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Advanced serum lipoprotein and glycoprotein profiling for cardiovascular event prediction in type 2 diabetes mellitus: the LIPOCAT study

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Abstract

Background Traditional risk factors cannot accurately predict cardiovascular events (CVE) in type 2 diabetes (T2D). The LIPOCAT study aimed to prospectively evaluate the clinical utility of advanced lipoprotein characteristics and glycoproteins to predict future cardiovascular events (CVE) in a large cohort of subjects with type 2 diabetes mellitus (T2D).

Methods From four different Spanish prospective cohorts, a total of 933 T2D subjects were selected to form the LIPOCAT study. Advanced 1H-Nuclear Magnetic Resonance (1H-NMR) analysis included lipoprotein (Liposcale®) and glycoprotein (Glycoscale) profiling. Random forest classification models and Area Under the Receiver Operating Characteristics (AUROC) analysis were used to assess the differential contribution of advanced variables in predicting CVE. Validation was performed using an external cohort.

Results Out of 933 T2D subjects, 104 reported a CVE during follow-up. Analysis of Liposcale®/Glycoscale uncovered elevations in the circulating VLDL-cholesterol(C), remnant IDL-triglycerides (TG) and LDL-TG in subjects with CVE, along with glycoproteins (Glyc) A and B. Moreover, the incorporation of advanced Liposcale® variables to a base model constructed with traditional risk factors significantly improved the prediction of CVE, as evidenced by 1.5-fold increase in the C statistic (AUROC), reaching AUROC values of 0.756. In the independent validation cohort, similar improvements in AUROC values were observed by adding the advanced variables to the traditional models.

Conclusions Advanced 1H-NMR analysis revealed previously hidden lipoprotein and glycoprotein characteristics associated with CVE in T2D subjects.

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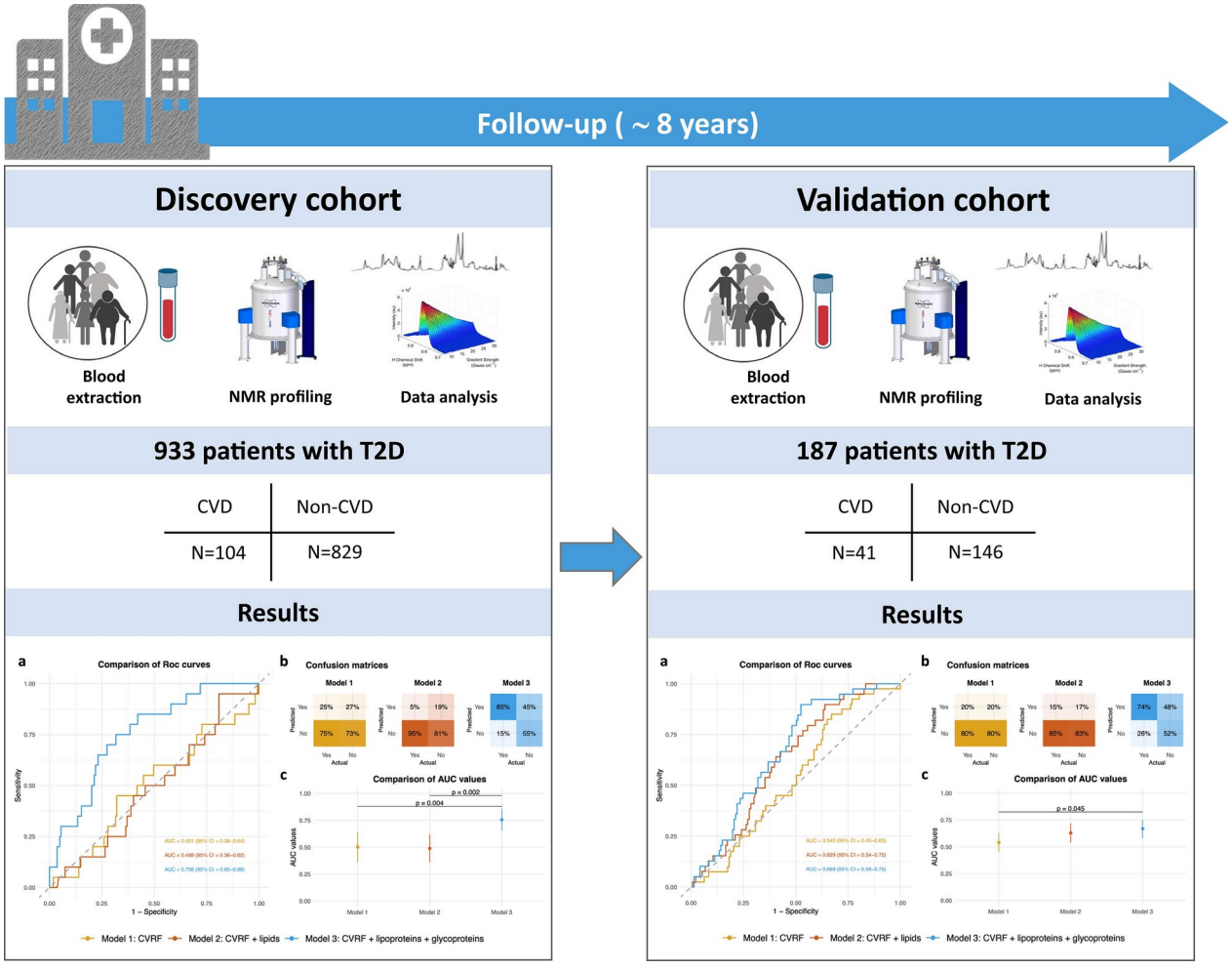
Graphical abstract



Keywords Biomarker, Lipoproteins, Glycoproteins, Type 2 diabetes, Cardiovascular events, Nuclear magnetic resonance spectroscopy, Diabetes mellitus

Background

Type 2 diabetes mellitus (T2D) is a prevalent chronic condition and a significant global public health concern [1]. Individuals with T2D face an increased risk of cardiovascular disease (CVD) [2]. The ability to accurately predict future cardiovascular events (CVEs) is vital for enabling targeted or precision prevention of this main complication. CVD risk prediction models have been designed to discriminate between individuals at lower or higher future risk, however, current models frequently fail to offer sufficient specificity to be used for clinical purposes, especially in T2D subjects [3]. Therefore, the identification of novel diagnostic markers for CVE in T2D subjects has become a subject of intense research.

Conventional serum lipid analysis frequently lacks the sensitivity to identify subjects with T2D at risk of CVE [4]. In this regard, the use of advanced nuclear magnetic resonance (1H-NMR) approaches, i.e., Liposcale®, has been successful in uncovering such hidden lipoprotein alterations associated with preclinical atherosclerosis in newly diagnosed T2D patients that were not captured by the traditional lipid profile analysis [5]. Therefore, advanced analysis of the lipoprotein profile may allow the identification of hidden candidate biomarkers for a more comprehensive CVD risk prevention strategy.

Clinical chronic, low-grade inflammation also contributes to many of the adverse complications of T2D, including CVD [6]. Indeed, low-grade inflammation is one of the key targets for the clinical management of subjects with T2D to reduce the CVD risk [7]. Studies using Glycoscale 1H-NMR spectra have identified a specific inflammation-responsive signal in serum or plasma

samples termed Glyc A [8], which has been positively associated with different cardiometabolic conditions [9]. In line with this, Glyc A has also been identified as a reliable biomarker of cardiometabolic risk [10]. Indeed, Glyc A has been associated with an elevated incidence of CVD in recent independent clinical trials over other traditional CVD risk factors [11–15]. The detection of Glyc A has also been linked to incident T2D, even after adjusting for other traditional risk factors of T2D and hypersensitive C-reactive protein (hsCRP) [16, 17]. Overall, these findings might suggest that Glyc A could be considered as a new independent biomarker of chronic, low-grade inflammation related to cardiovascular burden in T2D subjects.

Taken together, quantitative characteristics of lipoprotein and glycoprotein profiles may be clinically relevant predictive biomarkers for risk assessment of CVE, especially in subjects with T2D, who are more prone to develop atherogenic dyslipidemia and inflammation. Therefore, we aimed to characterize the serum 1H-NMR-lipoprotein and glycoprotein profiles in 933 subjects with T2D collected from four different Spanish prospective population-based cohorts. Furthermore, we also aimed to assess the added predictive value for CVE prediction, if any, provided by the incorporation of advanced, non-traditional lipoprotein (i.e., Liposcale®) and glycoprotein characteristics (i.e., Glycoscale). Finally, we externally validated the CVE prediction models with an independent cohort including 187 individuals with T2D.

Methods

Study population

The LIPOCAT study was prospectively built up using four independent cohorts recruited from several areas of Catalonia in the north-east of Spain. Subjects with T2D matched for age, sex and body mass index (BMI) who had a serum sample available were selected and a follow-up update was performed to determine the cardiovascular events and all-cause mortality. The four cohorts that form the LIPOCAT study were previously described: *Peripheral Arterial Disease study (PERART/ARTPER)* [18, 19], *Chronic Liver Disease-Fibro Scan cohort* (FIBROSCAN) [21], Diabetes Mellitus study (DM) [22], DIABIMCAP Study (Carotid Atherosclerosis in Newly Diagnosed Type 2 Diabetic Individuals) [24]. We used an independent cohort, the Di@bet.es [25], to validate the results. The characteristics of the five cohorts are detailed in the Supplementary file 1.

The data collection procedures for all cohorts have been extensively detailed in previous publications [19, 21, 24, 25]. Key clinical variables recorded included age, sex, smoking habit, systolic blood pressure (SBP), diastolic blood pressure (DBP), glycated hemoglobin (HbA1c), and lipid profile. T2D was defined by glucose > 126 mg/dL, HbA1c > 6.5%, ongoing treatment or a medical diagnosis. Dyslipidemia was defined by a cholesterol level > 200 mg/dL, ongoing treatment or a medical diagnosis. Hypertension was defined by SBP > 140 mmHg or DBP > 90 mmHg or treatment or a medical diagnosis. Remnant cholesterol was calculated according to the consensus European Atherosclerosis Society (EAS) formula: remnants cholesterol = triglycerides (TG) * (VLDL-C/VLDL-TG) [31]. Additionally, remnant cholesterol was calculated using variables from Liposcale®, using the formula: remnants cholesterol = VLDL-C + IDL-C. Standardized protocols were followed for the measurement of weight, height, and blood pressure. Blood samples were collected in a fasting state, analyzed using standardized methods to determine biochemical parameters. For the Liposcale® test and the glycoprotein profile, blood samples were collected in a fasting state into EDTA tubes, processed immediately after extraction, and stored at – 80 °C at the biobanks of the participant centers until analysis.

A comprehensive review of medical records, encompassing all available information from the healthcare system, was conducted to identify occurrences of all-cause mortality and CVEs during the follow-up update. Incident cardiovascular endpoints were identified using codes from the International Classification Diseases (ICD-9 and ICD-10). These codes were used to identify and track specific cardiovascular events, procedures, and mortality outcomes throughout the follow-up period. Specific diagnostic codes included: Acute myocardial infarction (410, I21, I22), Angina (413, 411.1, I20), Unstable angina (411.1, I20.0), Ischemic heart disease (410–414, I20, I25 excluding 414.10, 414.11, 414.12, 414.19), Stroke (431, 433, 434, 435, 438, I61, I63, I65, I69, G45, G46), Peripheral arterial disease (443.1, 443.8, 443.9, 444, I73.1, I73.8, I73.9, I74), Heart failure (40,201, 40,211, 40,291, 40,401, 40,403, 40,411, 40,413, 40,491, 40,493, 428, I50, I11.0, I13.0, I13.2). In addition, relevant procedures were monitored, including Coronary revascularization (00.66, 36.0, 36.1, 36.2, 36.3) and Revascularization of other vascular territories (39.50, 39.90, 39.79, 00.60, 00.61, 00.62, 00.63, 00.64, 00.65). For mortality assessment: Cardiovascular death (390–459, I00-I09 excluding 427.5, 435, 446, 459.0) and Overall mortality (001-E999, A00-Y89) were also documented. The events included ischemic heart disease (encompassing any documented diagnosis such as angina pectoris), stroke, heart failure, peripheral artery disease, revascularization procedures, and cardiovascular mortality. To ensure accuracy, CVEs were only confirmed if a treating physician had documented a new diagnosis corresponding to the event after hospital admission or in outpatient medical records. This meticulous approach aimed to maintain the integrity and reliability of the collected data. The study protocol for the LIPOCAT study was approved by the Ethics Committees

of University Hospital Germans Trias i Pujol (PI-18–039). All participants were informed about the study protocols and provided their consent to participate.

NMR molecular profiling

Three hundred µL of serum samples were shipped on dry ice to Biosfer Teslab (Reus, Spain) for the NMR analysis which included the lipoprotein profile based on the Liposcale® test [27] and the glycoprotein profile [28].

Serum samples were thawed overnight and prepared for NMR analyses. High-resolution 1H-NMR spectroscopy data was acquired on a Bruker 600 MHz spectrometer using LED Diffusion (Diff) experiments to detect larger molecules such as lipoproteins and glycoproteins, running at 37 °C in quantitative conditions.

The Liposcale® test was used to obtain the composition, the mean size and the number of lipoprotein particles of nine subtypes i.e. the main lipoprotein types (VLDL, LDL and HDL) further subdivided into large, medium and small particles for each type according to their respective size. Using the same NMR spectra, the general measurement of circulating glycoproteins was obtained by deconvoluting the specific region where glycoproteins resonate using analytical functions, and the area was quantified in proportion to the concentration of the acetyl groups of N-acetylglucosamine and N-acetyl galactosamine (Glyc A) and acetyl groups of N-acetylneuraminic acid (Glyc B). We analyzed the 1H-NMR spectral region where glycoproteins resonate (2.15–1.90 ppm) using several analytical functions, following a previously published procedure [28].

Statistical analysis

Continuous variables were tested for normality using the Kolmogorov–Smirnov test. Data are presented as median (25th percentile–75th percentile), and n and percentages for categorical variables. Differences between groups were analyzed using the non-parametric Mann–Whitney test or Student's parametric t-test for continuous variables and the chi-square test or Fisher's exact test for categorical variables.

We used random forests to construct the prediction models of CVE. Model parameters were adjusted using fivefold cross-validation, and the models were trained on 80% of the dataset and tested on the remaining 20% of data, based on a strategy that has proved to be more accurate for complex scenarios compared to other multivariate approaches [29]. The first model (Model 1) was performed with the traditional cardiovascular risk factors (CVRFs): sex, age, BMI, hypertension, dyslipidemia and smoking. Model 2 was built on top of Model 1 by further incorporating conventional lipid variables: total cholesterol (Total C), HDL-C, LDL-C, TG and remnant cholesterol using the EAS formula. Model 3, also built upon Model 1, incorporated advanced integrative variables of Liposcale®, including ratios and percentages of quantitative characteristics of lipoprotein metabolism. Specifically, the variables included the VLDL-TG-to-VLDL-C ratio, IDL-TG-to-IDL-C ratio, LDL-TG-to-LDL-C ratio, HDL-TG-to-HDL-C, the percentages of smaller VLDL, LDL and HDL, remnant cholesterol calculated using the Liposcale® formula and incorporating glycoproteins Glyc A and Glyc B. To assess model performance, we computed the accuracy and the area under the ROC curve (AUROC) for all models. Differences between AUROC values were assessed using DeLong's test for correlated ROC curves, with pairwise comparisons performed across models. Statistical significance was set at $p < 0.05$. Statistical analyses were performed using the R statistical software version 4.4.0 [30].

Results

Characteristics of the LIPOCAT cohort

In the total cohort of 933 T2D subjects drawn from the four distinct prospective cohorts, 104 individuals experienced at least one CVE during the follow-up period. Given the matching by age, sex and BMI, these variables did not differ between CVE groups i.e. with and without CVEs. Additionally, there were no differences in smoking habits between the groups (Table 1).

The proportion of T2D subjects receiving antihypertensive medication was significantly higher in those with a CVE than those without. Correspondingly, significant elevations in SBP were observed in the CVE group compared with the non-CVE subjects. The HbA1c levels were significantly higher in the CVE group, indicating suboptimal glycemic control. However, conventional total lipids did not differ between both groups, although total cholesterol was marginally elevated in the CVE group. No changes were observed in the serum concentrations of HDL- and LDL-cholesterol.

During the follow-up period among the CVE group, peripheral arterial disease was the most common event (29.8%), followed by heart failure (22.7%), stroke (21.2%), acute myocardial infarction, (18.3%), angina pectoris (17.3%), ischemic heart disease (16.3%) and coronary revascularization (15.4%). Cardiovascular mortality was 10.6% (Table 1).

Advanced lipoprotein and glycoprotein variables

The advanced analysis of serum lipoproteins using the NMR approach revealed a significant increase in total triglycerides in the CVE group. This increase was mainly due to significant increases of IDL- and LDL-triglycerides and VLDL-triglycerides (p-value = 0.054). Despite total cholesterol not differing between groups, NMR analysis uncovered significant elevations in the VLDL-cholesterol and marginally in the IDL-cholesterol (p-value = 0.058) in

Table 1 Clinical variables and incidence of cardiovascular events in the prospective T2D cohort (LIPOCAT)

Characteristics	All	non-CVE	CVE	*p*-value
	n = 933	n = 829	n = 104	
Clinical and demographic				
Age (years)	63.0 [57.0;68.2]	62.7 [57.0;68.1]	64.0 [56.0;69.0]	0.300
Sex (female)	402 (43.1%)	358 (43.2%)	44 (42.3%)	0.948
Obesity	474 (50.8%)	419 (50.5%)	55 (52.9%)	0.847
BMI (kg/m^2)	30.1 [27.4;33.8]	30.0 [27.4;33.8]	30.1 [27.5;33.4]	0.770
SBP (mm Hg)	136 [126;148]	135 [125;146]	144 [130;158]	**< 0.001**
DBP (mm Hg)	80.0 [73;87]	80.0 [73;87]	80.0 [71;86]	0.878
HbA1c (%)	6.80 [6.20;7.80]	6.80 [6.20;7.70]	7.30 [6.50;8.75]	**0.001**
Hypertension	673 (72.1%)	587 (70.8%)	86 (82.7%)	**0.015**
Dyslipidemia	685 (73.4%)	601 (72.5%)	84 (80.8%)	0.093
Smoking	351 (41.5%)	306 (41.0%)	45 (45.5%)	0.952
Conventional lipid profile				
Total triglycerides (mg/dL)	132 [95;181]	131 [94;179]	146 [101;189]	0.121
Total cholesterol (mg/dL)	191 [167;219]	190 [167;218]	197 [176;224]	0.060
LDL-C (mg/dL)	113 [91;137]	112 [91;136]	115 [98;140]	0.168
HDL-C (mg/dL)	48 [40;57]	48 [41;57]	47 [39;60]	0.882
Cardiovascular events and mortality				
All-cause mortality (Yes)	40 (4.29%)	26 (3.14%)	14 (13.5%)	**< 0.001**
Cardiovascular mortality (Yes)	11 (1.18%)	0 (0.00%)	11 (10.6%)	**< 0.001**
Acute myocardial infarction (Yes)	19 (2.04%)	0 (0.00%)	19 (18.3%)	**< 0.001**
Angina pectoris (Yes)	18 (1.93%)	0 (0.00%)	18 (17.3%	**< 0.001**
Ischemic heart disease (Yes)	17 (1.82%)	0 (0.00%)	17 (16.3%)	**< 0.001**
Stroke (Yes)	22 (2.36%)	0 (0.00%)	22 (21.2%)	**< 0.001**
Peripheral arteriopathy (Yes)	31 (3.32%)	0 (0.00%)	31 (29.8%)	**< 0.001**
Heart failure (Yes)	10 (2.71%)	0 (0.00%)	10 (22.7%)	**< 0.001**
Coronary revascularization (Yes)	16 (1.71%)	0 (0.00%)	16 (15.4%)	**< 0.001**

Bold values indicate statistical significance ($P < 0.05$)

Data are shown as the n (percentage) for qualitative variables and median [25th percentile–75th percentile] for quantitative variables. BMI, body mass index; CVE, cardiovascular event; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol. SBP, systolic blood pressure; DBP, diastolic blood pressure.

the CVE group. Interestingly, the serum concentration of LDL-cholesterol did not differ between groups (Table 2).

The triglyceride-to-cholesterol ratio calculated for each lipoprotein fraction revealed a significant increase only in the LDL, partly attributed to the relatively higher triglyceride content in the serum from subjects in the CVE group.

Regarding the particle size, our data showed that the circulating concentrations of larger HDL were significantly elevated in the CVE group (p-value = 0.007). Consistently, the small HDL-P-to-medium HDL-P ratio was concurrently decreased in the CVE group (p-value = 0.039). Medium VLDL was significantly increased in the CVE group. No significant differences were observed in other cardiovascular surrogates calculated from quantitative characteristics of lipoproteins, but the HDL-C-to-total triglycerides showed a trend (p-value = 0.063) towards being decreased in the CVE group compared with the non-CVE group.

Serum glycoproteins, especially Glyc A, are composite biomarkers of inflammation that can also be detected by [1H]-NMR. Median values of Glyc A and Glyc B were higher in T2D subjects with CVE compared with those without (Table 3).

Contribution of advanced lipoprotein and glycoproteins characteristics to CVE prediction

We developed three models with the overall aim of examining whether the addition of advanced variables (i.e., Liposcale® and Glycoscale) that were differentially changed in the CVE group (i.e., IDL-TG, LDL-TG and diameter, and glycated proteins) (Model 3) could improve CVE prediction over traditional CVRFs such as BMI, age, sex, hypertension, dyslipidemia, smoking habit (Model 1), and traditional CVRFs together with the conventional lipid profile (Model 2) (Fig. 1; Table 4). We used random forests to construct the prediction models of CVE.

Model 1: Designed with traditional CVRFs, random forest analysis identified BMI, age and hypertension as the most important variables for making accurate predictions (scores higher than 75%) (Fig. 1a).

Model 2: We incorporated the conventional lipid profile (i.e., total cholesterol, TG, LDL-C, and HDL-C) and estimated cholesterol remnants (EAS formula) to analyze their added effect on CVE risk. Random forest analysis again identified BMI as the most important variable. This analysis also revealed that age, total cholesterol, LDL-C, HDL-C, remnant cholesterol, and total triglycerides scored within the top quartile (> 75%) on the variable importance scale for accurate CVE prediction (Fig. 1b).

Model 3: We incorporated the advanced characteristics of lipoproteins (Liposcale®) and glycoproteins (Glycoscale) into Model 1. In this model cholesterol remnants were calculated using the Liposcale® formula. BMI, age,

Table 2 Advanced lipoprotein variables of the LIPOCAT study

Lipid and lipoprotein variables	All	non-CVE	CVE	*p*-value
	n = 933	n = 829	n = 104	
Total lipids				
Total cholesterol (mg/dL)	177 [155;198]	207 [154;198]	178 [163;198]	0.284
Total triglycerides (mg/dL)	104 [79;142]	103 [78;140]	122 [84;147]	**0.017**
Lipoprotein lipid distribution				
VLDL-C (mg/dL)	16.8 [11.1;25.0]	16.6 [10.9;24.3]	20.7 [12.2;26.6]	**0.031**
VLDL-TG (mg/dL)	59.3 [40.8;92.1]	58.5 [40.9;90.7]	72.8 [37.8;96.2]	0.054
VLDL-TG/VLDL-C ratio	3.77 [3.34;4.30]	3.77 [3.34;4.31]	3.77 [3.31;4.19]	0.605
IDL-C (mg/dL)	11.3 [8.04;15.2]	11.2 [8.00;15.2]	12.7 [8.72;15.4]	0.058
IDL-TG (mg/dL)	10.9 [8.14;13.9]	10.8 [8.06;13.8]	11.9 [9.06;15.0]	**0.013**
IDL-TG/IDL-C ratio	0.95 [0.87;1.06]	0.95 [0.87;1.06]	0.94 [0.86;1.06]	0.879
LDL-C (mg/dL)	92.7 [78.4;109.0]	82.9 [78.5;109.0]	91.3 [77.9;107.0]	0.715
LDL-TG (mg/dL)	13.1 [10.1;16.3]	12.9 [9.91;16.2]	14.3 [11.6;17.7]	**0.002**
LDL-TG/LDL-C ratio	0.14 [0.12;0.16]	0.14 [0.11;0.16]	0.15 [0.13;0.18]	**< 0.001**
HDL-C (mg/dL)	50.9 [43.8;59.5]	50.9 [43.8;59.5]	51.6 [43.9;60.2]	0.994
HDL-TG (mg/dL)	17.8 [13.4;22.3]	17.6 [13.2;22.2]	18.7 [14.8;23.0]	0.084
HDL-TG/HDL-C ratio	0.34 [0.26;0.44]	0.34 [0.26;0.43]	0.37 [0.28;0.45]	0.084
Lipoprotein size distribution				
VLDL diameter (nm)	42.2 [42.0;42.3]	42.2 [42.0;42.3]	42.2 [42.0;42.3]	0.789
VLDL-P (nmol/L)	45.4 [31.0;67.9]	44.4 [31.0;67.2]	56.7 [30.2;71.8]	0.052
Large (nmol/L)	1.17 [0.91;1.58]	1.15 [0.91;1.56]	1.32 [0.91;1.64]	0.090
Medium (nmol/L)	4.77 [3.21;7.36]	4.74 [3.21;7.16]	5.58 [3.43;9.27]	**0.038**
Small (nmol/L)	39.3 [26.9;58.9]	38.7 [26.9;58.7]	49.2 [26.7;61.1]	0.060
LDL diameter (nm)	20.8 [20.6;20.9]	20.8 [20.6;20.9]	20.9 [20.7;21.0]	0.078
LDL-P (nmol/L)	705 [609;817]	708 [607;819]	696 [617;804]	0.960
Large (nmol/L)	96.8 [83.5;111.0]	96.4 [83.1;111.0]	99.7 [89.9;114.0]	0.105
Medium (nmol/L)	188 [145;238]	188 [144;237]	188 [147;241]	0.871
Small (nmol/L)	423 [369;477]	423 [369;478]	414 [367;477]	0.498
HDL diameter (nm)	8.20 [8.15;8.24]	8.19 [8.15;8.24]	8.22 [8.17;8.26]	**0.004**
HDL-P (nmol/L)	29.2 [24.7;34.4]	29.2 [24.7;34.5]	29.4 [24.9;34.1]	0.927
Large (nmol/L)	0.29 [0.25;0.32]	0.28 [0.25;0.32]	0.29 [0.27;0.32]	**0.007**
Medium (nmol/L)	8.83 [7.82;9.81]	8.79 [7.80;9.76]	9.10 [8.08;10.0]	0.070
Small (nmol/L)	20.2 [16.3;24.5]	20.3 [16.3;24.6]	19.8 [16.2;23.9]	0.413
Calculated CV surrogates				
Small HDL-P-to-medium HDL-P	2.27 [1.95;2.68]	2.28 [1.98;2.72]	2.19 [1.87;2.55]	**0.039**
LDL-P-to-HDL-P	24.4 [19.8;29.6]	24.3 [19.9;29.6]	24.7 [19.8;29.8]	0.714
Total particles-to-HDL-P	26.2 [21.4;31.6]	26.0 [21.4;31.5]	26.7 [21.5;31.8]	0.496
Non-HDL-P (nmol/L)	728 [635;841]	726 [634;841]	729 [650;832]	0.622
HDL-C/total TG	0.50 [0.34;0.71]	0.50 [0.34;0.71]	0.44 [0.30;0.67]	0.063
Cholesterol remnants (Liposcale®; mg/dL) †	28.7 [20.0;39.4]	28.5 [20.0;38.6]	32.7 [21.3;42.9]	**0.019**
Cholesterol remnants (EAS; mg/dL) ‡	29.1 [20.5;39.0]	28.6 [20.1;38.7]	33.5 [22.8;42.3]	**0.010**

Bold values indicate statistical significance ($P < 0.05$)

Data are shown as the median (25th percentile–75th percentile). † Remnant cholesterol EAS formula = triglycerides (TG) * (VLDL-C/VLDL-TG). ‡ Remnant cholesterol Liposcale® formula = VLDL-C + IDL-C. C, cholesterol; CV, cardiovascular; CVE, cardiovascular event; VLDL, very low-density lipoprotein; VLDL-P, VLDL particles; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; LDL-P, LDL particles; HDL, high-density lipoprotein; HDL-P, HDL particles; TG, triglycerides.

Table 3 Advanced glycoprotein variables of the LIPOCAT study

Characteristics	All	non-CVE	CVE	*p*-value
	n = 845	n = 746	n = 99	
Glyc A (μmol/L)	793 [710;899]	789 [707;896]	821 [739;931]	0.042
Glyc B (μmol/L)	385 [349;427]	384 [349;426]	393 [359;452]	0.007

Data are shown as the median [25th percentile–75th percentile]. CVE, cardiovascular event; Glyc A, glycoprotein A; Glyc B, glycoprotein B.

Glyc A, Glyc B, and all lipids scored over 75% on the variable importance scale for accurate CVE prediction (Fig. 1c).

The AUROC score calculated in Model 3 was 1.5-fold higher (AUROC: 0.756) compared with Model 1 (0.501) (Δ value = 0.255). Likewise, the balanced accuracy value estimated using Model 1, which was strictly built using only traditional risk factors, was 0.492, much lower than

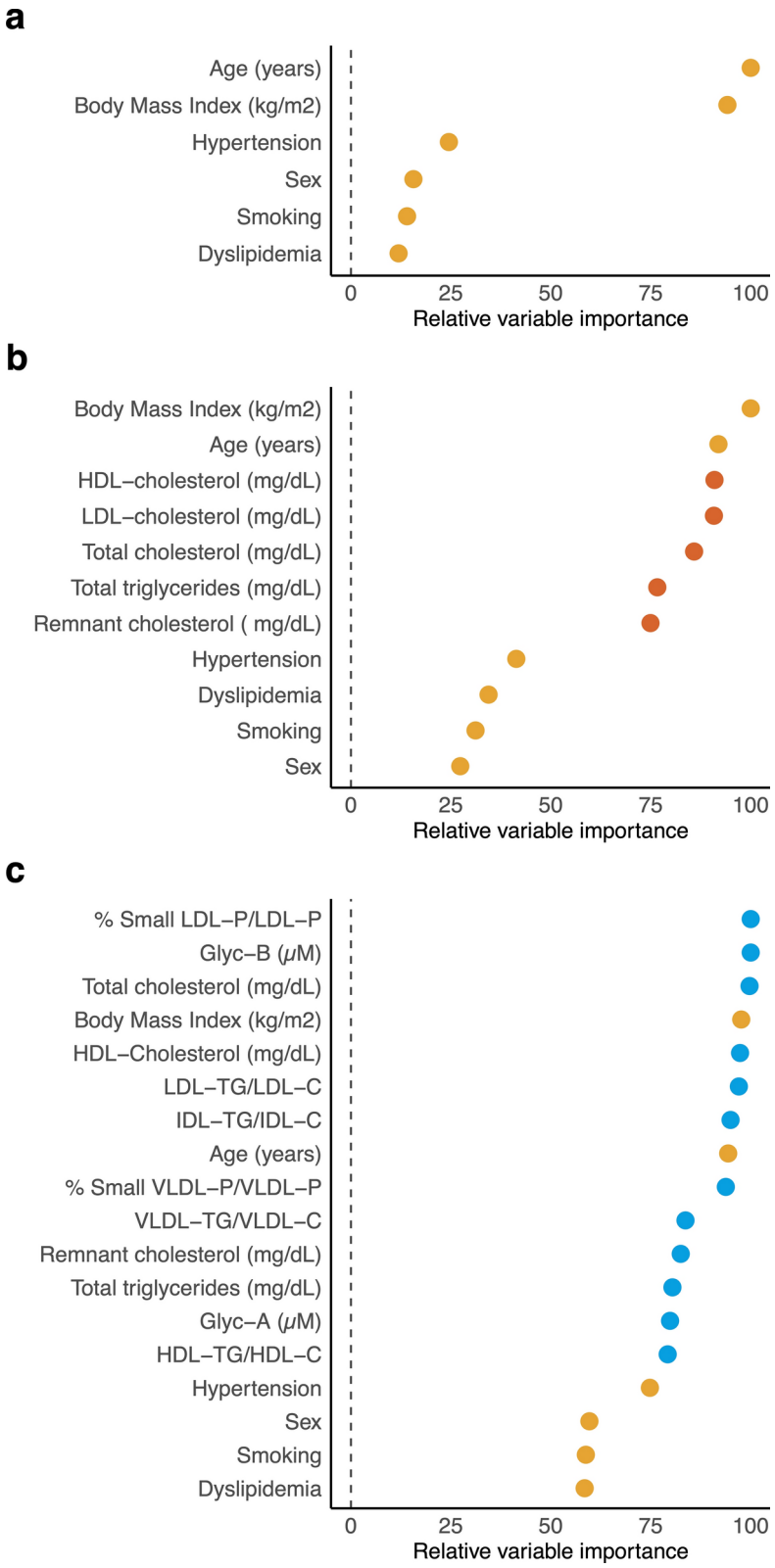


Fig 1. Random forest analysis for CVE prediction. Model 1: **a** Random Forest model of cardiovascular risk factors (including sex, age, BMI, hypertension, dyslipidemia, smoking habit) and **b** Model 2: Random Forest of Model 1 + conventional lipid profile (i.e., total triglycerides, total cholesterol, HDL-C and LDL-C, remnant cholesterol calculated by EAS formula = Triglycerides (TG) * (VLDL-C/VLDL-TG). **c** Model 3: Random forest of Model 1 + Liposcale® + Glycoproteins variables and remnant cholesterol calculated by the Liposcale® formula = VLDL-C + IDL-C. Yellow dots = traditional cardiovascular risk factors. Orange dots = conventional lipid profile. Blue dots = Liposcale® + Glycoproteins variables

Table 4 Performance Metrics of Predictive Models for Cardiovascular Events in the LIPOCAT study

Model description	Model	Accuracy	Sensitivity	Specificity	AUROC
Traditional CV risk factors (CVRF)	1	0.492	0.250	0.733	0.501 [0.36;0.64]
CVRF + conventional lipid profile †	2	0.432	0.050	0.815	0.489 [0.36;0.62]
CVRF + Liposcale® ‡ + Glycoscale	3	0.709	0.850	0.567	0.756 [0.65;0.86]

AUROC data are shown as the median [lower value; upper value] of the confidence interval. CVE, cardiovascular event; AUROC, area under the ROC curve. † Remnant cholesterol EAS formula = Triglycerides (TG) * (VLDL-C/VLDL-TG). ‡ Remnant cholesterol Liposcale® formula = VLDL-C + IDL-C. CVRF, cardiovascular risk factors (BMI, age, sex, hypertension, dyslipidemia, and smoking habit).

Model 3, which had the addition of Liposcale® and Glycoscale variables (0.709). Supplementary Table 1 details false-positive and false-negative rates for each model. (Table 4).

Internal validation cohort analysis

The addition of Liposcale® and Glycoscale variables to the traditional base model increased the prediction accuracy for CVE, as revealed by the use of confusion matrices. Internal analysis by comparing all models revealed that predictability improved from 25% (Model 1) to 85% (Model 3). Moreover, the rate of false negatives was significantly reduced when adding the advanced variables to the traditional ones (Model 3, 15%) compared to using only the traditional CVRFs (Model 1, 75%). However, the relative rate of false positives increased when using the Model 3 (45%) compared to Model 1 (27%).

The comparison of ROC curves (Fig. 2a)and AUC values (Fig. 2c) supported the added value of using advanced variables to predict CVE in the LIPOCAT cohort. Interestingly, the AUC values calculated in Model 3 were significantly increased compared with those calculated using Model 1 (p-value = 0.004) and Model 2 (p-value = 0.002).

To assess potential differences in predictive performance across cardiovascular event (CVE) types, we applied Model 3 (traditional CV risk factors, Liposcale®, and Glycoscale markers) to individual CVE subgroups. As shown in Table 5, the model demonstrated moderate to good discrimination across most event types, with (AUROC values ranging from 0.571 to 0.866).

To further evaluate the predictive performance of our models, we conducted additional analyses incorporating family history of cardiovascular disease, glycoproteins (Glyc A and Glyc B), HbA1c levels, and SCORE2 alongside traditional cardiovascular risk factors (CVRF). The inclusion of family history of cardiovascular disease

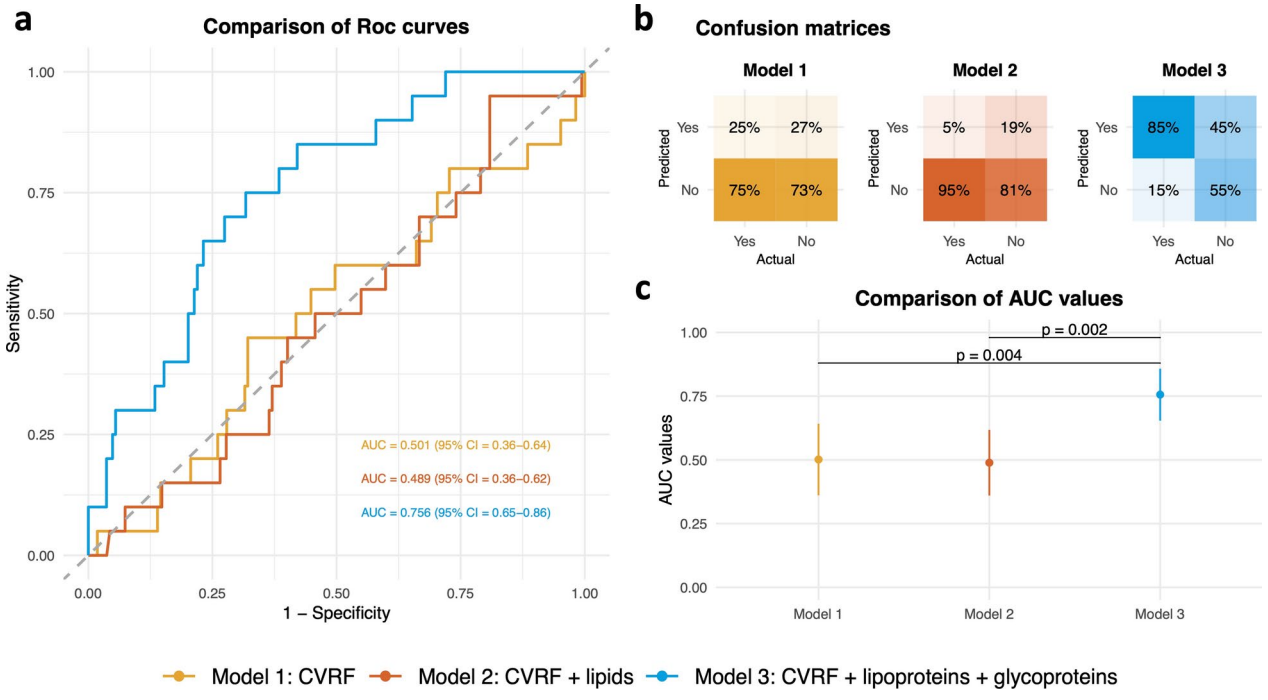


Fig 2. Predictability accuracy and ROC curves in the LIPOCAT cohort. **a**. ROC curves. **b**. Confusion matrices. **c**. AUC curves. Model 1: Traditional CV risk factors (CVRF); Model 2: Traditional CVRF + conventional lipid variables, including remnant cholesterol EAS formula = triglycerides (TG) * (VLDL-C/VLDL-TG). Model 3: Traditional CVRF + Liposcale®, + Glycoscale variables, including remnant cholesterol Liposcale® formula = VLDL-C + IDL-C

Table 5 Predictive performance of Model 3 across different cardiovascular event subgroups in the LIPOCAT cohort

Cardiovascular events	Balanced Accuracy	Sensitivity	Specificity	AUROC
All-cause mortality	0.607	0.50	0.71	0.738 (0.31–1.00)
Cardiovascular mortality	0.646	0.50	0.79	0.756 (0.34–1.00)
Acute myocardial infarction	0.666	0.67	0.66	0.815 (0.61–1.00)
Angina pectoris	0.653	0.67	0.64	0.705 (0.32–1.00)
Ischemic heart disease	0.668	0.67	0.67	0.709 (0.42–1.00)
Stroke	0.841	1.00	0.68	0.829 (0.70–0.96)
Peripheral arteriopathy	0.551	0.50	0.60	0.571 (0.31–0.83)
Heart failure	0.634	0.50	0.77	0.740 (0.34–1.00)
Coronary revascularization	0.829	1.00	0.66	0.866 (0.69–1.00)

slightly reduced the false negative rate; however, it did not significantly improve the overall model performance, as reflected by unchanged AUC values (0.756, Supplementary Table 2 and Supplementary Fig. 1). Upon adding glycoproteins to the traditional CVRF model (Model 4, Supplementary Fig. 2), the AUC increased to 0.629, indicating improved predictive performance compared to the base model using CVRF alone (Model 1). However, this improvement was not statistically significant in comparison to the model that incorporated both Liposcale® and Glycoscale variables (Model 3). Incorporation of HbA1c into the traditional CVRF and Liposcale® parameters (Model 4, Supplementary Fig. 3) resulted in an increased AUC (0.682) compared to the base model using CVRF alone (Model 1). However, this improvement was less pronounced than that observed in Model 3, which included both Liposcale® and Glycoscale variables. Consequently, the addition of HbA1c did not surpass the predictive performance of the model incorporating glycoproteins, and the overall conclusions of the study remain unchanged. Integrating the SCORE2 risk score into the traditional CVRF model (Model 4, Supplementary Fig. 4) led to an AUC increase to 0.678, reflecting improved predictive performance over CVRF alone. The subsequent addition of the HbA1c variable in Model 5 resulted in a slight enhancement in discrimination, though this did not substantially alter the AUROC values compared to Model 4. Importantly, both models demonstrated inferior predictive accuracy relative to Model 3, which consistently yielded the highest performance. Overall, Model 3, which integrates traditional CVRF with Liposcale® and Glycoscale variables, consistently demonstrated the best performance for CVE prediction across all analyses.

External validation cohort analysis

To validate the results from the LIPOCAT cohort, we tested the models and conducted an analysis using confusion matrices and calculated the AUROC values with an independent cohort comprising 187 subjects, of which 41 had a CVE. The clinical variables are detailed in Supplementary Table 3, while the Liposcale® and Glycoscale variables are provided in Supplementary Table 4. This validation process allowed us to assess the performance and generalizability of the models using a separate dataset.

Consistent with the LIPOCAT cohort, this validation cohort showed that the AUROC score values calculated when adding the Liposcale® and Glycoscale variables to traditional CVRFs were 1.2-fold higher (AUROC: 0.669) compared with the traditional base model (0.540) (Δ value = 0.129) (Fig. 3).

Our analysis also revealed that the balanced accuracy value estimated using the traditional base model, which was strictly built only using traditional CVRFs, was 0.499, much lower than that obtained by adding the Liposcale® and Glycoscale variables (0.631) (Table 6).

The addition of Liposcale® and Glycoscale variables to the traditional base Model 1 also increased the prediction accuracy for CVE in the external validation cohort (Fig. 3a), similar to the internal validation analysis results (Fig. 2a). The estimated accuracy was greater when using Model 3 (74%) compared with Model 1 (20%). The false negatives were also reduced when adding the advanced to the traditional CVRFs (Model 3, 26%) compared with the matrix built solely on traditional factors (Model 1, 80%). However, the relative rate of false positives remained much higher for Model 3 (48%) compared with Model 1 (20%).

The comparison of ROC curves (Fig. 3b) and AUC values (Fig. 3c) supports the added value of using advanced variables to predict CVE in this validation cohort, fully consistent with the data obtained using the LIPOCAT cohort.

Discussion

Conventional clinical lipid/lipoprotein variables do not show the full complexity of the altered lipid metabolism associated with increased cardiovascular burden in subjects with T2D [32]. Conversely, the relationship between

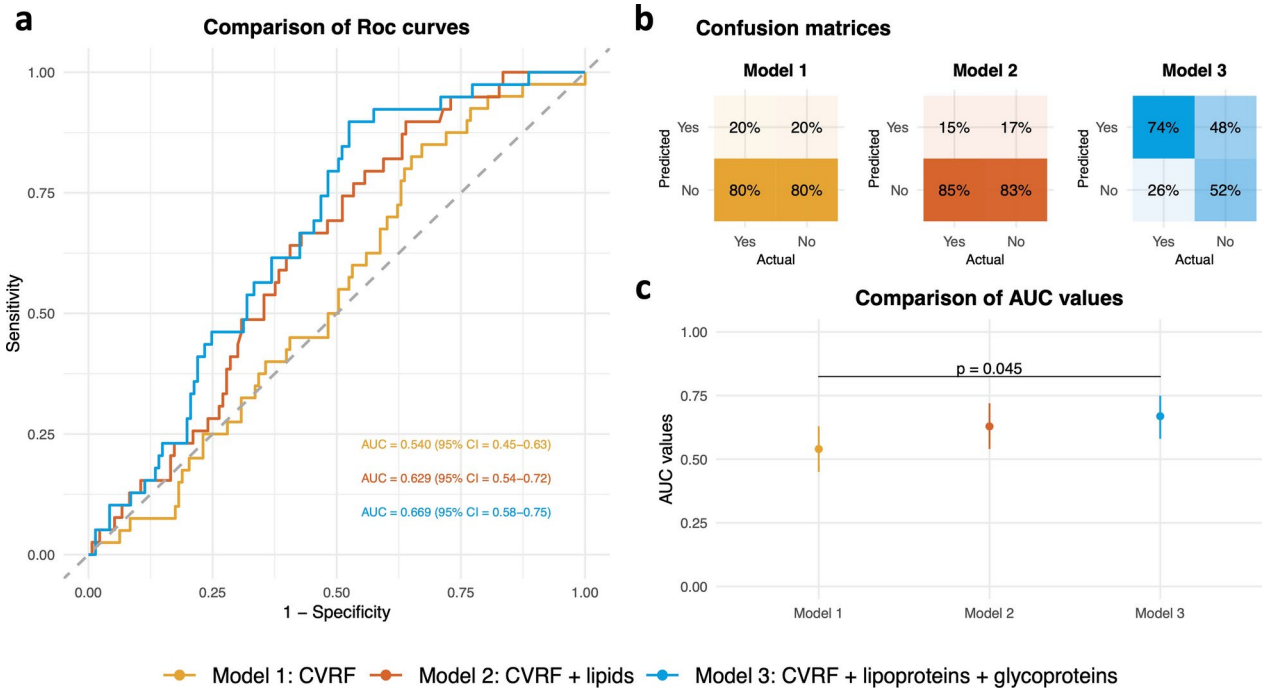


Fig. 3 Predictability accuracy and ROC curves in the validation cohort. **a**. ROC curves. **b**. confusion matrices. *c*. AUC curves. Model 1: Traditional CV risk factors; Model 2: Traditional CV risk factors + conventional lipid variables, including remnant cholesterol EAS formula = triglycerides (TG) * (VLDL-C/VLDL-TG). Model 3: Traditional CV risk factor + Liposcale®, + Glycoscale variables, including remnant cholesterol Liposcale® formula = VLDL-C + IDL-C

Table 6 Performance Metrics of Predictive Models for Cardiovascular Events in the validation cohort

Model description	Model	Accuracy	Sensitivity	Specificity	AUROC
Traditional CV risk factors (CVRF)	1	0.499	0.200	0.797	0.540 [0.45;0.63]
CVRF + conventional lipid profile †	2	0.494	0.154	0.835	0.629 [0.54;0.72]
CVRF + Liposcale® ‡ + Glycoscale	3	0.631	0.744	0.518	0.669 [0.58;0.75]

AUROC data are shown as the median [lower value; upper value] of the confidence interval. CVE, cardiovascular event; AUROC, area under the ROC curve. † Remnant cholesterol EAS formula = Triglycerides (TG) * (VLDL-C/VLDL-TG). ‡ Remnant cholesterol Liposcale® formula = VLDL-C + IDL-C. CVRF, cardiovascular risk factors (BMI, age, sex, hypertension, dyslipidaemia, and smoking habit).

advanced lipoprotein characteristics or glycoproteins and atherosclerosis has recently been reported in subjects with T2D [5, 33]. However, their contribution to CVE in this complex metabolic context has not been previously evaluated. In this study, we assessed the predictive potential of quantitative non-traditional variables analyzed by 1H-NMR for CVE across 933 T2D subjects enrolled from different cohorts. Advanced NMR methodologies enable the assessment of hidden, differentially expressed molecules related to lipoprotein metabolism and inflammation, which could be candidate biomarkers of CVE in subjects with T2D.

Our NMR data revealed elevations in total triglycerides, primarily due to significant increases in triglycerides in VLDL, IDL, and LDL, although VLDL-TGs were only marginally increased in the CVE group. These findings suggest impaired lipolysis, which could lead to the accumulation of cholesterol remnants in this group of subjects [31]. In support of this is the evidence showing an accumulation of VLDL and IDL in T2D subjects in the CVE group. In this respect, firstly, VLDL-C, and IDL-C albeit marginally, were increased in the CVE group. Secondly, due to the nature of circulating IDL particles, our [1H]-NMR approach did not classify this class of lipoproteins by size. Despite this limitation, medium and smaller VLDL-P were also increased in the CVE group; thus, their sum could be considered a novel correlate of such remnant lipoprotein. Lastly, the remnant cholesterol levels in the CVE group were significantly elevated compared with the non-CVE group. Moreover, the calculated levels of cholesterol remnants were consistent with those directly determined by the NMR approach. Indeed, our NMR analysis provided a more accurate estimation of serum cholesterol remnant concentrations than previous studies [34, 35], which simply calculated them by subtracting the LDL-C and HDL-C moieties from total cholesterol determined using conventional methods.

Although the atherogenicity of cholesterol remnants, which include both medium-to-smaller range of VLDL and IDL, is an old concept [36], there is renewed interest due to their predictive potential for CVE [31, 37]. The accumulation of cholesterol remnants in circulation is considered a potential risk factor for future CVE in T2D subjects [38]; consistent with our data. Specifically, elevations in circulating IDL are highly proatherogenic [39] and considered a risk factor for CVD [40]. In this context, we identified the IDL-TG-to-IDL-C ratio (Liposcale®) as a variable associated with CVE. However, no significant differences in the ratio were observed between the CVE and non-CVE groups.

Another important finding from our study was the elevated concentrations of LDL-TG in the CVE group compared with the non-CVE group. Our data align with other studies where LDL-TG has been recently identified as a risk factor for CVD [41, 42]. Since CVE parallels the development of atherosclerosis in T2D [43], increased triglyceride content in LDL might also be a potential candidate risk factor of CVE; however, LDL-TG did not appear among the advanced variables significantly linked to CVE.. In our cohort, despite the absence of significant differences in LDL-cholesterol levels, both groups (with and without CVE) exhibited a high proportion of small LDL particles, with over 50% of total LDL belonging to the small subclass (particle size < 21 nm). This lipid profile is characteristic of atherogenic dyslipidemia, a well-recognized contributor to increased cardiovascular risk in patients with type 2 diabetes. Our findings suggest that LDL particle composition, rather than LDL-cholesterol concentration alone, may be a more relevant marker of atherogenicity in this population.

Systemic chronic inflammation, a frequent feature in diabetes [44], is also related to an elevated risk of CVE in T2D subjects [45]. In this context, another set of variables differentially expressed in the CVE group was glycoproteins. Recent research has linked circulating NMR-derived glycoproteins with chronic inflammation in various clinical studies [28, 33], suggesting their potential as systemic biomarkers of inflammation. Supporting this notion, glycoprotein elevations has been associated with atherogenic dyslipidemia in subjects with T2D [33]. Importantly, their added predictive value above other traditional risk factors of CVE was unveiled in this study, along with advanced lipoproteins characteristics.

In the external validation cohort, the predictive performance of models incorporating advanced NMR-based lipid and glycoprotein variables did not significantly exceed that of models using traditional lipid parameters. This contrasts with findings from the LIPOCAT cohort, where NMR markers improved CVE prediction. The differences in event composition between cohorts likely contributed to this discrepancy. The external cohort predominantly experienced stroke and heart failure, with no recorded cases of atherosclerotic cardiovascular disease (e.g., myocardial infarction), where lipid metabolism plays a more critical role. Additionally, the smaller sample size and the absence of mortality events may have limited the statistical power to detect differences in model performance.

Although NMR is currently considered a relatively expensive technique, its scalability and clinical utility make it a valuable tool for cardiovascular risk assessment. It is particularly beneficial for patients with dyslipidemia and residual cardiovascular risk, especially those with metabolic disorders such as diabetes, obesity, and metabolic syndrome. The Spanish Society of Arteriosclerosis (SEA) has identified key patient groups who could benefit most from this technology [46]. A preliminary evaluation estimated that around 0.1% of the Spanish population (~48,000 individuals) would be eligible for NMR-based lipoprotein analysis. Despite its cost, a single NMR instrument can process up to 50,000 samples per year ensuring sufficient analytical capacity without requiring additional infrastructure for broad territories. Given that a single plasma sample can provide over 70 molecular parameters related to cardiometabolic and inflammatory risk, NMR profiling represents a powerful tool for improving risk stratification and guiding preventive strategies.

One of the main strengths of this study was the inclusion of a large population of age, sex, and BMI-matched T2D subjects with follow-up of CVE. This eliminated major confounding factors, providing a better evaluation of lipoprotein characteristics uncovered by advanced [1H]-NMR analysis. Another important strength was the improved predictive value provided by incorporating Liposcale® variables to traditional clinical variables linked to CVE, confirmed elegantly in an independent validation cohort. However, our study had some limitations. The study did not examine traditional factors like diabetes duration or kidney disease estimates for their effects on CVE risk. This limitation prevented us from directly comparing our developed models to the SCORE2-diabetes risk prediction algorithms, which are currently used to predict events in reference populations. As these variables were not consistently recorded across cohorts, they may have obscured the predictive value of advanced [1H]-NMR-derived variables in our study. Although our model exhibits a relatively high false-positive rate, its value lies in identifying individuals with a molecular profile associated with elevated cardiovascular risk, even if they have not yet experienced the event. A high-risk classification may indicate that the patient is in a subclinical or pre-event stage, suggesting they are closer to experiencing a cardiovascular event due to their molecular signature. This predictive capacity is particularly valuable

for early risk stratification, allowing for timely preventive interventions that could mitigate disease progression and reduce the likelihood of future adverse events.

Conclusions

Advanced 1H-NMR analysis reveal previously hidden lipoprotein and glycoprotein characteristics associated with CVE in T2D subjects. The enhanced prediction of CVE offered by these advanced variables, beyond traditional risk factors, suggests their potential as novel biomarkers for cardiovascular risk stratification in diabetes mellitus.

Abbreviations

CVD	Cardiovascular disease
CVE	Cardiovascular events
CVRFs	Cardiovascular risk factors
IDL	Intermediate-density lipoprotein
LDL	Low-density lipoprotein
HDL	High-density lipoprotein
NMR	1H-nuclear magnetic resonance
T1D	Type 1 diabetes mellitus
T2D	Type 2 diabetes mellitus
TG	Triglycerides
VLDL	Very-low-density lipoprotein

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12933-025-02636-5.

Supplementary file 1.

Supplementary file 2.

Supplementary file 3.

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Author contributions

NAm, EC, JF-N., and DM participated and designed the study. NAm, EC, JF-N, and DM conceptualized the study. All authors contributed to researched data; NAm, EC, NM-M, JJ and DM analyzed and interpreted data. All authors contributed to the discussion and reviewed the manuscript; NAm, EC, JJ, wrote the original draft of the manuscript; NAm, EC, JJ, MH, NA, JF-N., and DM reviewed/edited the manuscript; DM supervised the study. All authors read and approved the manuscript.

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Data availability

Proposals relating to the data access should be directed to the corresponding authors. To gain access, data requestors will need to sign a data access agreement.

Declarations

Ethics approval and consent to participate

The study protocol for the LIPOCAT Study was approved by the Ethics Committees of University Hospital Germans Trias i Pujol (PI-18–039). The associated cohorts were previously approved by the Ethics Committees of University Hospital Arnau de Vilanova (CEIC-1571, Diabetes Mellitus study), Ethics and Clinical Investigation Committee of IDIAP Jordi Gol (P16/014, ARTPER; P11/58, FIBROSCAN), Hospital Clinic of Barcelona (Clinic cohort ClinicalTrials.gov Identifier: NCT01898572, DIABIMCAP Study), and Carlos Haya Hospital (Di@bet.es), and all participants were duly informed about the study protocols and provided their consent to participate.

Consent for publication

Not applicable.

Competing interests

N. Amigó is a stock owner of Biosfer Teslab and has a patent of the lipoprotein profiling described in the present manuscript. Prof. Mauricio is a co-author of this study and an Editorial Board member of the Cardiovascular Diabetology journal. He did not participate in handling this manuscript during the submission or review processes. The rest of the authors declare that they have no competing interests.

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