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NARRATIVE REVIEW

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Increasing the complexity of lipoprotein characterization for cardiovascular risk in type 2 diabetes

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Abstract

The burden of cardiovascular disease is particularly high among individuals with diabetes, even when LDL cholesterol is normal or within the therapeutic target. Despite this, cholesterol accumulates in their arteries, in part, due to persistent atherogenic dyslipidaemia characterized by elevated triglycerides, remnant cholesterol, smaller LDL particles and reduced HDL cholesterol. The causal link between dyslipidaemia and atherosclerosis in T2DM is complex, and our contention is that a deeper understanding of lipoprotein composition and functionality, the vehicle that delivers cholesterol to the artery, will provide insight for improving our understanding of the hidden cardiovascular risk of diabetes. This narrative review covers three levels of complexity in lipoprotein characterization: 1—the information provided by routine clinical biochemistry, 2—advanced nuclear magnetic resonance (NMR)-based lipoprotein profiling and 3—the identification of minor components or physical properties of lipoproteins that can help explain arterial accumulation in individuals with normal LDLc levels, which is typically the case in individuals with T2DM. This document highlights the importance of incorporating these three layers of lipoprotein-related information into population-based studies on ASCVD in T2DM. Such an attempt should inevitably run in parallel with biotechnological solutions that allow large-scale determination of these sets of methodologically diverse parameters.

KEYWORDS
apoC-III, cardiovascular risk, diabetes, lipoprotein, LPS, NMR

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For affiliations refer to page 11.

Atherosclerotic cardiovascular disease (ASCVD) is the leading cause of mortality and morbidity in people living with type 2 diabetes mellitus (T2DM).[1] Adults with diabetes have a 2–4 times higher risk of suffering cardiovascular events than those without diabetes,[2] and the risk of dying from ASCVD is twice as high.[3] In addition to a higher prevalence of several classical risk factors, there are specific cardiovascular risk factors, including diabetes duration, coexistence of microvascular complications, higher prevalence of low-income, dyslipidaemia, obesity and/or abnormal fat distribution, among individuals with T2DM. This review focusses on the pathological accumulation of cholesterol in the arteries of individuals with normal or within the therapeutic target[4] low-density lipoprotein cholesterol (LDLc) and our incomplete understanding of this phenomenon characteristic of T2DM and other states of insulin resistance. The accumulation of cholesterol transported by lipoproteins in the arterial wall is an almost inescapable feature of ASCVD since only as little as 6%–8% of cases of myocardial infarction occur in the absence of atherosclerotic coronary artery disease.[5] However, it is not obvious that cholesterol-loaded atherosclerotic plaques are always the result of hypercholesterolaemia as more than half of the cases of myocardial infarction occur in individuals with desirable or within the LDL cholesterol concentration goal.[6] The causal link between dyslipidaemia and atherosclerosis in T2DM is complex, and our contention is that a deeper understanding of lipoprotein composition and functionality, the vehicle that delivers cholesterol to the artery, will provide the necessary insight to understand the hidden cardiovascular risk of diabetes. This narrative review covers three levels of complexity in lipoprotein characterization: 1—the information provided by routine clinical biochemistry, 2—advanced nuclear magnetic resonance (NMR)-based lipoprotein profiling and 3—the identification of minor components or physical properties of lipoproteins that can help explain arterial accumulation in individuals with normal LDLc levels, which is typically the case in subjects with T2DM.

1 | LIPID PARAMETERS THAT CAN BE OBTAINED IN A CLINICAL SETTING

1.1 | Triglycerides, HDLc, LDLc and Lp(a)

An increased prevalence of lipid abnormalities, which usually does not include elevated LDLc, contributes to the increased risk of ASCVD in individuals with T2DM. Typical analytical alterations include high triglyceride (TG) and free fatty acid levels and low high-density lipoprotein cholesterol (HDLc) concentrations.

On the other hand, HDLc has been suggested to have a U-shape association with cardiovascular disease and mortality in the general population,[7] and this association has also been found in T2DM.[8,9]

Nevertheless, approximately 40%–60% of subjects exhibit characteristics of the so-called diabetic or atherogenic dyslipidaemia and only approximately 25% are reported to be normolipidaemic.[10,11] Paradoxically, therapeutic interventions aimed at lowering TG levels[12,13] or increasing HDLc levels[14] have not consistently shown a decrease in cardiovascular events,[15] while therapy directed at lowering LDLc levels (even within the normal range) does, independently of the therapy used.[16,17] While this therapeutic strategy has been very effective, a large proportion of patients still have unexplained risk, the so-called residual risk, which requires further investigation.[1] Part of this residual risk can be explained by Lipoprotein (a) (Lp(a)) which is also an independent and causal risk factor for ASCVD in diabetes. The intriguing inverse association between Lp(a) concentration and risk of diabetes is still a matter of debate.[18]

1.2 | Apolipoprotein B, non-HDLc and remnant cholesterol

These findings all suggest an incomplete understanding of the mechanisms leading to cholesterol deposition and ASCVD in general and in T2DM. The accumulation of cholesterol in the arteries when the concentration of circulating LDLc is normal can be caused by other lipoproteins also transporting cholesterol, likely TG-rich lipoproteins (TRLs), since HDL has precisely the opposite function, or by physical or compositional characteristics of LDL, which make it prone to arterial infiltration and retention even when LDLc is present at normal concentrations. The pathophysiology of diabetic dyslipidaemia provides the biological basis for both possibilities (Figure 1). Briefly, the loss of insulin sensitivity in muscle and adipose tissue results in an increased flux of free fatty acids which, in turn, promotes hepatic overproduction of TRLs in the form of large VLDL1, causing moderate hypertriglyceridaemia (HTG). TG-rich lipoproteins are rapidly hydrolysed by lipoprotein lipase (LPL) and become smaller particles, known as remnant particles, which can be cleared by the liver or further metabolized to IDL and LDL. Insulin resistance-induced hepatic synthesis of large VLDL1 stimulates cholesteryl ester transfer protein (CETP) activity. CETP facilitates the transfer of triglycerides from triglyceride-rich lipoproteins to HDL and LDL in exchange for cholesteryl esters, resulting in an increase in triglyceride content of HDL and LDL. Triglyceride-enriched LDL particles then undergo further hydrolysis

via hepatic lipase, thereby reducing LDL particle size.[19] Overall, this pool of remnant particles transports the so-called, remnant cholesterol. This is aggravated by the delayed postprandial lipaemia characteristic of T2DM. Not only is there increased production and decreased catabolism of chylomicrons in T2DM,[20] but the intestinal synthesis of VLDL, approximately 25% of total VLDL, nearly doubles in T2DM and increases their residence time fivefold. This helps increase the pool of TRLs and remnant particles, including IDLs.[21]

This metabolic scenario derived from insulin resistance provides clues on four biological and clinical atherogenic characteristics of lipoproteins beyond the cholesterol concentration of the LDL particle:

1. Non-HDL cholesterol: Cholesterol transported by atherogenic lipoproteins other than LDL is an obvious parameter of interest when atheroma is present in normocholesterolaemic individuals (Figure 1A).
2. More circulating particles: Increased number of circulating particles due to an insulin resistance-driven increase in hepatic production and decreased clearance of TRLs. Again, this often occurs with normal LDL or total cholesterol levels (Figure 1B).
3. Smaller LDL and TRL particles: Smaller particles cross the endothelium more easily than larger particles, and these smaller particles are retained and oxidized in the extracellular matrix. VLDL remnants rapidly become smaller particles (<70 nm) that can enter the subendothelial space and be taken up by macrophages without any previous modification[22] (Figure 1B).
4. Cholesterol content of TRLs: Biology and clinical trials indicate that cholesterol, not TGs, is the atherogenic component of TRLs. TGs are hydrolysed and do not accumulate in the arteries, while TG-lowering therapies have shown to be ineffective at decreasing ASCVD mortality in diabetes.[13] Although most cholesterol is transported by LDL, in the nonfasting state, up to one-third of cholesterol can be carried by remnant lipoproteins.[23] The biological relevance of TRL remnant cholesterol in atherosclerosis has been determined by comparing a single TRL particle to a single LDL particle. TRLs can be taken up more easily by macrophages than LDL, as they do not require any previous modification, and each TRL remnant particle contains four times more cholesterol than a single LDL particle[24,25] (Figure 1C).

The immediate questions are which biomarkers can provide information on these four lipoprotein characteristics and whether there is evidence of their clinical relevance.

1. Non-HDL cholesterol: In T2DM, the non-HDLc (calculated as total cholesterol minus HDL cholesterol) is considered a more informative parameter than LDLc as it also takes into account the cholesterol transported by the TRLs produced in excess in T2DM.[26] In diabetes, non-HDL cholesterol may be a stronger predictor of ASCVD than either LDLc or TGs.[27] According to the current ESC/EAS guidelines, non-HDLc is the key lipid parameter in the assessment of cardiovascular risk in T2DM.
2. More circulating particles: Even though the exact number of lipoprotein particles can be assessed only through advanced NMR lipidomics (as discussed below), apolipoprotein B is still a reliable surrogate marker of the number of atherogenic lipoproteins. Because apoB is one of the largest proteins in circulation, only one apoB molecule is bound to each lipoprotein. Therefore, the more apoB there is, the more atherogenic particles there are. ApoB has been shown to be able to identify dyslipidaemic phenotypes associated with cardiovascular risk in normocholesterolaemic individuals with T2DM.[28] ApoB, however, does not allow distinction between TLRs (VLDL and IDL) and LDL or Lp(a). ApoB has been proven to be a better predictor of myocardial infarction and ASCVD than LDLc and non-HDLc[29,30]
3. Smaller LDL and TRL particles: Although the exact diameter of each lipoprotein subclass can be reliably assessed only by NMR lipidomics, an LDLc/apoB ratio lower than 1.2 has been used to estimate prevalence of small and dense LDL in T2DM.[31] The amount of cholesterol transported by small LDL particles can also be estimated by direct precipitation of LDL.[32]
4. Cholesterol content of TRLs: The exact content of TRL cholesterol can be determined by direct measurement of cholesterol in VLDL and IDL isolated by ultracentrifugation, direct automated determination[33] or advanced NMR lipidomics. Given that these techniques might not be easily accessible, remnant cholesterol can be alternatively calculated by subtracting LDLc and HDLc from total cholesterol. When LDLc is calculated by the Friedewald formula, the increase in TG in individuals with T2DM may render inexact results,[34] which may require specific adjustments.[35] Epidemiology, genetic epidemiology and randomized controlled trials have extensively proven the clinical relevance of remnant cholesterol in ASCVD.[36,37] In relation to T2DM, the identification of VLDLc as a stronger contributor than LDLc to myocardial infarction,[38] which is a significant contributor to the excess cardiovascular risk due to T2DM[39] or the identification, in the PREDIMED study,[40] of the low LDLc-elevated VLDLc category (typically observed in

T2DM) as the one with the highest ASCVD, are particularly clarifying.

Normality and reference values for the parameters described in this section can be found in Table 1.

2 | NUCLEAR MAGNETIC RESONANCE

From the pathophysiology described above, the number and size of particles appear to be two distinctive atherogenic characteristics of the lipoprotein profile in T2DM. While these parameters can be estimated only through routine biochemical methods, they can be precisely measured via NMR.

Therefore, NMR has emerged as a methodology with the potential to deepen our understanding of ASCVD by evaluating additional characteristics of lipoproteins known to play a role in residual cardiovascular risk. NMR lipoprotein measurements are based on the fact that esterified cholesterol and TGs carried by lipoproteins contain methyl groups in their molecular structure that, through the use of radiofrequency pulses, resonate at different frequencies depending on the size of the lipoprotein, thus generating a series of detectable signals. In this respect, the smaller the lipoprotein size is, the lower the resonance frequency of the lipids contained. NMR is a metabolomic profiling technique that offers rapid and simultaneous automatic quantification of the number and lipid content of particles of the three main classes of lipoproteins (VLDL, LDL and HDL), and of their subclasses according to size (large, medium and small) (Figure 2).

In addition, the fact that this technique allows the sample to be retrieved, stored for a long time and reused, should not be underestimated. NMR also allows for the determination of the cholesterol and TG content of each lipoprotein subclass, which establishes a collection of conventional and advanced parameters.

Overall, NMR is effective for metabolite quantification in large-scale epidemiological studies because it requires minimal sample preparation and processing, allowing high-throughput methods to be used. Thus, although this methodology is less accessible and has been suggested to be unfeasible for clinical practice,[41] its utility in assessing cardiovascular risk has been more accurately demonstrated in large cohorts.

2.1 | NMR lipoprotein profiling and risk of CVD in diabetes

Individuals with T2DM have an approximately twofold increase in CVD risk on average, which is subject to wide variation depending on the population and therapy.[42] Atherogenic dyslipidaemia,[43] which presents as a cluster of plasma lipid and lipoprotein abnormalities that are metabolically interrelated and triggered by an increase in large VLDL particles associated with atherogenic remnants, small dense LDL and small TG-rich dense HDL particles, is recognized as a major risk factor for the elevated risk.[44] Both LDL and HDL particles show variable compositional changes that are reflected in their functions and are independently associated with cardiovascular risk in individuals with T2DM.[45] Together, TRL remnants, small dense LDL and small dense HDL comprise the atherogenic lipid profile, which is also characterized by an increase in the ApoB concentration, and the number of ApoB-containing particles.

Several large-scale clinical outcome trials have shown that a decrease in LDLc levels is crucial for reducing cardiovascular morbidity and mortality.[46] However, using LDLc alone is insufficient, as approximately 50% of these events occur in individuals with normal LDL concentrations, and specific management of atherogenic dyslipidaemia might help reduce the high residual burden of ASCVD.[4] How to prevent the extra risk beyond the traditional risk factors in clinical practice is currently debated.

The IMPROVE-IT study[47] involving 18,144 patients provided evidence that lower LDLc (53 mg/dL) and apoB (70 mg/dL) result in better outcomes in subjects with diabetes after acute coronary syndrome, and apoB-targeted lipid-lowering therapies are recommended beyond LDLc. On the other hand, the LDL particle (LDL-P) count can also be useful as a target for treatment in subjects with diabetes. However, in the absence of robust prospective clinical trial evidence, there is a lack of uniform agreement regarding

FIGURE 1 (A) Non-HDLc accounts for the cholesterol content of TRLs (VLDL and VLDL remnants) and LDL. In diabetes, given the increased amount of circulating TRL triggered by insulin resistance, non-HDLc is a strong predictor of ASCVD. (B) VLDL, VLDL remnants and LDL particle concentrations are elevated in T2DM due to an increased synthesis of VLDL by the liver, a result of insulin resistance, and a decreased clearance of TRLs and LDL due to conformational changes or interference of apoC-III with apoE. These particles stay in circulation longer, increasing the risk for lipoprotein modification and accumulation in the intima. TRL and LDL of people with diabetes are TG-enriched. Hydrolysis of these TGs by HL or LPL results in smaller TRL and LDL, which can easily go through the arterial endothelium and accumulate in the intima. (C) Only a small proportion of TRL lipid cargo is cholesterol; however, since they are much larger than LDL, one TRL that accumulates in the intima contains 4 times more cholesterol per particle than LDL. ASCVD, atherosclerotic cardiovascular disease; HDLc, high-density lipoprotein cholesterol; LDL, low-density lipoprotein; TRL, TG-rich lipoprotein.

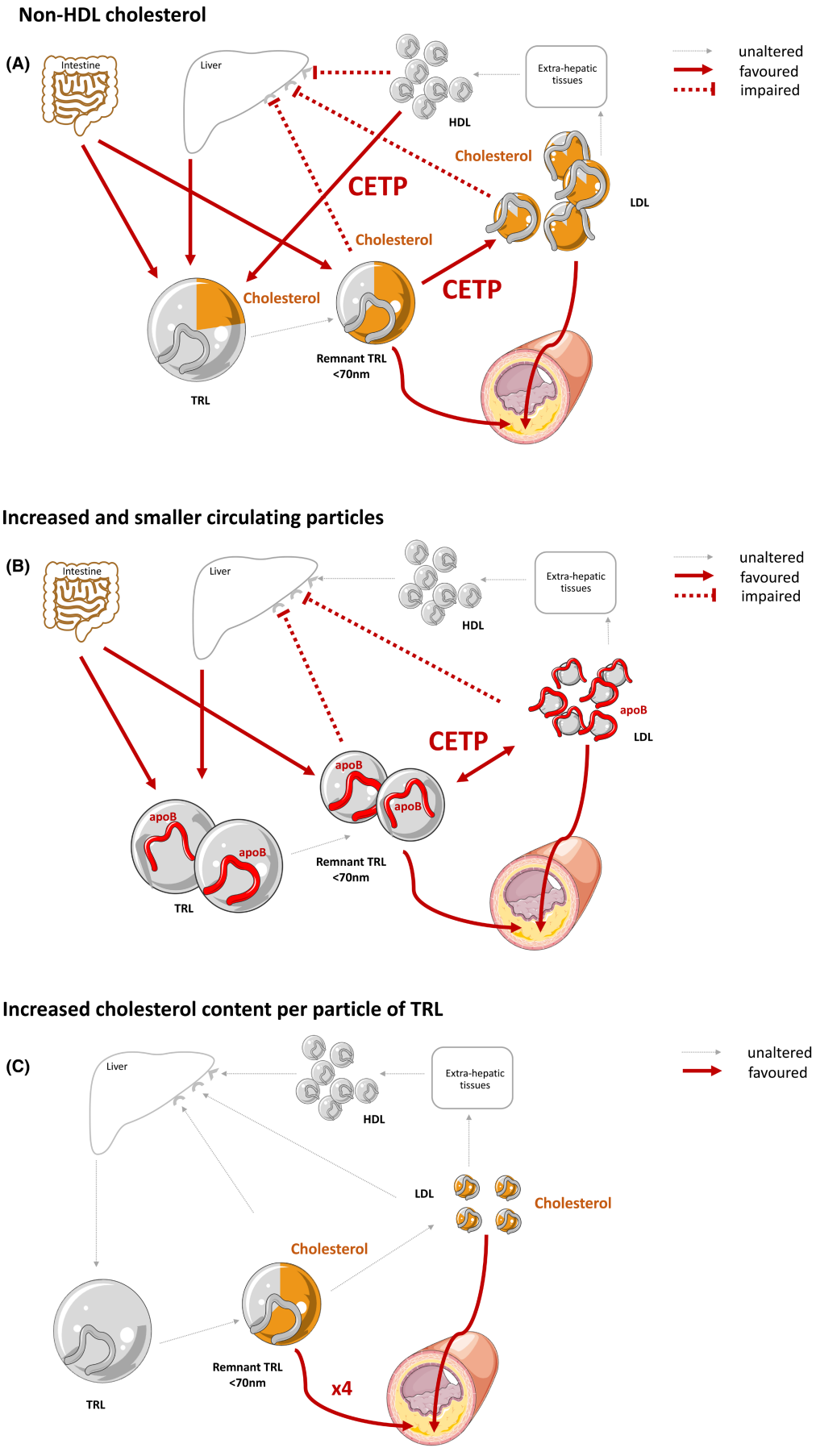


TABLE 1 Normality values and assessment methods of the proposed parameters.

Parameter	Normality values	Assessment method	Observation
Clinical setting[4,118,119]			
Triglycerides	<150 mg/dL (1.69 mmol/L)	• Enzymatic colorimetric assay (glycerol-phosphate oxidase-peroxidase)	–
LDLc	<55 mg/dL (1.4 mmol/L)	• Enzymatic colorimetric assay; • Friedewald formula	Very high CVR
	<70 mg/dL (1.8 mmol/L)		High CVR
	<100 mg/dL (2.6 mmol/L)		Intermediate CVR
HDLc	>50 mg/dL (1.29 mmol/L) in women	• Enzymatic colorimetric assay	>97 mg/dL (2.51 mmol/L) in women and >77 mg/dL (1.99 mmol/L) in men increase all-cause mortality[7]
	>40 mg/dL (1.03 mmol/L) in men		
Non-HDLc	<85 mg/dL (2.2 mmol/L)	• Total cholesterol minus HDL cholesterol	Very high CVR
	<100 mg/dL (2.6 mmol/L)		High CVR
	<130 mg/dL (3.4 mmol/L)		Intermediate CVR
Remnant cholesterol	<30 mg/dL (.78 mmol/L)	• Total cholesterol minus LDL cholesterol minus HDL cholesterol; • automated two-step determination (degradation of LDL and HDL + measurement of cholesterol content); • NMR • ultracentrifugation + cholesterol measurement	–
ApoB	<65 mg/dL (1.27 μmol/L)	• Turbidimetric immunoassays • Rate nephelometry	Very high CVR
	<80 mg/dL (1.56 μmol/L)		High CVR
	<100 mg/dL (1.95 μmol/L)		Intermediate CVR
Lp(a)	<80 nmol/L	• Turbidimetric immunoassays	–
Nuclear magnetic resonance			
Total VLDL particle concentration	41.27 (27–56) nmol/L	Nuclear magnetic resonance	Data obtained from 6.022 subjects from three different cohorts.[120–122] Total LDL particle concentration: according to the AACE guidelines for the management of diabetes it should be <1200 nmol/L in subjects with moderate risk and <1000 nmol/L in subjects with high risk[123]
Large VLDL particle concentration	1.09 (.73–1.35) nmol/L		
Medium VLDL particle concentration	4.33 (3.04–6.08) nmol/L		
Small VLDL particle concentration	35.69 (23–49) nmol/L		
Total LDL particle concentration	1313.71 (1120–1500) nmol/L		
Large LDL particle concentration	200.24 (170–230) nmol/L		
Medium LDL particle concentration	394.43 (310–500) nmol/L		
Small LDL particle concentration	709.21 (610–790) nmol/L		
Total HDL particle concentration	28.36 (24–32) μmol/L		
Large HDL particle concentration	.28 (.25–.32) μmol/L		
Medium HDL particle concentration	9.68 (8.5–11) μmol/L		
Small HDL particle concentration	18.31 (15–21) μmol/L		

Note: Parameters also mentioned in the manuscript but measured in lipoproteins only in research studies have not been included in this table due to the absence of reliable reference values. These include apolipoprotein C-III (measured by turbidimetric immunoassays, ELISA or Mass Spectrometry immunoassay in the case of its Glycoforms); Lipopolysaccharide (measured by Limulus amoebocyte assay (LAL), Endotoxin activity assay (EAA) or Mass spectrometry); Lipoprotein electronegativity (measured by Ultracentrifugation + Anion-exchange chromatography) and Oxidized LDL (measured by ELISA against a variety of epitopes).

Abbreviations: CVR, cardiovascular risk; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein; apo, apolipoprotein; Lp(a), lipoprotein (a); NMR, nuclear magnetic resonance.

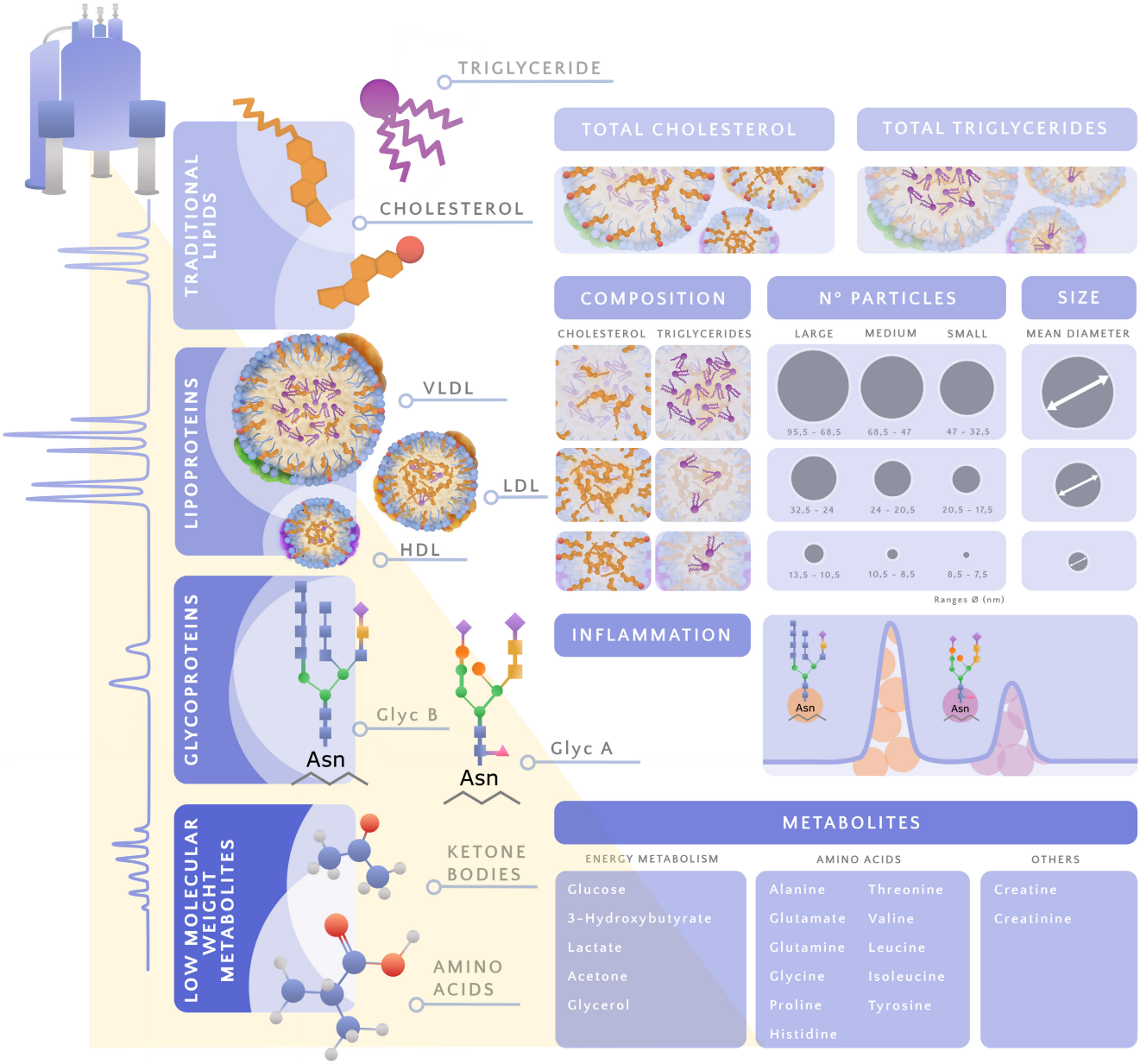


FIGURE 2 NMR spectroscopy allows for the quantification of lipoprotein particle concentrations in different lipoprotein subclasses (large, medium and small) within each main lipoprotein class (VLDL, LDL and HDL) including also their cholesterol and TG content. Interestingly, the acquisition of NMR spectra for lipoprotein profiling also contains additional signals from which data on useful biomarkers can be obtained. One of these signals correspond to the glycoproteins (GlycA, B and F) which are an integrated marker of low-grade inflammation derived from a composite signal of N-acetylmethyl group resonances from several acute phase proteins, thus integrating multiple inflammatory pathways. NMR spectra analysis also distinguishes the low molecular weight metabolites window (LMWM) that includes a wide range of small molecules such as different amino acids and metabolites related to glucose metabolism, renal function and ketone bodies that have been linked to various phases of the progression from pre-diabetes to T2DM. HDLc, high-density lipoprotein cholesterol; LDL, low-density lipoprotein; NMR, nuclear magnetic resonance; T2DM, type 2 diabetes mellitus.

the goal levels.[4] The suggested targets in nmol/L have been proposed to be <1200 for high-risk and <1000 for very high-risk individuals. Data for LDL-P in patients now described as at extreme risk have not been established.

In particular, the superiority of apoB or LDL-P for cardiovascular risk assessment is more evident when LDLc and LDL-P/apoB are discordant.[48] Patients with discordantly high LDL-P or apoB tend to have small, cholesterol-depleted LDL and normal LDLc levels, which are hallmarks of atherogenic dyslipidaemia and insulin resistance.[49] Therefore, consideration of particle number in metabolic-related patients helps identify high-risk individuals who would have a low cardiovascular risk according to their LDLc levels.[50]

Furthermore, although simple measurements of apoB and LDL-P were found to be comparably associated with cardiovascular risk, incorporating LDL particle concentration into clinical guidelines is largely based on availability, which currently favours apoB. However, (i) if other aspects of the lipoprotein analysis provided by the NMR spectroscopy assay are evaluated, including access to VLDL and HDL particle concentrations and lipid composition, and (ii) if its availability increases through the placement of instruments in clinical laboratories, it is possible that this approach could eventually emerge as a valuable method of lipoprotein quantification for cardiovascular risk management.

For T2DM and metabolic-related conditions such as insulin resistance or prediabetes course with HDL alterations, previous reports have shown that HDL particle number (HDL-P) is more predictive of cardiovascular risk than HDLc[51,52] in population-based studies and in subjects with T2DM.[53] Furthermore, NMR spectroscopy, which is inherently able to simultaneously measure a wide range of lipoprotein parameters, may add further value in cardiovascular risk assessment by complementing the known cardiovascular risk factor LDL-P with a more comprehensive metabolomic profiling approach to ASCVD. Although substantial additional research is needed in this area, the NMR approach may provide additional future benefits.

Representative population values for the parameters described in this section can be found in Table 1.

3 | EXAMPLES OF FUNCTIONALLY IMPORTANT MINOR COMPONENTS AND PHYSICAL PROPERTIES

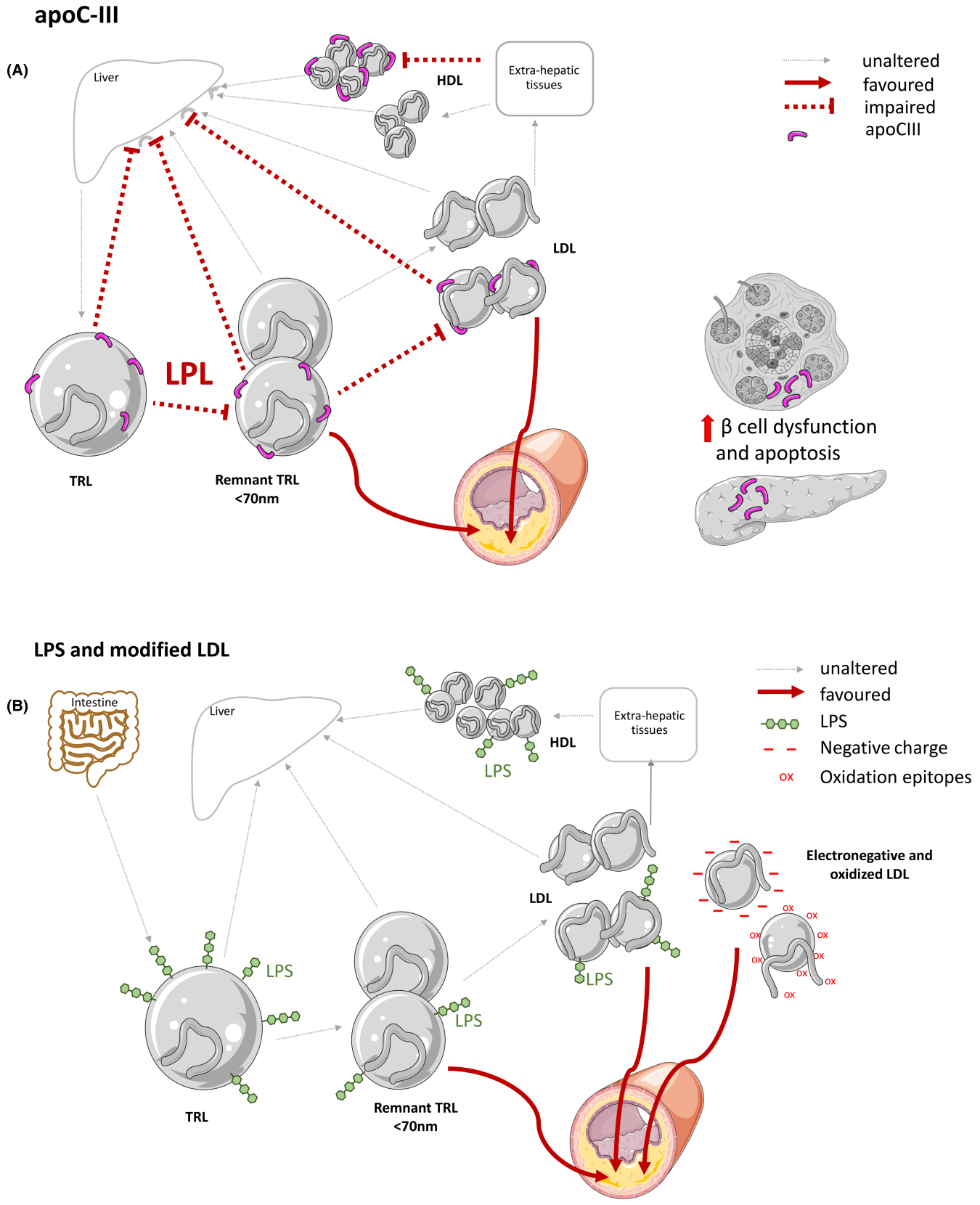
Routine lipid and lipoprotein biochemistry analyses provide the full set of parameters that are most extensively and consistently associated with ASCVD in the general population, namely, the lipid concentrations of the different lipoproteins. However, these studies do not include lipoprotein characteristics that have proven to be relevant in T2DM, namely, the size and concentration of each lipoprotein subclass, which can be determined by NMR. However, despite this information, the cause of atherosclerosis in a significant part of the population has not fully elucidated. The third section of the present review focusses on minor components or physical characteristics of lipoproteins, which can help clarify unexplained residual risk. Candidate elements are those bound to lipoproteins at very low concentrations but with solid evidence of functional implications for atherosclerosis; however, these elements, have almost always been studied separately. We believe that incorporating selected minor components into a global panel that includes traditional and NMR parameters would significantly increase our understanding of atherosclerosis development even in the context of normolipidaemia. Although the number of potentially important lipoprotein components is extensive, we will address this point by describing apolipoprotein C-III (apoC-III), lipopolysaccharide (LPS) and lipoprotein electronegativity.

3.1 | APOC-III

ApoC-III is a small protein that is transported on the surface of lipoproteins at very low concentrations; nevertheless, it has been reported to be an independent risk factor for CVD.[54,55] People carrying loss-of-function mutations in the APOC-III gene exhibit reduced TG levels and a reduced incidence of cardiovascular events.[56,57] This may be because, although apoC-III is found at very low concentrations in circulation, it plays key roles in the metabolism of lipoproteins that cause or enhance ASCVD; for example, apoC-III inhibits lipoprotein lipase; inhibits the apoE-mediated uptake of lipoproteins by the liver receptors LDLR and LRP1[58]; induces the aggregation of LDL particles and their binding to proteoglycans[59]; or triggers inflammation by inducing the expression of adhesion molecules[60] and activation of the NLRP3 inflammasome and subsequent secretion of interleukin-1β.[61] ApoC-III also induces the expression of adhesion molecules on endothelial cells, leading to the infiltration of circulating monocytes into the subendothelial space[62] (Figure 3A).

The presence or absence of apoC-III in lipoproteins alters their characteristics: HDL particles lacking apoC-III are associated with a higher risk of diabetes and coronary heart disease, but apoC-III-containing HDL do not have such protective effect.[63–65] Similarly, light LDL particles

FIGURE 3 (A) ApoC-III impacts lipoprotein clearance by the liver, and TG hydrolysis by LPL and enhances LDL aggregation and binding to proteoglycans in the arterial wall. The autocrine production of apoC-III in pancreatic islets triggers β-cell dysfunction and apoptosis. Two LDL and HDL subclasses can be defined by the presence or absence of apoC-III; in both cases, those containing apoC-III are more proatherogenic. (B) Intestinal LPS leakage is increased in T2DM. LPS bound to lipoproteins that accumulate in the intima can enhance inflammation within atherosclerotic plaques. A small fraction of circulating LDL are electronegative (LDL(−)), which are more proinflammatory, impact the endothelial integrity and are associated with plaque vulnerability. Oxidized LDL (oxLDL) also contribute to endothelium damage, inflammation and promote foam cell formation.



with apoC-III result in a greater conversion to dense LDL than light LDL particles without apoC-III.[66]

Importantly, in the context of diabetes, the expression of apoC-III is modulated by insulin and glucose. Both glucose and a lack of insulin inside the cell enhance the transcription of apoC-III via different transcription factors.[67,68] In addition, VLDL (and, more specifically, apoC-III), can also exacerbate insulin resistance by inducing

endoplasmic reticulum stress and inhibiting insulin signalling.[69] Thus, apoC-III is involved in a positive feedback loop in which both apoC-III transcription and insulin resistance are enhanced.

Understanding the role of apoC-III in ASCVD also includes measuring its glycoforms, which originate from O-glycosylation of the Thr94. These modifications can modulate apoC-III function and differences in the proportions of certain apoC-III glycoforms are associated with differences in lipid and lipoprotein profiles regarding TG levels, LDL particle size and TRL uptake.[70–73] Measuring not only total apoC-III but also the apoC-III content of lipoprotein subclasses and the relative distribution of apoC-III glycoforms can provide a better understanding of dyslipidaemia and cardiovascular risk in people with diabetes. Currently, plasma apoC-III levels can be easily assessed using common turbidimetric assays, although methods with greater sensibility (such as ELISA) are needed to measure apoC-III levels in lipoprotein fractions. Obtaining reliable measurements of apoC-III glycoforms is challenging and requires affinity immobilization of apoC-III and isoform quantification via mass spectrometry.[70,74]

3.2 | Lipopolysaccharide

LPS is a component of the outer membrane of gram-negative bacteria that induces a strong immune response in innate immune cells by binding to cluster of differentiation 14 (CD14) and activating Toll-like receptor 4 (TLR4) signalling.

A high-fat diet can cause alterations in the gut microbiota and increase gut permeability leading to the translocation of LPS through the intestinal wall to the circulation (endotoxaemia).[75,76] These alterations are associated with systemic, low-grade inflammation, which is a hallmark of T2DM, and have been related to metabolic disorders such as obesity and metabolic syndrome.[77,78]

Approximately 90% of the LPS found in the bloodstream is bound to lipoproteins. There is no consensus on the exact distribution of LPS between lipoprotein fractions, although most of the studies reported a higher proportion of lipoprotein-bound LPS in the LDL and HDL fractions.[79–83] However, since there are fewer VLDL particles than LDL or HDL particles in circulation, the LPS per particle ratio is higher in VLDL.[83] Lipoprotein-bound LPS is cleared by the liver, metabolized and excreted into the bile[84,85]; thus, regarding systemic inflammation, the binding of LPS to lipoproteins reduces the immune response to endotoxaemia.[86–89] However, in the context of ASCVD, LPS has been found in human atherosclerotic plaques and plaque regions with more LPS showed increased macrophage activation, while no LPS was detected in nonatherosclerotic arteries.[90] In line with these findings, circulating LPS levels are associated with an increased incidence of major adverse cardiovascular events[91] and in vitro studies suggest that LPS plays a role in triggering platelet aggregation.[92] Therefore, regarding the atherosclerotic plaques, LPS in lipoproteins that accumulate in the intima may be proatherogenic (Figure 3B).

This proatherogenic role of LPS could be especially relevant in people with T2DM, since they exhibit increased endotoxin levels in circulation compared to those of healthy controls.[93] Similarly, the baseline LPS concentration in circulation is also higher in people who develop diabetes than in nonincident controls.[94]

Several methods for measuring LPS levels in circulation have been used for the assessment of sepsis. Popular methods such as the limulus amoebocyte assay (LAL) or endotoxin activity assay (EAA) are rapid and are based on the reactivity induced by LPS molecules.[95,96] Other more complex methodologies, such as those using mass spectrometry, directly detect the LPS and are more sensitive, with less interference from other molecules, although they are more difficult to use in routine analysis.[97]

3.3 | LDL modifications: electronegativity and oxidation

In addition to the cargo of lipoproteins, some intrinsic characteristics of lipoprotein particles (such as particle number and size, reviewed above) are also biomarkers of cardiovascular risk. Other less common measures include lipoprotein electrical charge and the concentration of negatively charged LDL [LDL(−)], which are increased in people with T2DM.[98]

Although LDL(−) represents only a very small proportion of total LDL (10%), abundant evidence supports its pro-atherogenic role. Increased LDL(−) levels in individuals with T2DM are important in regard to increased cardiovascular risk since LDL(−) is associated with increased plaque vulnerability[99] and has numerous proatherogenic effects in vitro, namely, impairment of endothelial integrity and apoptosis of endothelial cells,[100] cardiomyocyte damage and apoptosis,[101] activation of macrophages[102] and polarization to the M1 phenotype.[103] Moreover, in people with diabetes, LDL(−) from patients with poor glycaemic control induced a greater proinflammatory response in monocytes than LDL(−) from controls or patients with good glycaemic control[104] (Figure 3B).

Diabetes is associated with increased oxidative stress, which can lead to the oxidation of LDL particles. Oxidized LDL (oxLDL) contributes to atherosclerosis by activating and damaging the endothelium, inducing inflammation, attracting monocytes, and providing lipids in a way

that promotes foam cell formation. Circulating oxLDL is known to be higher in type 2 diabetic patients than in control subjects.[105] Additionally, plasma levels of oxLDL are predictors for future events in diabetic patients with coronary artery disease,[106] and in diabetic males, there is an association of oxLDL/LDL with CHD.[107] We also showed that the oxLDL/HDL ratio is associated with vascular disease in diabetic patients.[108] It is of special interest that antidiabetic drugs were clinically associated with a suppressive effect on oxLDL in patients with impaired glucose tolerance. Thus, the anti-inflammatory, antioxidant and anti-apoptotic properties of antidiabetic agents reverse the effects induced by oxLDL, which can be beneficial in controlling atherosclerosis in diabetic patients.[109]

3.4 | Other putative nonconventional biomarkers of lipoproteins

As mentioned above, apoC-III, LPS, LDL(−) and ox-LDL are just few examples of lipoprotein features with substantial evidence of their impact on ASCVD beyond lipid concentrations. There might be other equally valid candidates, such as angiopoietin-like protein 3 (ANGPTL3). ANGPTL3 has emerged as a promising molecular target for reducing remnant lipoprotein levels and CVD risk,[110] and loss-of-function mutations in ANGPTL3 are associated with decreased plasma insulin and HOMA-IR.[111]

These parameters are not measured in clinical practice due to obvious methodological limitations. Overcoming such limitations is one of the challenges for the implementation of new biomarkers for better identification of residual risk. Another significant challenge is the selection of potentially relevant biomarkers among the numerous proteins, enzymes, physicochemical modifications (e.g., the microplastics and nanoplastics recently identified in carotid artery plaques[112]) or miRNAs that can be associated with lipoproteins. Notably, lipoprotein-bound miRNAs are small noncoding RNAs that can bind to target sites on mRNA and regulate gene expression at the mRNA level; increasing amounts of miRNAs are being identified in many different species with numerous target genes.[113] Lipoproteins, especially HDL but also VLDL and LDL, have been proven to stabilize and transport miRNAs in the circulation and deliver them to recipient cells, and differences in the miRNA profile have been found in patients with familial hypercholesterolaemia.[114,115]

Proteomic studies on lipoproteins have identified numerous proteins (beyond the known apolipoproteins related to lipid metabolism) carried by VLDL, LDL and especially HDL. Those carried by VLDL and LDL were associated with human diseases such as atherosclerosis and coagulation disorders,[116] while a larger and more diverse set of proteins was identified in HDL, as reviewed previously.[117] While the number of potential biomarkers is overwhelming, the rationale for selecting them is straightforward, as we focussed on biomarkers with convincing scientific evidence of their implications for ASCVD.

4 | CONCLUSIONS

The rationale for this narrative review lies in the observation that atherosclerosis can occur even in the absence of high LDLc concentrations. Hence, factors other than LDLc play a crucial role in the accumulation of cholesterol in the arterial wall. Gaining a comprehensive understanding of these characteristics is essential for elucidating cardiovascular residual risk. In the context of diabetes, recent advancements have allowed for the precise measurement of atherogenic lipoprotein features, including the particle number and diameter of lipoprotein subclasses, which were previously approximated only through routine lipid analyses. Notably, NMR technology now provides reliable and accurate measurements in this regard. We propose the integration of a third level of lipoprotein study, focussing on minor components and physicochemical properties consistently associated with ASCVD risk. These factors have not been collectively examined alongside traditional biomarkers. The limited exploration of these alternative biomarkers is largely attributed to the technical complexity involved in their analysis. Undoubtedly, addressing this methodological challenge is crucial for advancing our comprehension of residual risk within the context of diabetes.

AUTHOR CONTRIBUTIONS

JR and JPB were involved in conception of the manuscript. PR, MG, JR and NA were involved in literature retrieve and writing of the first draft of the manuscript. PR, JR, JG, MG and NA were involved in conception and design of the figures. FA, MB, JAG, APP, EO, JIM, JPB and VS were involved in critical review of the entire manuscript. All authors approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare they have no conflict of interest.

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ANNEX: CARDIOVASCULAR DISEASE GROUP OF THE SPANISH SOCIETY OF DIABETES

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