves as an indirect indicator of triglyceride breakdown, primarily in adipose tissue [95].

Measuring glycerol and β-hydroxybutyrate may slightly under- or overestimate whole-body triglyceride breakdown. Some TG are only partially metabolized or used locally in tissues, while ketones can also come from amino acids, and glycerol may enter circulation via the gut or from lipoprotein metabolism [96]. Most lipases in adipose tissue, the gut, and blood vessels mainly produce 2-monoacylglycerol, not glycerol, due to their bond preferences [97]. Glycerol production is minimal in the gut and slow in the vasculature. In contrast, adipocytes fully break down triglycerides into glycerol and free fatty acids, mak-

Table 2 | Postprandial Markers and Cardiovascular Risk.

Markers	Clinical involvement	Ref.
Fasting lipid profile (TG, TC, HDL-C, LDL-C)	– Traditional CVD risk assessment tool; – Limited predictive value compared to postprandial lipids; – ~47.5% of ACS patients shows abnormal lipid levels in the fasting state.	[66, 67]
Postprandial TG	Stronger correlation with cardiovascular events than fasting TG.	[65, 69]
Remnant lipoproteins	– Rich in cholesterol; – <70 nm, can cross endothelium and enter subendothelial space; – Activate MCP-1 and PAI-1 expression; – Promote inflammation, apoptosis, and lesion formation.	[70-75]
ApoB	One molecule per particle VLDL, IDL, LDL, Lp(a).	[16, 76]
ApoB100/apoAI ratio	– Correlates with CVD risk better than LDL/HDL ratio; – Strong predictor of cardiovascular events.	[77]
Anti-apoB100 antibodies (IgG/IgM)	Reflect immune response to plaque instability and atheroma vulnerability.	[16, 78]
OFTT (Oral Fat Tolerance Test)	– Measures lipid metabolism after fat meal; – Not standardized; – Not widely used in clinics like OGTT.	[79]
GLP-1	– Potential early biomarker of metabolic dysfunction; – Is reduced in T2D; – Findings inconsistent.	[65, 87-89]
Glycoprotein acetylation (GlycA)	– Responds to dietary intake and correlates with TG peaks and visceral fat; – Promising marker for metabolic inflammation and cardiometabolic risk.	[4, 90, 91]
Plasma glycerol and β-hydroxybutyrate	– Increased risk of cardiovascular, cancer, and other mortality, independent of plasma TG levels and BMI; – Reliable markers of whole-body TG breakdown.	[92-95] [96]

ing them the main source of plasma glycerol and ketogenesis substrates; both reasonable markers of triglyceride metabolism [92].

In **Table 2** are summarized the postprandial potential markers and their clinical implication in cardiovascular development.

Current therapeutic agents for postprandial lipids

Targeting postprandial lipids with specialized therapies could enhances the control and the reduction of cardiovascular complications.

The FDA recently approved **angiopoietin-like 3 protein (ANGPTL3) inhibitors**, a new class of lipid-lowering drugs. By increase LPL and endothelial lipase activity, these agents improve VLDL remnant clearance and lower LDL-C levels despite of LDLR function. They are mainly used for homozygous familial hypercholesterolemia and mixed dyslipidemia. ***Evinacumab*** and ***vupanorsen*** are the first drugs in this class [98]. Evinacumab, a monoclonal antibody targeting ANGPTL3, lowers LDL-C by enhancing clearance of apoB lipoproteins, reducing LDL-C by 25%, ApoB by 31%, and non-HDL-C by 46% at 20 mg/kg monthly. Over 18 months, reductions reached 45.5% for LDL-C, 38.8% for ApoB, 48.4% for non-HDL-C, and 57.2% for triglycerides, with no major side effects. In contrast, vupanorsen, an antisense oligonucleotide targeting ANGPTL3 mRNA, shows modest lipid-lowering effects in T2D patients, reducing LDL-C by 18%, ApoB by 12%, and non-HDL-C by 9% compared to placebo [99].

Conclusion

The link between metabolic imbalance and cardiovascular disease is now well established, with postprandial hyperglycemia and hyperlipidemia emerging as key contributors to vascular dysfunction. These postprandial disturbances initiate a cascade of effects that induce atherosclerosis and cardiovascular modifications, primarily through endothelial dysfunction. Excess nutrient intake leads to low-grade systemic inflammation which plays a central role in this process, driven by pro-inflammatory cytokines and inflammasome activation, further amplifying endothelial injury and oxidative stress, immune cells implications that accelerate the progression of cardiovascular disease.

Throughout the years, a series of changes in the metabolism of triglyceride-rich lipoproteins and their clinical implications have been described. In recent years, both epigenetic factors and non-traditional postprandial risk factors such as telomere shortening and CHIP have also been identified, targets that should be further explored.

In this review, we highlighted the changes that occur following food intake and how these alterations impact lipoprotein metabolism, which together with inflammation contribute to the development of cardiovascular diseases. Additionally, we provided an overview of certain molecules that may serve as potential early biomarkers for the onset of cardiovascular diseases and their complications. Further research is needed to fully understand the complex interplay between postprandial inflammation, atherosclerosis, and cardiovascular disease. Identifying the specific mechanisms and developing targeted therapies to reduce postprandial inflammation could be crucial in preventing and treating cardiovascular disease.

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Comparing the predictive value of genetic determinants and measured plasma levels of Lipoprotein(a) in cardiovascular risk assessment: evidence from a large-scale UK Biobank study

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Keywords
Lipoprotein(a); genetic variants; plasma concentration; cardiovascular risk assessment

ABSTRACT

Introduction: Lipoprotein(a) [Lp(a)] is a genetically influenced lipoprotein causally associated with atherosclerotic cardiovascular disease risk. This study compares the predictive value of genetically determined versus directly measured Lp(a) levels for major coronary events (MCE).

Methods: From UK Biobank data, participants with complete genetic and plasma Lp(a) data, including LPA variants rs3798220 and rs10455872, were selected. Cox proportional hazards models were employed to estimate the risk of MCE, associated with both Lp(a) genetic score (0, 1, or ≥2 minor alleles) and measured Lp(a) levels.

Results: Among 410,194 participants (mean age 57.25, 54% females), both Lp(a) genetic score and measured levels were independently associated with a stepwise increase in MCE risk. Within each genetic score group, increasing measured Lp(a) quintiles were associated with higher MCE. However, for individuals with similar measured Lp(a), MCE risk did not differ by genetic score.

Conclusions: Directly measured Lp(a) levels offer superior cardiovascular risk prediction, supporting the practice of measuring Lp(a) levels at least once in adulthood.

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Introduction

Atherosclerotic cardiovascular disease (ASCVD) remains a leading cause of morbidity and mortality worldwide. Lipoprotein(a) [Lp(a)] is a lipoprotein subclass that has gained significant attention due to its strong association with an increased risk of ASCVD [1-3]. The concentration of plasma Lp(a) is primarily genetically determined, with 70–90% of its variability attributed to differences in the number of repeats in the DNA sequence encoding kringle IV type 2 (KIV-2), with the *LPA* gene playing a pivotal role [1]. Despite its strong genetic basis, Lp(a) levels vary widely among individuals, influencing cardiovascular risk assessment. While genetic variants associated with Lp(a) identifies predisposition, direct plasma Lp(a) measurement is gaining clinical relevance despite certain limitations [4]. Given its established causal role in cardiovascular diseases, it is crucial to determine whether genetic assessment or direct measurement provides a more accurate prediction of cardiovascular risk. By comparing the cumulative lifetime risk of major coronary events (MCE) based on genetic variants and measured plasma levels of Lp(a), we aim to identify the most effective approach for cardiovascular risk stratification, ultimately guiding clinical decision-making and improving patient outcomes.

Methods

This was a prospective observational cohort study based on data from the UK Biobank, a large, population-based biomedical database and research resource. Participants with complete genetic and principal component data who self-identified as being of white ancestry were evaluated. Only subjects genotyped for the *LPA* gene with available measured plasma levels of Lp(a) were included.

We used the number of inherited minor alleles of genetic variants rs3798220 (Ile4399→Met) and rs10455872 (intronic A/G polymorphism) to calculate a genetic score for each participant, with the reference group defined as participants with no copies of either mi-

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nor allele (Lp(a) score equal to 0), the second group defined as participants with one minor allele (Lp(a) score equal to 1), and the third group defined as participants with at least two minor alleles (Lp(a) score equal to 2).

Plasma Lp(a) concentration was measured in nmol/L at study enrolment using an immunoturbidimetric method on the Beckman Coulter AU5800 platform (Randox Bioscience, UK) [5]. The primary outcome for the study was major coronary events (MCE), defined as the first occurrence of either a fatal or non-fatal myocardial infarction (MI), or coronary revascularization. Cox proportional hazards models adjusted for age, sex, and the first 10 principal components of ancestry were used, with age as the time scale (hazard ratio [HR] and 95% confidence interval [95% CI]) to evaluated the effect of Lp(a) on MCE risk. Cumulative lifetime risk of MCE was plotted, using Kaplan-Meier curves.

All analyses were performed using Stata (version 17; StataCorp). A 2-tailed p-value less than 0.05 was considered statistically significant.

Results

A total of 410,194 participants were included in the study, with a mean (SD) age at enrolment of 57.25 (8.03) years; 54% were of female sex. The median [IQR] Lp(a) level in the overall population was 18.70 [7.40–72.90] nmol/L. Despite the same genetic determinants, participants exhibited substantial variability in measured Lp(a) levels: among individuals with an Lp(a) score of 0 (N=334,182), the median Lp(a) concentration was 13.56 [6.20–35.00] nmol/L, increasing to 146.3 [104.80–200.20] nmol/L for those with a score of 1 (N=72,087) and to 261.80 [190.21–336.00] nmol/L for those with a score of 2 (N=3,925). Notably, only 5.55% of individuals with an Lp(a) score of 0 had measured Lp(a) levels exceeding the cut-off of 125 nmol/L (which is considered elevated [2]), with this proportion increasing to 63.04% for those with a score of 1 and to 90.80% for those with a score of 2.

A clear stepwise increase in the risk of MCE was observed with rising genetic Lp(a) score. Compared to individuals with an Lp(a) score of 0, those with a score of 1 had a hazard ratio (HR) of 1.47 (95% CI 1.42–1.53, p <0.001), while those with a score of 2 had an even higher risk, with an HR of 1.86 (95% CI 1.67–2.08, p <0.001). We than stratified participants within each genetic score group into quintiles based on their measured plasma levels of Lp(a). Using the lowest quintile as the reference, we found a progressive increase in MCE risk from the first to the highest quintile within the same genetic score value (HR from 0.96 [95% CI 0.91–1.01] to 1.41 [95% CI 1.35–1.48] for Lp(a) score of 0; HR from 1.11 [95% CI 0.99–1.23] to 2.34 [95% CI 2.13–2.57] for Lp(a) score of 1; HR from 0.98 [95% CI 0.67–1.44] to 1.63 [95% CI 1.15–2.31] for Lp(a) score of 2).

When the lifetime risk of MCE was assessed across different genetic determinants (using individuals with an Lp(a) score of 0 as the reference group) in subjects matched for similar median Lp(a) plasma concentrations (**Figure 1**), the risk was found to be comparable.

Discussion

This large-scale study provides critical insights into the predictive value of Lp(a) in assessing the risk of MCE and the comparative utility of Lp(a) genetic determinants versus directly measured concentrations in clinical practice. Our findings demonstrate that, despite the strong genetic basis of Lp(a) variability, measured Lp(a) levels offer superior predictive value for cardiovascular risk assessment.

Specifically, measured Lp(a) concentrations showed a stronger association with MCE compared to genetic Lp(a) score, indicating that direct measurement provides a more accurate risk stratification tool. This finding is particularly relevant because Lp(a) levels are stable over a lifetime, requiring only a single measurement in adulthood to offer a reliable estimate of long-term cardiovascular risk [6]. Additionally, Lp(a) measurement is more accessible and cost-effective than genetic testing, making it a practical option for routine clinical use. However, genetic testing retains its value in specific scenarios, particularly for identifying individuals with a familial predisposition to elevated Lp(a) levels, which may be useful when measured Lp(a) values are borderline or inconclusive.

This study has several limitations. First, the observational nature of the analysis precludes definitive conclusions about causality. Although extensive adjustments were made, residual confounding cannot be ruled out. Second, the study population included only individ-

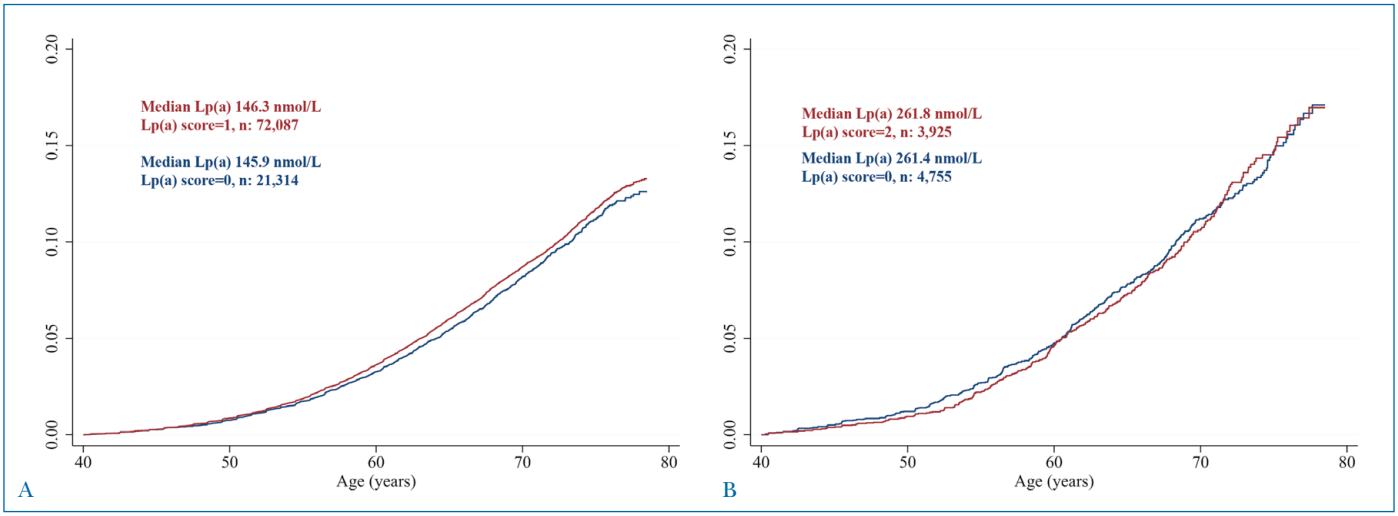

Figure 1 | **Survival curves showing the lifetime risk of major coronary events by Lp(a) score values among participants with comparable median Lp(a) concentrations.**
Panel A compares the lifetime risk for subjects with Lp(a) score of 1 to those with Lp(a) score of 0. Panel B compares the lifetime risk for subjects with Lp(a) score of 2 to those with Lp(a) score of 0.

uals of white ancestry, limiting the generalizability of the findings to other ethnic groups, especially considering that Lp(a) levels and their genetic determinants vary across populations. Third, Lp(a) was measured only once at baseline, although its lifelong stability mitigates this limitation. Finally, genetic scoring was limited to two well-established *LPA* variants, and additional *loci* may contribute to Lp(a) variability and associated risk, which were not captured in this analysis.

In conclusion, our study strongly supports prioritizing direct Lp(a) measurement for cardiovascular risk assessment, even when genetic data are available. When Lp(a) levels are unknown, clinical testing should be the first-line approach, given its greater predictive accuracy, simplicity, and cost-effectiveness in guiding preventive and therapeutic strategies.

Author contributions

E.O., F.G., and A.L.C. were responsible for the study concept and design. A.L.C was responsible for study management and data collection. E.O., F.G., and M.C. provided methodological and statistical knowledge and performed the analysis. A.L.C. and M.C. contributed to the interpretation of the results. E.O., and F.G. wrote the article. A.L.C. and M.C. critically revised for important intellectual content and approved the final article.

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Conflict of interest

All authors declare no support from any organization for the submitted work; no other relationships or activities that could appear to have influenced the submitted work.

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Ethical statement

Ethical approval was not required for this study.

Data Sharing Statement

The data that support the findings of this study are available on reasonable request from the corresponding author, F.G.

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Evaluation of the Effect of Lomitapide Treatment on Major Adverse Cardiovascular Events (MACE) in Patients with Homozygous Familial Hypercholesterolemia: Study Protocol of the LILITH Study

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ABSTRACT

Homozygous Familial Hypercholesterolemia (HoFH) is a rare genetic disorder characterized by markedly elevated low-density lipoprotein cholesterol (LDL-C) levels from birth, leading to premature and severe cardiovascular disease. Lomitapide, an inhibitor of microsomal triglyceride transfer protein (MTP), effectively lowers LDL-C in HoFH patients. However, data on its impact on major adverse cardiovascular events (MACE) remain limited, and randomized controlled trials are not feasible due to the rarity of the condition and ethical constraints. This article presents the protocol of the LILITH study (Evaluation of the Effect of Lomitapide Treatment on Major Adverse Cardiovascular Events in Patients with Homozygous Familial Hypercholesterolemia), a multicenter, observational, retrospective–prospective cohort study. The study aims to compare the incidence of MACE during the first three years of lomitapide treatment with that observed in the three years preceding treatment, within the same cohort of adult HoFH patients (target N=72). Clinical data, including MACE, lipid levels, liver function, safety outcomes, and concomitant lipid-lowering therapies, will be collected. The primary analysis will apply McNemar's test to assess changes in MACE incidence pre- and post-treatment. This methodological approach enables the evaluation of long-term cardiovascular outcomes in a real-world setting for a rare disease population.

Keywords
Homozygous Familial Hypercholesterolemia; Lomitapide; Methodology; Observational Study; Major Adverse Cardiovascular Events; Rare Disease

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Introduction

Homozygous Familial Hypercholesterolemia (HoFH) is a rare and life-threatening genetic disorder characterized by extremely elevated plasma levels of low-density lipoprotein cholesterol (LDL-C) from birth. The chronic and severe LDL-C burden leads to accelerated atherosclerosis and early-onset cardiovascular disease, often manifesting within the first two decades of life, with a significant impact on life expectancy and quality of life [1-5]. In light of this high-risk profile, the recently published international guidelines underscore the importance of early diagnosis and the attainment of ambitious LDL-C targets (<1.4 mmol/L or even <1.0 mmol/L in the presence of established cardiovascular disease) [1]. In order to achieve these rigorous therapeutic goals, guidelines advocate a stepwise, mechanism-oriented treatment approach. Initial interventions focus on LDL receptor–dependent agents, such as high-intensity statins, ezetimibe, and PCSK9 inhibitors. In cases where the response is deemed to be suboptimal, it is advised that escalation to receptor-independent therapies is considered. Among these, lomitapide, a microsomal triglyceride transfer protein (MTP) inhibitor, occupies a pivotal position due to its unique mechanism of action and demonstrated efficacy in this patient population [1, 6]. Lomitapide is a small molecule inhibitor of the microsomal triglyceride transfer protein (MTP) that effectively reduces the production of apoB-containing lipoproteins in the liver and intestine. Its mechanism of action bypasses LDL receptor pathways, making it particularly suitable for HoFH patients with minimal or absent receptor

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function. Lomitapide has received approval from both the FDA and EMA for use in HoFH, and has demonstrated substantial LDL-C reductions of approximately 50% in both pivotal phase 3 trials and real-world clinical settings [7-1 2]. While the lipid-lowering efficacy and safety of lomitapide are well established [1 3], robust data on its impact on major adverse cardiovascular events (MACE) are still lacking [9, 1 4]. Given the rarity of HoFH (estimated prevalence 1:300,000–500,000) and the ethical constraints of withholding effective therapy, randomized controlled trials (RCTs) are unfeasible in this setting. In order to address this critical evidence gap, the LILITH study was designed as a multicentre, observational, retrospective–prospective cohort study. The aim of the study is to evaluate the incidence of MACE in HoFH patients receiving lomitapide in their routine clinical settings.

Methods

Study design and rationale

LILITH is an observational, multicenter, open-label study with both retrospective and prospective data collection phases. The core design involves comparing the incidence of MACE within the same cohort of patients during two distinct 3-year periods: the three years immediately preceding the initiation of lomitapide treatment (pre-treatment period) and the first three years of lomitapide treatment (treatment period). This intra-patient comparison design was chosen to minimize confounding by indication and by stable patient characteristics, which is crucial in observational studies, especially when a concurrent control group is unavailable, as is typical in rare diseases like HoFH. The study involves multiple lipid centers across Europe and potentially other regions, coordinated by Fondazione SISA (Sponsor). The assignment of patients to lomitapide treatment is determined by routine clinical practice and is independent of study participation. No additional diagnostic or monitoring procedures beyond standard care and lomitapide prescribing information are mandated by the protocol.

Study population

Eligible participants are adult patients (≥18 years) with a clinical or genetic diagnosis of HoFH who have been treated with lomitapide

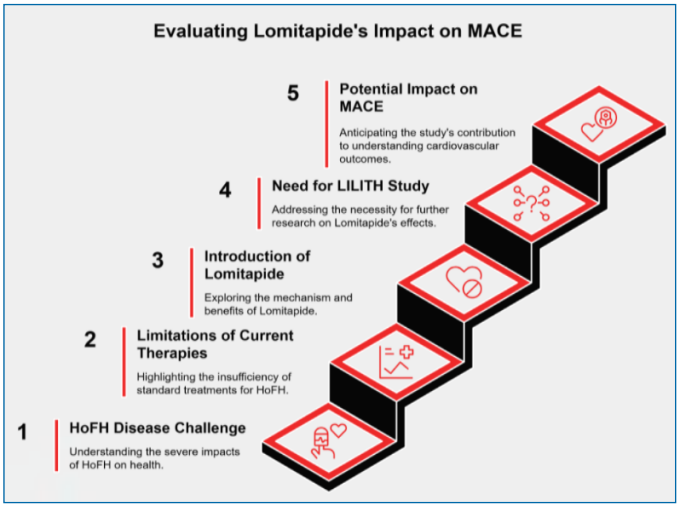

Figure 1 | Schematic representation of the rationale and context of the LILITH study: from the clinical challenge posed by HoFH and the limitations of current therapies, through the introduction of lomitapide, to the need to evaluate the impact of treatment on MACE.

(at any dosage according to prescribing information) for at least 12 months at the time of enrollment. Crucially, patients must have available medical records covering the three years prior to starting lomitapide to allow for retrospective MACE assessment. Patients receiving lomitapide as part of a clinical trial or receiving other investigational agents (other than lomitapide) are excluded. Written informed consent is mandatory prior to any data collection for the study. The target sample size is approximately 72 patients, based on power calculations using preliminary MACE data from European HoFH cohorts [8, 9] (see Section 2.6).

Study periods and data collection

The study encompasses three main periods for data collection:

- **Pre-Treatment period (retrospective):** Data covering the 3 years prior to the first lomitapide prescription (baseline visit) are collected retrospectively from patient medical records. Key data include demographics, medical history (including HoFH diagnosis confirmation), MACE, lipid profiles (total cholesterol, HDL-C, triglycerides, calculated LDL-C), liver function tests (ALT, AST, GGT), and details of all prior lipid-lowering therapies (including LA). Data are collected for baseline and at least one time-point in each of the three preceding years (Y-3, Y-2, Y-1).
- **Treatment period (retrospective/prospective):** Data are collected for the first three years following lomitapide initiation (Y+1, Y+2, Y+3). Data from the first 12 months are collected retrospectively at enrollment. Subsequent data up to Year 3 may be collected retrospectively or prospectively, depending on the patient's enrollment date relative to their lomitapide start date. For patients who have already completed 3 years of treatment at enrollment, data collection can be extended up to 5 years (Y+4, Y+5). Data collected include MACE events, Adverse Events (AEs), Serious Adverse Events (SAEs), Adverse Drug Reactions (ADRs), lipid profiles, LFTs, concomitant medications (especially lipid-lowering therapies), vital signs, weight/BMI. Specific safety monitoring follows lomitapide prescribing information. Exploratory data include liver imaging (ultrasound/MRI for steatosis, elastography/Fibroscan for stiffness, where available per clinical practice), additional biomarkers (ApoB, Lp(a), hsCRP, etc. at baseline and Y+3/Y+5 if available), dietary habits, and medication adherence questionnaires.
- **Post-Study Follow-up:** For patients contributing prospective data, a phone call is made 30 days after their final visit (end of Y+3 or Y+5) to capture any ongoing SAEs or new ADRs/special situations.

Data are entered by site personnel into a secure, web-based electronic Case Report Form (eCRF) compliant with regulatory standards. Source data verification and monitoring are performed by a designated Contract Research Organization (CRO). Medical terms are coded using MedDRA and WHO-ATC dictionaries.

Study Endpoints

- **Primary Endpoint:** The primary outcome is the incidence of MACE during the first three years of lomitapide treatment compared to the incidence during the three years prior to initiation. MACE is defined as a composite of hospitalization for stable or unstable angina, acute myocardial infarction, coronary or carotid revascularization, aortic valve replacement, nonfatal ischemic stroke, transient ischemic attack (TIA), and cardiovascular death. Both first and recurrent events within each period are captured.
- **Secondary Endpoints:**
  - Incidence of 3-point MACE (CV death, nonfatal MI, nonfatal ischemic stroke).

Table 1 | LILITH study protocol procedures and data collection timeline relative to lomitapide initiation.

Protocol Procedures	Pre-Lomitapide			Baseline	On-Lomitapide					
Study time	**Year -3**	**Year -2**	**Year -1**	**First prescription**	**Year 1**	**Year 2**	**Year 3**	**Year 4**	**Year 5**	**Post Study Follow up Day 30 +/- 7 Phone call**
Informed Consent						X	X	X	X	
Inclusion/Exclusion Criteria						X	X	X	X	
Genotype	X									
Demographic Data (sex, age, ethnicity, height)	X									
Demographic Data (weight, BMI)	X	X	X	X	X	X	X	X	X	
Physical Examination and vital signs	X	X	X	X	X	X	X	X	X	
Medical History	X									
MACE	X	X	X	X	X	X	X	X	X	
Plasma lipids (Total Cholesterol, HDL Cholesterol, Triglycerides, LDL-C)	X	X	X	X	X	X	X	X	X	
Liver function test (ALT, AST, GGT)	X	X	X	X	X	X	X	X	X	
Laboratory†				X		X		X		
SAEs	X	X	X	X						
Adverse Events, ADRs, SAEs and Special Situations					X	X	X	X	X	X
Prior and Concomitant Medications	X			X	X	X	X	X	X	
Prior and Concomitant Lipid Lowering Therapies	X	X	X	X	X	X	X	X	X	
Liver MRI or ultrasound				X			X		X	
Liver Elastography				X			X		X	
Medication Adherence Scale						X	X	X	X	
Food frequency questionnaire						X	X	X	X	

  - Incidence of 4-point MACE (3-point MACE + coronary revascularization).
  - Changes in plasma lipids (LDL-C, TC, HDL-C, TG) and LFTs (ALT, AST, GGT) at Y+1, Y+2, Y+3 versus baseline.
  - Changes in concomitant lipid-lowering therapies, including discontinuation or frequency reduction of LA and use of other agents like PCSK9 inhibitors or evinacumab, at Y+1, Y+2, Y+3 versus baseline.
  - Lipid and LFT values during the pre-treatment period, particularly at times when new therapies were initiated.
- **Exploratory Endpoints:** These include changes in additional biomarkers (ApoB, Lp(a), hsCRP, FIB-4, CK-18F etc.), liver steatosis/stiffness assessed by imaging, dietary patterns, and medication adherence, where available. For patients with extended follow-up (Y+4, Y+5), descriptive analysis of MACE, clinical, biochemical, and safety data will be performed.

MACE Adjudication

Given the critical nature of the primary endpoint (MACE) and the potential subjectivity in classifying cardiovascular events based only on local documentation, the protocol mandates a centralized and independent adjudication process. All potential MACE identified by site personnel in the medical records, both in the pre-treatment and treatment periods (3 or 5 years), are reported centrally. An independent Clinical Events Committee (CEC), composed of three expert cardiologists external to the study and without conflicts of interest, will blindly review all relevant source documentation for each potential event (e.g., discharge summaries, ECG reports, imaging results, cardiac enzymes, etc.). The CEC will rigorously apply the predefined MACE endpoint definitions established in the protocol and decide by consensus whether the event meets these criteria. This standardized process is crucial to ensure the reliability, consistency, and objectivity of the primary outcome assessment across different centers and over time.

Statistical Analysis Plan

The primary statistical analysis will compare the proportion of subjects experiencing at least one MACE event during the 3-year treatment period with the proportion of the same subjects experiencing at least one MACE during the 3-year pre-treatment period. McNemar's test for paired nominal data will be used for this comparison, which is the appropriate statistical test for evaluating changes in a dichotomous variable (presence/absence of MACE) measured twice in the same subject (before and after the intervention). The analysis will be two-sided, with a statistical significance level (alpha) set at 0.05. All patients for whom valid MACE data are available for both observation periods will be included in this primary analysis. Secondary continuous endpoints (such as lipid levels and liver enzymes) will

be summarized using standard descriptive statistics (mean, standard deviation, median, interquartile range). Changes from baseline (time of first lomitapide prescription) to the various follow-up time points (Y+1, Y+2, Y+3, and, if applicable, Y+5) will be analyzed using statistical tests for paired data, such as the paired t-test (if data follow a normal distribution) or the Wilcoxon signed-rank test (for non-normally distributed data). Changes in secondary categorical endpoints (e.g., discontinuation of LDL apheresis) will be described using absolute frequencies and percentages, and comparisons between pre- and post-treatment periods may utilize McNemar's test or similar methods for paired categorical data. Safety analyses will include descriptive summaries of the frequency and type of AEs, SAEs, ADRs, and changes in safety-related laboratory parameters (especially ALT, AST, GGT). Exploratory endpoints will be analyzed primarily descriptively. The handling of missing data will be detailed in the Statistical Analysis Plan (SAP). The planned sample size of 72 patients was calculated to provide approximately 80% power to detect a difference in MACE incidence between the two observation periods, assuming event rates similar to those observed in preliminary analyses of European cohorts (e.g., a reduction from ~23% in the pre-lomitapide period to ~11.5% in the post-lomitapide period), with a concordance probability (absence of events in both periods or presence in both) of 88%, using a two-sided McNemar's test at an alpha level of 0.05 [9]. A detailed Statistical Analysis Plan (SAP), specifying all planned analyses, analysis populations, and statistical methodologies, will be finalized before database lock.

Ethical Considerations

The LILITH study protocol has been written in full compliance with the ethical principles enshrined in the Declaration of Helsinki and with the international guidelines for Good Clinical Practice (ICH GCP). Before patient enrollment or any study-specific data collection can begin at a given site, the protocol, the patient informed consent form, and all other participant-facing materials must receive formal approval from the competent independent Ethics Committee (EC) or Institutional Review Board (IRB) for that site, and/or the national regulatory authority, according to local regulations. All participants must provide written, free, and voluntary informed consent after receiving comprehensive explanations about the study. Consent must be obtained before performing any procedure or collecting any information specifically required by the protocol. The confidentiality of patients' personal data is ensured through the use of a unique anonymized subject identification code and secure data management and storage practices, in compliance with data protection regulations as the GDPR in the European Union.

Discussion

The LILITH study employs a specific methodological approach—an observational, multicenter design with retrospective and prospective components and an intra-patient comparison—to evaluate the long-term impact of lomitapide treatment on MACE incidence in the rare and complex HoFH patient population. This design was chosen as a pragmatic and feasible alternative to an RCT, considered unfeasible in this setting for the reasons previously outlined (disease rarity, ethics). The main strength of the intra-patient comparison lies in its inherent ability to effectively control for stable individual-level confounders, i.e., patient characteristics that do not change or change very slowly over time, such as genetic background, disease severity at diagnosis, sex, and chronic comorbidities established before the start of the pre-treatment observation period. This type of control is particularly valuable in observational studies. However, this design is not without potential limitations. A significant limitation is the risk of time-dependent confounding: changes in other therapies (e.g., introduction of new lipid-lowering drugs other than lomitapide), lifestyle, or the status of other comorbidities that may occur differentially between the pre-treatment and treatment periods, and which could influence MACE risk independently of lomitapide. Another potential limitation is the risk of bias in retrospective data collection, such as missing data or incomplete documentation of events, although the use of relatively objective endpoints like MACE (especially if hospitalized) and, crucially, the centralized and independent adjudication process, were implemented precisely to mitigate this risk and standardize outcome assessment. Selection bias is also possible if patients who survive and remain on lomitapide treatment for 3 years systematically differ from those who initiate it. The combination of retrospective and prospective data collection offers a balance between the ability to assess outcomes relatively quickly (by leveraging historical data) and the opportunity to collect more detailed and potentially higher-quality prospective data on aspects like treatment adherence, adverse events, and dietary habits, which may be difficult to reconstruct accurately retrospectively.

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TechNote - Minimum Preanalytical Information for the Publication of Studies on Circulating Cell-Free microRNA-based Biomarkers

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ABSTRACT

Circulating cell-free microRNAs (miRNAs) are emerging as promising biomarkers with broad potential for clinical applications. However, pre-analytical variability significantly affects miRNA quantification and hampers reproducibility across studies. This work aims to define the Minimum Preanalytical Information required for the publication of studies on circulating cell-free miRNA-based biomarkers. We review key pre-analytical factors that influence circulating miRNA levels quantified using RT-qPCR. Critical variables include blood collection timing, sample type, centrifugation protocols, transport and storage conditions, hemolysis, lipemia, medication, physical activity and pathogen inactivation methods. We introduce a standardized checklist to promote methodological transparency and inter-study comparability. The final aim is to enhance the reliability of miRNA-based biomarker research and support its successful translation into clinical practice.

Keywords
Biomarker; microRNA; pre-analytical variables; RT-qPCR

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Background

Circulating cell-free microRNAs (miRNAs) have emerged as a promising tool with potential applications in clinical decision-making [1]. Their accessibility through non-invasive or minimally invasive methods, combined with the availability of biobanked samples and measurement techniques, has facilitated the extensive exploration of these short transcripts for biomarker development.

Despite extensive research efforts, integrating miRNA-based biomarkers into routine clinical practice remains challenging. A major barrier to the successful translation of circulating miRNAs into reliable biomarkers is the lack of reproducibility across studies from different laboratories. Variability can be introduced at several stages of the process from sample collection, storage, sample processing, RNA isolation, miRNA quantification and data analysis [2].

To improve reproducibility in RNA-based biomarker research, the scientific community is actively working toward methodological standardization and the implementation of quality control measures

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[3,4]. The establishment of guidelines for best practices and standard operating procedures (SOPs) ensures consistency across studies. Additionally, transparent and detailed reporting of methods and results is critical to facilitate replication and identify potential sources of variability. The methods section of a scientific paper is pivotal in this respect, providing information about the experimental protocols. This section must include enough detail to allow readers to assess the reproducibility and robustness of the study, such as descriptions of the study population, sample types, experimental design, data acquisition and analysis protocols.

In this TechNote, we examine and discuss the pre-analytical parameters that should be detailed in the methods section of studies on circulating cell-free miRNA-based biomarkers, with a particular focus on plasma and serum samples and the gold-standard RT-qPCR methodology. This TechNote also proposes a comprehensive checklist covering the minimum pre-analytical information that should be reported in publications focused on circulating cell-free miRNA-based biomarkers (**Table 1**) [based on the MIQE guidelines [5]].

The TechNote has been developed under the auspices of the AtheroNET COST Action (CA21153, https://atheronet.eu/), which represents an international and interdisciplinary network dedicated to accelerating the utilization of multiple omic technologies to bring novel paradigms in prevention, diagnosis and treatment of atherosclerotic cardiovascular disease [6].

Minimal Preanalytical Information

Timing for Blood Sampling and Processing

The timing of blood sample collection is a key pre-analytical variable, typically governed by standardized protocols recommending an 8- to 12-hour fasting period in the early morning. This helps minimize the impact of postprandial states (e.g., lipemia) and circadian fluctuations. However, timing presents a particular challenge in studies involving acute cardiovascular events, as variability in patient admissions, treatment initiation and diagnostic procedures significantly impact the timing of sample collection (see Section 2.7 on Medication).

The interval between blood collection and subsequent processing (e.g., plasma/serum preparation, aliquoting and storage) should also be considered. Processing delays alter miRNA levels, although the degree of impact varies between individual miRNAs. A study analyzing 179 miRNAs in plasma samples processed at 30 minutes, 2, 6 and 24 hours post-collection demonstrated that processing time was a major source of variability [7]. Out of the total panel of miRNAs analyzed, 53 showed significant changes, including commonly used endogenous controls such as miR-16-5p, miR-25-3p and miR-223-3p.

To reduce variability and improve data reliability, samples should be processed within consistent timeframes using standardized protocols. This approach minimizes potential changes in circulating miRNA levels due to differences in storage time or conditions.

Sample Type

Clearly identifying the sample type is essential to account for the effects of different anticoagulants and stabilizers on miRNA quantification. Various blood collection tubes are available, including those designed for serum (with clot activator and separation gel) and plasma (e.g., EDTA, sodium citrate, heparin, oxalate). Additionally, specialized systems developed for molecular diagnostics are available. BD Vacutainer® PPT™ is a closed system allowing separation and storage of undiluted EDTA plasma in the primary blood collection tube. Other examples include the PAXgene® Blood RNA Tube, Streck RNA Complete BCT®, and cf-DNA/cf-RNA Preservative Tubes (Norgen Biotek Corp), which aim to preserve RNA integrity. Consistency on the type of collection tubes is important to avoid introducing pre-analytical variability, since tubes containing RNA preservatives will result in different stability of the RNA, even under short processing times, from those that do not.

While tubes coated with RNA preservatives may appear ideal, recent findings from the exRNAQC consortium reveal a nuanced reality. In a comprehensive study on biofluids (serum and plasma) across three different time intervals post-blood collection, the impact of blood collection tubes on downstream extracellular RNA (exRNA) sequencing was evaluated [8]. Intriguingly, preservation tubes marketed for stabilizing extracellular nucleic acids for 7 to 14 days exhibited compromised stability over time compared to non-preservation tubes. The study concluded that the tested preservation tubes are unsuitable for exRNA analysis within the examined time intervals. Citrate tubes were recommended for extracellular analysis and pro-

Table 1 | A comprehensive checklist for the publication of circulating cell-free miRNA-based biomarker studies.

Item	Description	Level of Importance
1. Timing for Blood Sampling and Processing	Specify the time of day when samples were collected and the time span for blood sample processing	1
2. Sample Type	Clearly describe the type of sample and blood collection tubes	1
3. Centrifugation Protocol	Provide information on the centrifugation protocol	1
4. Sample Transport and Storage	Detail the conditions and procedures for sample transportation Specify the conditions and duration of storage Detail the number of freeze-thaw cycles	2
5. Hemolysis	Report on the assessment and presence of hemolysis	1
6. Lipemia	Report on the assessment and presence of lipemia	1
7. Medication	Document the use of medications taken by sample donors	2
8. Exercise	Record physical activity prior to blood sampling	3
9. Sample Pretreatment	Detail the specific procedures applied to biological samples	1

This checklist is designed to delineate the minimum pre-analytical information that should be addressed and reported in the Methods section of publications reporting results from circulating cell-free miRNA-based biomarker studies. This checklist was developed based on the MIQE guidelines [5].

cessing tubes within four hours of blood draw were advised for exRNA analysis.

The choice of blood derivatives for miRNA investigation significantly influences study outcomes, as evidenced by the fact that different blood derivatives yield distinct profiles. Serum and plasma miRNA content differs due to the release of miRNAs during the coagulation processes in serum, limiting comparisons between these sample types [9]. Regarding blood processing timing (see Section 2.1), evidence indicates that, for certain miRNAs, variance due to delayed sample handling may be partially attributed to *in vitro* platelet activation [10]. Furthermore, differences in hemolysis levels have been observed based on the type of anticoagulant used, with notable variation reported among EDTA, citrate and CTAD [10].

It is important to note that heparin interferes with PCR reactions [11], as it binds calcium and magnesium ions, essential components of the PCR master mix. Consequently, when isolating RNA for RT-qPCR, heparin-containing tubes should be avoided [12]. Citrate, on the other hand, may dilute plasma and potentially increase hemolysis.

Centrifugation Protocol

Providing a detailed description of the centrifugation conditions used to obtain plasma or serum is critical to ensure reproducibility and precision in miRNA research. Protocols for obtaining serum, plasma or platelet-poor plasma (PPP) vary widely among laboratories, especially in clinical settings. The process of obtaining plasma (platelet-rich plasma; PRP) typically involves centrifugation of whole blood at 1,200 to 2,000 g for 10 to 15 minutes. For PPP, an additional centrifugation step is required, with protocols varying in duration (10 to 20 minutes) and speed (2,000 to 5,000 g). Establishing the optimal protocol for obtaining plasma prevents contamination by platelet-enriched RNAs. Platelets, known to harbor a significant amount of RNAs, release these transcripts during activation and destruction, causing notable changes in the transcriptomic landscape. Indeed, Krammer et al. [13] demonstrated that miRNA profiles in PRP cluster closely with those in platelets. Additionally, the study of Mitchell et al. [14] showed that freeze–thaw cycles influence plasma miRNA profile, if not prepared as PPP. The reason for this is that miRNAs are released from the residual blood cells and contaminate the sample [15].

Efforts have been made to standardize centrifugation procedures. The International Society on Thrombosis and Haemostasis [16] defined the protocol for obtaining PPP. Whole blood samples should be double-centrifuged: firstly, at 2,500 g for 15 min at room temperature. Afterwards, plasma should be collected in a plastic tube, leaving 1 cm of plasma above the buffy layer and cautiously handled to avoid disturbance and centrifuged for a second time at 2,500 g for 15 min at room temperature and collected afterwards into a new plastic tube, leaving approximately 100 µL at the bottom of the plastic tube. For miRNA analysis, Chan et al. [17] propose a dual-spin protocol for processing PPP: an initial spin at 1,500 g for 15 minutes followed by a second spin at 2,500 g for 15 minutes, both conducted at room temperature. While some protocols suggest a second centrifugation at 10,000 (or even 16,000) g, the feasibility of employing such high-speed centrifuges in clinical settings may be limited. In summary, careful consideration of centrifugation parameters ensures rigorous blood processing protocols.

It is also important to consider the impact of centrifugation parameters when specific miRNA carriers, such as lipoproteins, are being analyzed. Lipoproteins share biophysical properties with exosomes, which complicates the interpretation of miRNA profiles [18].

Sample Transport and Storage

Transporting samples is a significant aspect, particularly when blood collection is conducted at clinics distant from the research center or in multicenter studies with samples sent from several centers to a central facility. Transport extends the time before blood processing, increasing the risk of introducing variability if not managed under proper conditions [19]. Glinge et al. [20] examined the effects of physical disturbance during transport on miRNA stability. Their study found that one hour of disturbance did not affect miRNA levels, but eight hours of disturbance led to a decrease in miR-1-3p and miR-21-5p levels in separated plasma and in miR-21-5p levels in serum whole blood. This highlights the importance of minimizing prolonged physical disturbance during transport to maintain the integrity of samples.

RNA integrity relies on careful management of storage conditions. RNA is highly vulnerable to degradation from RNase activity, making immediate processing or appropriate storage essential. While cooling samples to 4°C temporarily slows enzyme activity, it is important to note that this temperature could activate platelets. For long-term preservation of RNA, storage at -80°C is considered the gold standard, particularly for extended periods. However, this presents challenges in clinical settings where access to this type of freezers may be limited.

In a study conducted by Chan et al. [17], the stability of miRNAs in serum samples was rigorously assessed under various storage conditions. Samples were stored at 25°C for 3 days, 4°C for 3, 7 and 30 days, -20°C for 3, 7, 30, 90, 180, 270 and 360 days and -80°C for 3, 7, 30, 90, 180, and 360 days. The findings indicated long-term storage at -80°C significantly extended miRNA stability, though an exception was observed in whole blood samples stored for nine months. In contrast, plasma samples demonstrated stable miRNA levels, even with extended storage. Freeze-thawing up to four cycles affected only one out of eight miRNAs [21], suggesting that freeze thawing should be avoided since the effect will not uniformly affect all miRNAs.

To mitigate these stability issues, it is recommended that plasma or serum samples be aliquoted before being stored at -80°C. This prevents repeated freeze-thaw cycles, which degrades miRNA. Proper aliquoting and storage practices ensure that samples remain intact and suitable for reliable miRNA analysis even after extended periods.

Hemolysis

Hemolysis, resulting from the rupture of red blood cells (during blood collection and processing), is a confounding factor that alters the circulating miRNA pool. This disruption releases a large quantity of intracellular RNA into the plasma or serum, leading to potentially misleading results in miRNA analysis. miRNAs that are highly concentrated in red blood cells, including miR-20b-5p, miR-363-3p and miR-451, serve as effective biomarkers for detecting hemolysis and assessing sample contamination [17]. Their presence in plasma or serum may not reflect true extracellular levels but rather contamination due to hemolysis.

In light of this, it is important to include routine checks and, whenever possible, quantification of hemolysis levels [22]. The method employed for hemolysis monitoring and whether hemolysis affects the miRNA candidate should be disclosed in the publication. As the field advances, continuous attention to hemolysis assessment will undoubtedly enhance the robustness of pre-analytical considerations in miRNA studies.

Lipemia

There is a notable lack of studies that comprehensively evaluate the impact of lipemia on circulating miRNAs. Lipoproteins, which

act as important extracellular carriers of miRNAs [23], could influence the circulating miRNA profile, particularly under conditions of elevated lipoprotein levels, such as the postprandial state. Among them, high-density lipoprotein (HDL) particles play a pivotal role contributing significantly to the regulation of the circulating miRNA landscape [24]. Recent evidence further indicates that HDL particles are also capable of transporting long noncoding RNAs (lncRNAs), suggesting that lipemia could affect a broader spectrum of circulating noncoding RNAs (ncRNAs) [25]. Despite these potential interactions, the direct influence of lipemia on ncRNA isolation and analysis remains insufficiently explored.

Lipemia is often indicative of underlying metabolic or cardiovascular diseases and presents a particular challenge in clinical studies since it cannot always be effectively controlled through standard 12-hour fasting protocols. Consequently, it is essential to disclose the presence of lipemia in the samples and, whenever feasible, quantify its extent and be aware of differences in circulating miRNAs that may be attributed to differences in lipoprotein levels.

Medication

Certain acute therapies administered in clinical situations, such as during emergency hospitalizations for myocardial infarction or prior to surgical/diagnostic interventions (e.g., coronary angiography, elective percutaneous coronary intervention, percutaneous balloon angioplasty or surgical myocardial revascularization), may influence the circulating miRNA profile. One key example is anticoagulation therapy with heparin, often administered upon admission but before blood sampling. While the necessity of these practices is acknowledged in clinical care, it is important to document and report their use with precision.

Heparin is known to interfere with miRNA quantification, particularly when using qPCR [26,27]. This interference is especially relevant in patients undergoing treatments for conditions such as myocardial infarction, where the timing and dosage of heparin therapy significantly affects blood sample composition. The concentration of heparin in the blood at a given time point depends on several factors, including the dosing schedule and individual pharmacokinetics, which introduces variability in miRNA measurements.

To mitigate the confounding effects of heparin, different heparinase enzymes could be used (either to plasma or RNA) prior to miRNA quantification. Heparinase treatment has been shown to effectively counteract the influence of heparin, thereby enhancing the accuracy of miRNA quantification [28,29]. As such, it is important to acknowledge and report the use of heparin in study subjects, including details on the timing of administration relative to blood sampling, heparin dosage and the method used to neutralize its effects.

Beyond heparin, other commonly prescribed cardiovascular medications may also influence circulating miRNA levels. For example, statin therapy has been shown to modify the expression profile of plasma exosome-derived miRNAs [30]. Likewise, antiplatelet agents such as aspirin affect platelet-derived miRNAs, thereby altering circulating miRNA profiles [31]. Recognizing these potential effects is essential, and future studies should systematically document concomitant pharmacological treatments to account for their possible impact on circulating ncRNA measurements.

Exercise

Existing research underscores the substantial influence of acute or chronic exercise on circulating miRNAs associated with cardiovascular health [32,33]. The analysis by Barber et al. [34] involved 20 previously sedentary adults from the HERITAGE Family Study and measured the expression of 53 miRNAs related to cardiovascular disease in serum collected at baseline and after 20 weeks of endurance exercise training. The study found that regular exercise resulted in significantly decreased mean serum expression of nine miRNAs (let-7b-5p, let-7e-5p, miR-7-5p, miR-25-3p, miR-29b-3p, miR-29c-3p, miR-92a-3p, miR-93-5p and miR-486-5p) and increased mean expression of five miRNAs (miR-27b-3p, miR-126-3p, miR-142-3p, miR-146a-5p and miR-221-3p).

These findings highlight how regular physical activity, even moderate or casual exercise, alters the circulating profile of specific miRNAs. In this context, it becomes recommended to record and disclose all forms of physical activity prior to blood sampling.

Sample Pretreatment

In specific situations, biological samples require pretreatment procedures to ensure safety during handling, particularly when dealing with pathogens that present significant health risks, such as during the recent COVID-19 pandemic. In such cases, a previous step involves the inactivation of infectious agents to mitigate transmission risks without compromising the integrity of RNA for subsequent molecular analyses.

For RNA isolation, reagents containing guanidine salts and phenol are widely employed and serve the dual purpose of inactivating pathogens and preserving RNA integrity. These reagents effectively prevent RNA degradation and maintain the reliability of molecular profiling. However, alternative inactivation protocols have also been proposed, including heat treatment at temperatures up to 70°C and the use of surfactants like Triton X-100.

Despite their potential for safe sample handling, the effects of these alternative inactivation methods on RNA isolation efficiency and downstream detection processes remain unclear. Heating, for example, may affect RNA structure or integrity. Given this uncertainty, it is crucial to carefully document the inactivation method used in sample processing.

Conclusions

The successful translation of circulating miRNAs into reliable clinical biomarkers depends on strict adherence to Good Laboratory Practice (GLP) principles. Following standardized procedures and ensuring meticulous sample handling greatly improves the reliability and reproducibility of results.

To enhance transparency and facilitate reproducibility, it is essential that all relevant information be thoroughly and accurately documented in the Methods section of scientific publications. By adopting and prioritizing these best practices, the scientific community strengthens confidence in the clinical utility of circulating miRNAs and accelerate their integration into diagnostic and prognostic workflows.

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Conflict of interest

YD has filled patents related to the use of miRNAs for diagnostic and therapeutic purposes and is a member of the Scientific Advisory Board of the molecular diagnostic company Firalis SA.

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Progress toward Implementing Multiomic Approaches in Atherosclerotic Cardiovascular Disease: Update from the 4th AtheroNET Meeting in Sarajevo (Bosnia and Herzegovina)

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ABSTRACT

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The COST Action CA21153 "Network for Implementing Multiomic Approaches in Atherosclerotic Cardiovascular Disease Prevention and Research" (AtheroNET; https://atheronet.eu) was launched in October 2022 with the mission to accelerate the application of multiomics in atherosclerosis research and to foster collaboration among experts from diverse disciplines. Born from the pressing need to advance atherosclerosis management and overcome existing challenges, AtheroNET creates a collaborative arena where innovation thrives through dialogue. The initiative was spearheaded by Prof. Miron Sopić (Faculty of Pharmacy, University of Belgrade), the primary proposer of the Action. The Action is led by Chair Prof. Paolo Magni (Università degli Studi di Milano) and Vice-Chair Prof. Yvan Devaux (Luxembourg Institute of Health), with strategic support from a dedicated leadership team:

- **Grant Holder Scientific Representative:** Dr. Ines Potočnjak.
- **Science Communication Coordinator:** Prof. Georgios Karagigas.
- **Grant Awarding Coordinator:** Dr. Susana Novella.
- **WG1** - Pathophysiological mechanisms: applying multiomics to uncover novel pathogenic players and processes; Leader: Prof. Dimitris Kardassis.
- **WG2** - Personalized clinical models: translating omics insights into improved management of ASCVD; Leader: Prof. Alberico Catapano.
- **WG3** - Standardization and harmonization: developing SOPs and guidelines to enhance reproducibility across omics research; Leader: Dr. Marie Mardal.
- **WG4** - Data integration and ML/AI: optimizing algorithms for integrating complex multiomic datasets; Leader: Dr. Aleksandra Gruca.
- **WG5** - Dissemination and communication: sharing advances with scientists, clinicians, patients, and the public; Leader: Prof. Georgios Karagigas.

Since its inception, AtheroNET has grown to 475 members from across Europe, embracing COST's core values of inclusiveness and excellence across geography, gender, and career stage. A significant proportion of members come from Inclusive Target Countries (n=282), with strong representation of women (n=277) and early-career researchers (n=223).

The 4th MC/WG Meeting – Sarajevo, Bosnia and Herzegovina

On 14-16 May 2025, AtheroNET COST Action held its fourth in-person Management Committee and Working Group meeting in Sarajevo

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(Bosnia and Herzegovina), hosted by Kanita Karaduzović-Hadžiabdić at the International University of Sarajevo. The event gathered over 80 participants, including 44 MC members from across the EU (**Figure 1**). Hybrid participation ensured broad access, and 16 young researchers and investigators (YRIs) were selected to showcase their work through oral and poster presentations, four of which are published along with this report.

The scientific programme of the 4th AtheroNET MC/WG meeting was crafted to reflect the breadth and depth of multiomics research in atherosclerosis, guiding participants on a journey from fundamental biological mechanisms to clinical translation. Presentations and discussions explored how molecular insights into inflammation, vascular biology, metabolic regulation, and tissue-specific changes can be uncovered and integrated using advanced omic technologies. Throughout the meeting, emphasis was placed on linking these discoveries to tangible improvements in patient care – from refining diagnostic markers and identifying novel therapeutic targets, to building predictive models that can guide personalized prevention strategies. A key feature of the programme was its focus on integration of disciplines, datasets, and perspectives. Rather than viewing biology, clinical science, and computational methods as separate domains, the meeting highlighted how their convergence is essential to unlocking the full potential of multiomics. Discussions repeatedly returned to the importance of harmonizing experimental approaches, ensuring reproducibility, and making effective use of artificial intelligence for data interpretation. This cross-cutting theme fostered a sense that the field is moving beyond isolated discoveries toward cohesive frameworks that can be adopted broadly and applied in real-world clinical settings.

A highlight of the programme was the keynote lecture delivered by Prof. Børge G. Nordestgaard, current President of the European Atherosclerosis Society (EAS). His presentation offered a comprehensive and thought-provoking perspective on residual lipid risk in atherosclerotic cardiovascular disease, stressing the critical importance of early and effective lipid-lowering strategies while pointing toward emerging therapeutic opportunities targeting triglyceride-rich remnant particles. The lecture not only synthesized the latest evidence but also set these insights in the context of global prevention strategies. Importantly, it reflected the close alignment between AtheroNET and the EAS in their shared mission to improve cardiovascular outcomes through research excellence, knowledge dissemination, and fostering the next generation of experts.

Roundtable sessions are a central feature of AtheroNET meetings, specifically designed to foster collaborative, open discussions that drive progress toward the Action's set deliverables. They provide a platform for targeted, multidisciplinary dialogue on topics that require integration of perspectives across basic, clinical, and computational research. Unlike formal presentations, these discussions encourage open exchange, critical questioning, and the identification of concrete, actionable steps. They also serve as incubators for collaborative projects, enabling participants to align methodologies, share resources, and explore emerging concepts in real time.

WG1 Roundtable – Perivascular Adipose Tissue and the Exposome in Atherosclerosis

This Roundtable was chaired by Dimitris Kardassis, Tijana Mitić, and Nuria Amigo. The discussion, led by Sònia Benítez González and Francesc Jiménez Altayó, explored how perivascular adipose tissue may function as an active modulator of vascular biology, influencing inflammation, vascular tone, and plaque development. This perspective challenges the traditional view of adipose tissue as a passive structural component. The conversation then broadened to the concept of the "exposome" – encompassing factors such as smoking, menopause, air pollution, and vascular ageing – and considered how multiomic approaches and machine learning could be leveraged to disentangle their complex, interrelated effects.

WG2 Roundtable – ApoB-Containing Lipoproteins in CVD: Integrating Omics

Led by Luis Masana and Liv Tybjærg Nordestgaard, this roundtable examined how multiomic strategies can refine our understanding of ApoB-containing lipoproteins in the pathogenesis of cardiovascular disease, also in light of the recent research and clinical interest in circulating lipoprotein (a) measurement and pharmacological management and the opportunity of lipidomics approaches in this area. Discussion was centered on how to uncover mechanisms underlying residual cardiovascular risk, with participants weighing the opportunities such integration offers against the methodological and interpretive challenges it poses.

WG3 Roundtable – Reproducibility in microRNA Biomarker Research

Chaired by Miron Sopić and David De Gonzalo Calvo, this session addressed the critical challenge of reproducibility in biomarker studies, with microRNA-based diagnostics serving as a focal example. AtheroNET is currently preparing its first large-scale inter-laboratory "ring trial," aimed at standardising pre-analytical, analytical, and post-analytical protocols for circulating microRNA analysis. The roundtable provided an opportunity to examine each step of the workflow in detail, drawing on the diverse experiences of participating laboratories. Discussions focused on identifying sources of variability, aligning

Figure 1 | Participants to 4th AtheroNET Meeting in Sarajevo (Bosnia and Herzegovina).

best practices, and working toward a consensus protocol that will be uniformly applied during the ring trial. This harmonised approach is expected to enhance the reliability, comparability, and translational value of microRNA biomarker research across the network.

WG4 Roundtable – ML/AI Pipelines for Multiomic Integration in ASCVD Prevention

Moderated by Artemis Hatzigeorgiou and Aleksandra Gruca, this roundtable considered the design of machine learning and AI pipelines for integrating multiomic data into personalised prevention models for atherosclerotic cardiovascular disease. Discussions highlighted the need for harmonised data structures, rigorous validation frameworks, and transparent algorithms to ensure clinical interpretability. The exchange exemplified AtheroNET's interdisciplinary ethos, drawing equally from computational sciences and clinical cardiology to chart a path toward implementation.

Recognising that its diverse and multidisciplinary membership offers fertile ground for high-impact collaboration, AtheroNET established a dedicated Grant Application Committee (Eric Biessen, Miron Sopić, Johannes Schmit, David De Gonzalo Calvo, Noemi Rotllan, and David Kreil). The committee's role is to actively monitor upcoming funding calls, identify opportunities aligned with AtheroNET's scientific priorities, and catalyse the formation of competitive EU-level consortia. This proactive approach has already borne fruit, resulting in the submission of two MSCA Doctoral Network proposals, five MSCA Postdoctoral Fellowship applications, one EpiPERMed proposal, and two Pathfinder calls. The Sarajevo meeting provided a valuable platform to advance this strategic agenda. A dedicated roundtable led by Erik Biessen and Miron Sopić brought together senior investigators and early-career researchers to exchange ideas on how to position AtheroNET's expertise within the evolving European funding landscape. Discussions centred on identifying high-priority research themes, mapping potential partnerships across disciplines and countries, and aligning proposals with the thematic priorities of upcoming Horizon Europe calls. Particular attention was given to opportunities in late 2025 and 2026, with the aim of maintaining momentum and ensuring AtheroNET remains at the forefront of multiomics-driven cardiovascular research at the EU level. This session not only strengthened the network's collective readiness to respond to competitive calls but also reinforced the culture of collaborative grant development as a core pillar of the Action's long-term impact.

AtheroNET places a strong emphasis on nurturing the next generation of scientists who will carry forward progress in atherosclerosis research. This commitment is reflected in the significant number of Young Researchers and Innovators (YRIs) involved in the Action (223 members), as well as in a range of initiatives designed to empower them both scientifically and professionally. In collaboration with the MSCA Staff Exchange CardioSCOPE, AtheroNET has organised 15 webinars on diverse and highly relevant topics, providing YRIs with direct access to leading experts in the field. These sessions are intended to transfer cutting-edge knowledge, expose early-career researchers to different perspectives, and inspire new lines of inquiry. Building on this effort, the MSCA SE *CardioSCOPE* project and AtheroNET jointly organised a 2024 training school, *"Omics for Precision Medicine in ASCVD"*, held in Heraklion, Crete, and will co-organise another in September 2025 in Milan, *"Shaping Scientific Careers in Cardiovascular Research: Within and Beyond Academia"*. Complementing these training initiatives, and inspired by the success of similar activities in the COST Action CardioRNA, AtheroNET has established its own Journal Club – a monthly event led by Melody Chemaly, Ignazio Fernando Hall, and Christina Pagiatakis. The Journal Club invites first or lead authors of high-impact publications in atherosclerosis to present their work, explain the scientific concepts and ideas underpinning it, and share insights into the peer review and revision process. This initiative not only promotes scientific literacy but also offers a rare behind-the-scenes view of academic publishing for emerging researchers. To further support hands-on research experience and international collaboration, AtheroNET funded 18 Short-Term Scientific Missions (STSMs) and 5 ITC congress grants to YRIs in 2024-2025. These mobility grants enabled YRIs to work in leading laboratories across Europe, strengthening their technical skills, expanding their professional networks, and fostering cross-institutional collaborations. At the Sarajevo meeting, YRIs who had completed STSMs presented their findings in dedicated sessions, complemented by 10 poster presentations from other early-career members. The meeting was concluded with awards for the best presentations, given to Melody Chemaly (Karolinska Institute), Veronika Boichenko (University of Verona), and Mannekomba Roxane Diagbouga (University of Geneva), who will be invited to return to the next AtheroNET meeting to update the network on their continuing research progress.

Beyond its rich scientific content, the 4th AtheroNET meeting in Sarajevo was also a celebration of the social bonds and friendships that make this network so vibrant. AtheroNET gatherings are not only about exchanging data and discussing methodologies – they are also an opportunity to reconnect with old colleagues, welcome new members into the community, share personal experiences, and forge lasting international friendships. This time, participants enjoyed a memorable guided tour of Sarajevo, a city poised at the crossroads of East and West, where Ottoman, Austro-Hungarian, and modern influences blend seamlessly. Its multicultural heritage and unique character seemed to mirror the very essence of AtheroNET – a network built on diversity, inclusiveness, and the blending of perspectives for a common goal. The closing dinner gathered all participants into a joyful celebration, where conversation turned to laughter and to music and dance beneath the shared rhythm of science and friendship.

Looking Ahead

As AtheroNET enters its final year, the focus sharpens on delivering the Action's ambitious objectives. The rapid evolution of single-cell and spatial omics, coupled with the transformative potential of AI, positions the network to break new ground in atherosclerosis research. The coming months will be dedicated to completing a series of concrete milestones that will define the Action's legacy.

One of the flagship outputs, the Cardio2Share database, is in the process of being launched and populated. This resource will serve as a centralised repository for multiomic and clinical data, facilitating data sharing, integration, and re-use across the network and beyond. In parallel, guidelines for the preparation of blood-based samples for multiomic analysis are being finalised, aiming to standardise protocols and ensure reproducibility across laboratories. The miRNA ring trial – the first large-scale inter-laboratory effort within AtheroNET – is being implemented to harmonise pre-analytical, analytical, and post-analytical steps in biomarker research.

Alongside these technical and infrastructural deliverables, AtheroNET is actively catalysing the formation of new research consortia around targeted scientific questions. These initiatives are designed to probe the intricate mechanisms underlying atherosclerosis and to develop innovative tools for personalised prevention and treatment. By consolidating know-how, fostering collaboration, and addressing unmet needs, AtheroNET is shaping the future of multiomics in cardiovascular science – advancing from current best practice toward bold, new horizons.

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Progress toward Implementing Multiomic Approaches in Atherosclerotic Cardiovascular Disease: Update from the 4th AtheroNET Meeting in Sarajevo (Bosnia and Herzegovina)

Functional and metabolomic characterization of human cell models to address obesity and metabolic dysfunction- associated steatotic liver disease pathogenetic mechanisms

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Introduction and Aim: Obesity and metabolic dysfunction-associated steatotic liver disease (MASLD) are global health concerns linked to an increased risk of atherosclerotic cardiovascular disease (ASCVD). Despite progresses in elucidating relevant pathophysiological mechanisms involved in obesity and MASLD, a comprehensive understanding toward ASCVD risk is missing due to the lack of reliable *in vitro* models reproducing obesity-related dysfunctional adipose tissue and MASLD.

This study focuses on developing and characterizing innovative *in vitro* models recapitulating key features of these conditions assessing novel molecular signatures for potential drug targeting.

Methods: Human-derived SW872 adipocytes, dysfunctional SW872 (SW872-OA) obtained by 7-days 100 µM oleic acid (OA), 17-days spontaneously differentiated SW872 (SW872-AUTO), human hepatoma HepG2 cells, and a HepG2-based steatotic-like model induced with 7-days 100 µM OA (HepG2-OA), were selected. All *in vitro* models were characterized using ^{1}H NMR spectroscopy, light-microscopy, spectrophotometry, flow cytometry, and RT-qPCR.

Results and Discussion: Metabolomic and lipidomic analysis allowed to identify specific metabolite signatures associated with dysfunctional patterns in both SW872 and HepG2 cell models. SW872- OA, SW872-AUTO, and HepG2-OA models showed increased lipid/triglycerides accumulation, confirmed by light-microscopy observations, and spectrophotometry quantification. HepG2-OA cells exhibited elevated triglyceride synthesis genes expression. Functional assays revealed reduced glucose uptake and elevated ROS production across all dysfunctional models. Trolox antioxidant treatment mitigated ROS levels in SW872 and HepG2 cells with minor effects on SW872-OA, SW872-AUTO, and HepG2-OA. Gene expression analysis (GEA) showed significant upregulation of oxidative stress-related genes in HepG2-OA cells. GEA in SW872-OA and SW872-AUTO cells revealed upregulation of genes involved in adipocyte differentiation, inflammation, and glycemic homeostasis.

Conclusion: These results showed that OA-treatment and SW872-spontaneous differentiation generate *in vitro* models recapitulating dysfunctional adipose tissue and MASLD.

Relevance for AtheroNET: Dysfunctional SW872 and HepG2 cell models show specific molecular, metabolomic and lipidomic signatures which may translate to innovative pharmacological targets for obesity, MASLD, and ASCVD.

Effects of Pro-Resolving Lipid Mediators on Nitric Oxide and Prostacyclin Pathways in Human Endothelial Cells

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Introduction and Aim: Inflammation plays a central role in the development and progression of atherosclerosis. Pro- resolving lipid mediators, including Resolvins (RvE1) and Maresins (MaR1) are key regulators of the resolution phase of inflammation. Despite their known anti-inflammatory effects, their impact on nitric oxide (NO) and prostacyclin (PGI2) levels in human umbilical vein endothelial cells (HUVECs) remains underexplored. This study investigates the effects of RvE1 and MaR1 on vascular inflammation, focusing on NO and PGI2 in HUVECs.
Methods: An in vitro inflammatory model was established by incubating HUVECs with lipopolysaccharides (LPS, 100 µg/mL) and interleukin-1 beta (IL-1β, 100 ng/mL) for 24 hours. RvE1 and MaR1 (100 nM) were then applied under the same conditions. Levels of tumor necrosis factor-alpha (TNF-α), total nitrite-nitrate, and the stable PGI2 metabolite 6- keto-PGF1α were measured in the incubation medium using ELISA.
Results and Discussion: LPS and IL-1β significantly increased TNF-α levels, confirming the inflammatory response. RvE1 and MaR1 significantly reduced nitrite-nitrate levels, suggesting their role in mitigating vascular inflammation. However, no significant changes were observed in 6-keto-PGF1α levels (n=3-4, p>0.05). These findings highlight the selective effects of RvE1 and MaR1 on different pathways involved in endothelial inflammation.
Conclusion: This preliminary study demonstrates that RvE1 and MaR1 appear to reduce nitrite-nitrate levels, their lack of impact on 6-keto-PGF1α suggests distinct pathway-specific actions that warrant further investigation. Future studies with larger sample sizes, varied inflammatory conditions, are essential to confirm these findings and better understand their therapeutic potential.
Relevance to AtheroNET: This research aligns with AtheroNET's goal of exploring novel anti-inflammatory strategies, emphasizing the importance of pro-resolving lipid mediators in vascular health.

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Proteomic analysis for prediction of type 2 diabetes identifies cardiovascular disease-related proteins

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Introduction and Aim: Cardiovascular disease (CVD) and type 2 diabetes (T2D) are interconnected chronic conditions which cause significant global health challenges and mortality. T2D is a risk factor for CVD characterized by insulin resistance and beta-cell dysfunction. This study aims to investigate the association between the blood plasma proteome and T2D in the Trøndelag Health (HUNT) Study.
Methods: The HUNT Study is a population-based cohort with four waves of enrollment beginning in 1984. 5,402 samples from HUNT3 were analysed with SomaScan including 3,221 on SomaScanv4.0 (~5,000 proteins) and 2,181 on SomaScanv4.1 (~7,000 proteins). T2D was defined based on self-reported disease from questionnaires. Binary logistic regression analysis was performed to test the associations of protein concentrations with T2D. Models included adjustment by sex, age, waist-hip-ratio (WHR), smoking status, and comorbidities.
Results and Discussion: We identified 584 proteins that are significantly associated with T2D. From these proteins, CILP2 and MXRA8 are associated with decreased risk, while PLXB2 and NFASC are associated with increased risk for having T2D. The most significant pathway from GO enrichment analysis includes proteins related to lipid catabolic process (FDR adjusted p-value: 0.000054). Indeed, several proteins significantly associated with T2D are known for their role in lipid metabolism, for instance Adiponectin (OR 0.55; 95% CI 0.46 - 0.65), Apo A – IV (1.57;1.37 - 1.81), Apo B (0.63; 0.55 - 0.73), Apo C – I (0.73; 0.64 - 0.84), and Apo D (0.49; 0.38 - 0.63).
Conclusion: The proteins and pathways identified represent insights into the underlying molecular mechanism of T2D and may serve as potential biomarkers for risk prediction and prevention for CVD.
Relevance for AtheroNET: The findings suggest that early identification of these proteins could mitigate cardiovascular risks in individuals with or at risk for T2D.

A validated and reproducible LC-MS/MS analytical method to quantify an emerging cardiovascular risk biomarker, trimethylamine N-Oxide (TMAO), in human plasma

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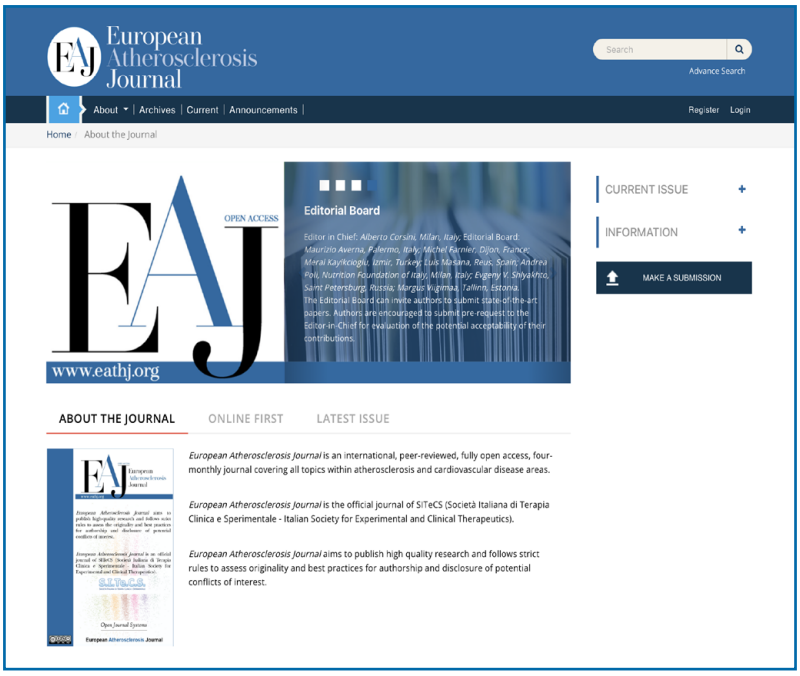
Introduction and Aim: The interplay between nutrition patterns, gut microbiota-derived metabolites and cardiovascular diseases (CVDs) has recently become a field of intensive research. Gut microbiota-derived metabolites represent an attractive source of biomarkers for CVDs, including atherosclerotic cardiovascular disease (ASCVD) and metabolic diseases. Trimethylamine (TMA), a gut-derived metabolite, undergoes oxidation in the liver, resulting in trimethylamine N-oxide (TMAO). Recent evidence from *in vivo* studies and clinical trials has correlated TMAO with increased CVD risk and occurrence due to the atherogenic potential of TMAO. TMAO has been quantified by exploiting mass spectrometry (MS) techniques, coupled either with liquid (LC) or gas chromatography (GC), and nuclear magnetic resonance (NMR) spectroscopy. However, standardization parameters and conditions still represent a critical issue when dealing with data reproducibility and robustness.
Hence, this study aimed at developing a validated and highly reproducible method for the quantification of TMAO in human-derived plasma.
Methods: A high-performance LC (HPLC) system (Shimadzu) coupled to tandem MS (MS/MS) (Sciex QTRAP 6500+) was selected, along with multiple reaction monitoring (MRM) modality and electrospray ionization (ESI) in positive polarity. Calibration curve was obtained spiking a healthy subject serum sample with different TMAO concentrations in the ng/mL range.
Results and Discussion: The HPLC-MS/MS method was optimized according to the different MS parameters and by selecting the most appropriate column for HPLC assessment. Method validation was performed evaluating the intra-/inter-assay accuracy and precision. The coefficient of variation (CV%) always resulted below 20%.
Conclusion: The HPLC-MS/MS method showed to be robust and reproducible according to standardization requirements, and useful to assess ASCVD risk in a cohort with subclinical atherosclerosis, food intake record and microbiome evaluation.[1]
Relevance to AtheroNET: TMAO data from characterized CVD patient cohorts, obtained in a reproducible and standardized manner, could be integrated by artificial intelligence/machine learning-based approach to improve CVD risk stratification toward precision medicine.

[1]Baragetti,A, et al. Gut Microbiota Functional Dysbiosis Relates to Individual Diet in Subclinical Carotid Atherosclerosis. Nutrients 2021, 13, 304. https://doi.org/ 10.3390/nu13020304.



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