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Precipitated sdLDL: An easy method to estimate LDL particle size

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Abstract

Background: LDL-C lowering is the main measure in cardiovascular disease prevention but a residual risk of ischemic events still remains. Alterations of lipoproteins, specially, increase in small dense LDL (sdLDL) particles are related to this risk.

Objective: To investigate the potential use of sdLDL cholesterol concentration (sdLDL-C) isolated by an easy precipitation method and to assess the impact of a set of clinical and biochemical variables determined by NMR on sdLDL concentration.

Methods: sdLDL-C and NMR lipid profile were performed in 85 men samples. Association among them was evaluated using Pearson coefficients (r_{xy}). A multivariate regression was performed to identify the influence of NMR variables on sdLDL-C.

Results: A strong association between sdLDL-C and LDLLDL-P (r_{xy} = 0.687) and with LDL-Z (r_{xy} = −0.603) was found. The multivariate regression explained a 56.8% in sdLDL-C variation (P = 8.77.10-12). BMI, ApoB, triglycerides, FFA, and LDL-Z showed a significant contribution. The most important ones were ApoB and LDL-Z; a 1nm increase (LDL-Z) leads to decrease 126 nmol/L in sdLDL-C.

Conclusion: The association between sdLDL-C, LDL-Z, and LDL-P is clear. From a large number of variables, especially LDL-Z and apoB influence on sdLDL-C. Results show that the smaller the LDL size, the higher their cholesterol concentration. Therefore, sdLDL-C determination by using this easy method would be useful to risk stratification and to uncover cardiovascular residual risk.

KEYWORDS

atherosclerosis, lipoprotein precipitation, nuclear magnetic resonance lipid profile, residual cardiovascular risk, small dense LDL

Abbreviations: ALT, alanine aminotransferase; Apo, CIII apolipoprotein CIII; ApoA-I, apolipoprotein A; ApoB, apolipoprotein B; ApoE, apolipoprotein E; AST, aspartate aminotransferase; BMI, body mass index; CRP, creatinine, c-reactive protein; CVD, Cardiovascular diseases; CVRFs, cardiovascular risk factors; FFAs, free fatty acids; Fib, fibulin; GPT, glutamate-pyruvate transaminase; HDL, high-density cholesterol; IDL-C, intermediate-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; LDL-P, LDL particles; LDL-Z, LDL size; LPA, lipoprotein A; MUFAs, monounsaturated fatty acids; NMR, nuclear magnetic resonance; PUFAs, polyunsaturated fatty acids; r_{xy}, Pearson coefficients; sdLDL, small dense LDL particles; sdLDL-C, small dense LDL particles cholesterol; Tg, triglycerides; Vit A, vitamin A; Vit E, vitamin E; VLDL-C, very low-density lipoprotein cholesterol; ρ, Spearman correlation coefficients.

1 | INTRODUCTION

Cardiovascular diseases (CVD) are the leading cause of mortality in the occidental countries being subclinical atherosclerosis the triggering factor for most of these events.[1] Premature atherosclerotic disease commonly occurs in individuals with atherogenic dyslipidemia who share a phenotype characterized by centripetal obesity, insulin resistance, and physical inactivity. In addition, they also have a lipid profile characterized by an increase in triglycerides (Tg) and small dense lipoproteins (sdLDL) concentrations. Also, a decrease in concentration of high-density cholesterol (HDL) and normal to moderately elevated low-density lipoprotein cholesterol (LDL-C) are generally observed in these patients.[2]

Reducing the incidence of cardiovascular events requires the early detection of cardiovascular risk factors (CVRFs), such as LDL-C concentration, nowadays the most important therapeutic target.[3] However, it has been demonstrated that lowering LDL-C concentration is not enough to prevent all ischemic events even in patients without CVRFs.[4] People with LDL-C concentrations lower than 3.4 mmol/L suffer from subclinical atherosclerosis. In fact, it has been shown that in 20% of patients whose treatment results in reductions in LDL-C to 1.81 mmol/L or less continue to show atherosclerotic plaque progression.[5] Therefore, it seems that measuring LDL-C alone does not provide enough information for CVD early detection, and treatment strategy based on lowering LDL-C concentration would be not enough for preventing all premature ischemic events. Nowadays, in some American scientific societies the LDL-C therapeutic threshold value is simply being lowered.[6] Nevertheless, the latest data suggest that LDL-C, by itself, cannot explain all aspects of atherosclerotic disease,[7,8] summarizing that LDL-C is not a good predictor of cardiovascular events in all cases.[9]

The number of particles, their size, and cholesterol concentration in different subclasses of lipoproteins are three factors that could be useful to estimate the residual cardiovascular risk unexplained by LDL-C concentrations alone.[8-10] It is well known that sdLDL particles are more atherogenic. Due to their small size and high density, these particles can easily infiltrate the vascular endothelium and promote atheroma formation.[11] The concentration of cholesterol derived from sdLDL (sdLDL-C) particles is related to the thickening of the intima media[7,12] and to a higher risk of cardiovascular disease.[13,14] Despite sdLDL particles are considered a new CVRF and its measurement is recommended by guidelines, sdLDL analyses are not being easily implemented in the clinical routine.[15] The principal reason is that quantifying sdLDL concentration is expensive and time consuming. An alternative method using Magnesium Heparin as surfactant was developed to precipitate lipoproteins in a way that facilitates the enrichment of sdLDL particles.[16]

It was well known that divalent cations and polyanions precipitate apolipoprotein B containing lipoproteins. Basing in this fact, Hirano et al tested different combinations of polyanion and divalent cations and compared the results with the reference method, the ultracentrifugation. They achieved to find the combination which showed the best correlation with results obtained by the ultracentrifugation. They concluded that the combination of 150 U/mL Heparin-Na and 90 mmol/L $MgCl^2$ allow to precipitate selectively lipoproteins with density <1.044 g/mL.

However, until 2017 there were not reference values established by this method.[17]

Nuclear magnetic resonance (NMR) can be used to estimate the number and diameter of lipoprotein particles from different subclasses.[18,19] This method is consolidated to measure LDL particles number (LDL-P) and their diameters (LDL-Z). However, discrepancies are often observed between LDL-P and the concentration of cholesterol derived from these particles (LDL-C).[20-22] LDL-P is more linked to atherothrombosis and is a better predictor of coronary events than LDL-C alone in some cases.[13,23] NMR lipid profile provides better risk stratification for subclinical atherosclerosis than traditional lipid profile.[24] New consensus guides for cardiovascular risk assessment recommend that lipid-lowering therapies should be guided by LDL-P and not by LDL-C.[25]

Taking into account the literature, LDL-P has been established as a predictor of cardiovascular events. However, the relationship between LDL-P, LDL-Z, and LDL-C is still unclear.

The aim of this study is to investigate the relationship between sdLDL-C obtained by precipitation and characteristics established by NMR in order to know whether performing the sdLDL-C concentration measurement could be useful as a complementary tool in cardiovascular risk assessment.

2 | MATERIALS AND METHODS

2.1 | Subjects

Eighty-five patients from a population selected in Reus (Spain) and Clermont-Ferrand (France) were included in this study. All 85 participants were 19- to 75-year-old male non-smokers who were enrolled in the VITAGE study. The exclusion criteria were as follows: enolism and history of familial hypercholesterolemia and chronic diseases, such as diabetes, cancer, heart failure, and arterial hypertension. Demographic and dietary variables were collected using a questionnaire. These variables included: age, BMI, systolic and diastolic blood pressure, alcohol consumption (g/d), and diet (kcal/d). Informed consent was obtained from all participants, after which blood samples were drawn. Samples were collected in tubes containing the anticoagulant EDTA-K4 and were centrifuged immediately for 15 minutes at 1500 g and at 4°C. The plasma was separated and stored at −80°C until analyzed. In order to guarantee the study reliability, all measurements, which differ from the results obtained before samples freezing more than 3% for Cholesterol and 4% for HDL, were excluded of the study.

The research protocol was approved by the ethics committees of both participating hospitals.

Each patient had their biochemical profiles assessed. This included plasma concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), glutamate-pyruvate transaminase

(GPT), creatinine, c-reactive protein (CRP), albumin, transthyretin, glucose, Free Fatty Acids (FFAs), polyunsaturated fatty acids (PUFAs), monounsaturated fatty acids (MUFAs), fibulin (Fib), vitamin A (Vit A), and vitamin E (Vit E). A complete lipid profile for each patient was also attained including plasma concentrations of cholesterol, very low-density lipoprotein cholesterol (VLDL-C), intermediate-density lipoprotein cholesterol (IDL-C), triglycerides (Tg), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), apolipoprotein A (ApoA-I), apolipoprotein B (ApoB), apolipoprotein E (ApoE), apolipoprotein CIII (ApoCIII), and lipoprotein A (LPA). In addition, atherogenic indices were calculated using the following formula: IA = LDL-C/HDL-C. LDL-Z and LDL-P and sdLDL-C were also measured (Table 1).

2.2 | Determining sdLDL-C

As a first step, sdLDL particles were isolated. This was carried out using a previously reported precipitation method adapted for use in our laboratory to process samples containing elevated concentrations of triglycerides. This method has been validated comparing the results obtained with the reference method, the ultracentrifugation.[16,17] Interassay imprecision of the precipitation method was calculated measuring 10 aliquots of 2 samples, one of them with pathological values of sdLDL-c and the other one with values within the reference interval for sdLDL-c. The study has been carried out during 2 weeks, and the aliquots were stored at −80C°. Interassay imprecision estimated by the variation coefficient (CV %) was 9%, 2%.

The surfactant magnesium heparin was prepared as a solution of 150 U/mL of Heparin-Na^+ (Sigma Aldrich H3393) and 90 mmol/L of $MgCl_2$. 300 μL of Magnesium Heparin was added to each 300 μL of plasma and incubated for 10 minutes at 37°C. The mixture was then kept at 0°C for 15 minutes and centrifuged in at 21 913 *g* for 15 minutes at 4°C (6K15 SIGMA centrifuge). At this point, lipoproteins with a density <1.044 g/mL were precipitated. The supernatant contained HDL and sdLDL particles that had densities of between 1.044 and 1.063 g/mL. Supernatant HDL-C and total cholesterol were measured using a Cobas 8000 modular analyzer (Roche® Diagnostics by homogeneous assays. The linearity range for cholesterol (Cholesterol gen.2) is 0.08-20.7 mmol/L and for HDL (HDL-Cholesterol plus 3rd generation) is 0.08-3.12 mmol/L.

Cholesterol concentration is determined enzymatically using cholesterol esterase and cholesterol oxidase in Cobas 701, Roche Diagnostics®. HDL is also determined by a homogeneous enzymatic assay in Cobas 701, which involves the same enzymes modified with polyethyleneglycol (HDL-Cholesterol plus 3rd generation). In these conditions, hydrogen peroxide is liberated and oxidizes 4-aminoantipyrine to a quinoneimine, which is directly proportional to HDL concentration.

Since supernatant only contained HDL and sdLDL particles, the sdLDL-C was calculated by subtracting the HDL-C from the total cholesterol concentration.

TABLE 1 Demographic variables and lipid and biochemical profile

Variable	Units	Average (range)
Age	years	45.8 (19-75)
BMI	kg/cm^2	25.1 (19.3-30)
Systolic pressure	mm Hg	124 (100-140)
Diastolic pressure	mm Hg	78 (60-96)
Alcohol	g/d	13 (0-100)
Diet	kcal/d	2240 (999-4347)
ALT	μkat/L	0.40 (0.16-1.55)
AST	μkat/L	0.39 (0.25-0.80)
GPT	μkat/L	0.35 (0.1-1.3)
Creatine	μmol/L	91.2 (57-120)
CRP	mg/L	6.2 (6-28.3)
Albumin	mg/L	44 (34-49)
TTR	g/L	0.28 (0.19-0.37)
Glucose	mmol/L	5.3 (4.2-6.8)
Fib	ng/L	0.48 (0-2)
Vit A	μmol/L	1.81 (1.15-2.81)
Vit E	μmol/L	30.12 (18.14-48.42)
Cholesterol	mmol/L	1.67 (1.13-2.63)
Tg	mmol/L	0.47 (0.52-2.76)
LDL-C	mmol/L	2.65 (1.40-4.28)
HDL-C	mmol/L	1.27 (0.76-2.39)
IDL-C	mmol/L	0.19 (0.01-0.56)
VLDL-C	mmol/L	0.32 (0.06-1.62)
Apo A	mg/dL	129.8 (95-195)
Apo B	mg/dL	72.2 (37-122)
Apo E	mg/dL	3.49 (1.6-5.8)
Apo CIII	mg/dL	16.03 (9.4-22.8)
Lpa	mg/L	25.22 (2-144)
FFAs	ng/mL	10 802 (6929-21506)
PUFAs	ng/mL	16.08 (6.19-52.49)
MUFAs	ng/mL	49.90 (22.62-80.32)
AI	n.a.	2.20 (1.04-4.80)
LDL-Z	nm	21.13 (19.1-22.7)
LDL-P	nmol/L	684 (0-2432)
sdLDL-C	nmol/L	350 (0-1260)

Abbreviations: AI = LDL-C/HDL-C, atherogenic index; ALT, alanine amino transferase; ApoA-I, apolipoprotein A; ApoB, apolipoprotein B; ApoCIII, apolipoprotein CIII; ApoE, apolipoprotein E; AST, aspartate aminotransferase; BMI, body mass index; CRP, c-reactive protein; FFA, free fatty acids; Fib, fibulin; GPT, glutamate-pyruvate transaminase; HDL-C, high-density lipoprotein cholesterol; IDL-C, intermediate-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; LDL-P, particle concentration; LDL-Z, LDL diameter; Lpa, lipoprotein A; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; sdLDL-C, small, dense LDL cholesterol concentration; Tg, triglycerides; TTR, transthyretin; Vit A, vitamin A; Vit E, vitamin E; VLDL-C, very low-density lipoprotein.

2.3 | NMR characterization of LDL particles

The NMR analyses were carried out with the Vantera® analyzer (LipoScience. Inc). This equipment is approved for in vitro diagnoses by the US Food and Drug Administration (FDA). This technique was used to determine the LDL diameters and estimate particle concentrations (LDL-Z and LDL-P). NMR spectra were acquired by 400 MHz 1H NMR spectrometers included in the Vantera Clinical Analyzer. The methyl signal envelope appearing between 0.718 and 0.914 ppm was analyzed using the LipoProfile-3 algorithm. The contribution of each subcomponent was determined by linear least squares Results were expressed as LDL-P in nmol/L and LDL-Z in nm. [26]

2.4 | Statistical analyses

2.4.1 | Validation of precipitation technique to measure sdLDL-C

First of all, a correlation study was carried out in order to assess the association between LDL-Z, LDL-p, and sdLDL-C by precipitation. The NMR lipid profile data are use as the gold standard. Outliers were identified using the Bland-Altman concordance analysis of paired data. The association of sdLDL-C with LDL-Z and LDL-P was determined by calculating the Pearson correlation coefficient (r_{xy}) with a confidence interval of 95% [95% CI] as well as levels of significance.

2.4.2 | Assessing the impact of a set of clinical and biochemical variables on sdLDL concentration

To study the relation between LDL-Z and sdLDL-C, a multivariate regression analysis adjusted for control variables was performed. Here, the dependent variable (Y) was the sdLDL-C, and the independent variable (X) was LDL-Z.

Those variables which contribute significantly to the sdLDL-C concentration in univariate analysis were included in the multivariate model. Significance levels were set to 0.05.

By using correlation matrices and calculating Spearman correlation coefficients (ρ), collinearities between variables were evaluated. Those variables with a $\rho > 0.5$ were discarded. A multivariate regression model was constructed using the selected variables. The adjusted coefficient of determination (adjusted R^2) was calculated to determine the proportion of variation in sdLDL-C concentration that could be explained by the adjusted model. B and β coefficients were used to assess the contribution of each variable to the multivariate regression model. A higher β coefficient was indicative of a greater contribution.

The analysis was adjusted for the control variables. The SPSS v.17.0 software package was used in all statistical analyses. Results with a *P*-value < .05 were considered statistically significant.

3 | RESULTS

3.1 | Subjects

All 85 participants included in the study completed a questionnaire. Demographic, biochemical, and lipid variables are listed in Table 1. The average age was 45.8 (19-75) years.

3.2 | Validation of precipitation technique to measure sdLDL-C

Only three outliers were discarded. The correlation study showed that there was a direct linear association between sdLDL-C and LDL-P (r_{xy} = 0.687 [0.555-0.785]; $P = 2.1 \times 10^{-7}$, (Figure 1). In addition, there was an inverse association between sdLDL-C and LDL-Z (r_{xy} = −0.603 [−0.723-0.447]; $P = 1.6 \times 10^{-12}$; (Figure 2). Both associations were statistically significant.

3.3 | Impact of a set of clinical and biochemical variables on sdLDL concentration

To evaluate the impact between a set of clinical and biochemical variables on sdLDL, a multivariate analysis was carried out. Results of the univariate analysis are laid out in Table 2. Variables found to be significant in the univariate analyses were BMI, ALT, Vit E, Tg, LDL-C, HDL-C, VLDL-C, IDL-C, ApoA-I, ApoB, ApoE, ApoCIII, FFAs, and LDL-Z. These variables were included in the multivariable model. By convention, age was also included.

Three pairs of variables were found to be collinear. These were as follows: LDL-C and ApoB; VLDL-C and Tg; and HDL-C and ApoA-I. LDL-C, VLDL-C, and ApoA-I contribute less to sdLDL-C concentration variations and were excluded in later analyses.

The multivariate regression model was found to be significant ($P = 8.77 \times 10^{-12}$; R^2 = 0.568). The model explains 56.8% of sdLDL-C variation. Regression coefficients were calculated for each variable included in the multivariate analysis (see Table 3). B values were significant for BMI, ApoB, Tg, FFAs, and LDL-Z. ApoB and sdLDL-C concentrations were the two variables, which contributed most to the model (β = 0.415) and LDL-Z (β = −0.394). The way of LDL-Z significantly influence on sdLDL-C concentration is by a 1nm increase in LDL-Z leads to a 126 nmol/L reduction in sdLDL-C concentration.

4 | DISCUSSION

In this article, we investigated the relationship between the cholesterol derived from precipitated sdLDL particles (sdLDL-C) and the characteristics of LDL lipoproteins, measured by NMR. From a cohort of 85 frozen samples belonging to men enrolled in the VITAGE study,[27] the sdLDL-C concentration was measured and its association with all variables collected was investigated.

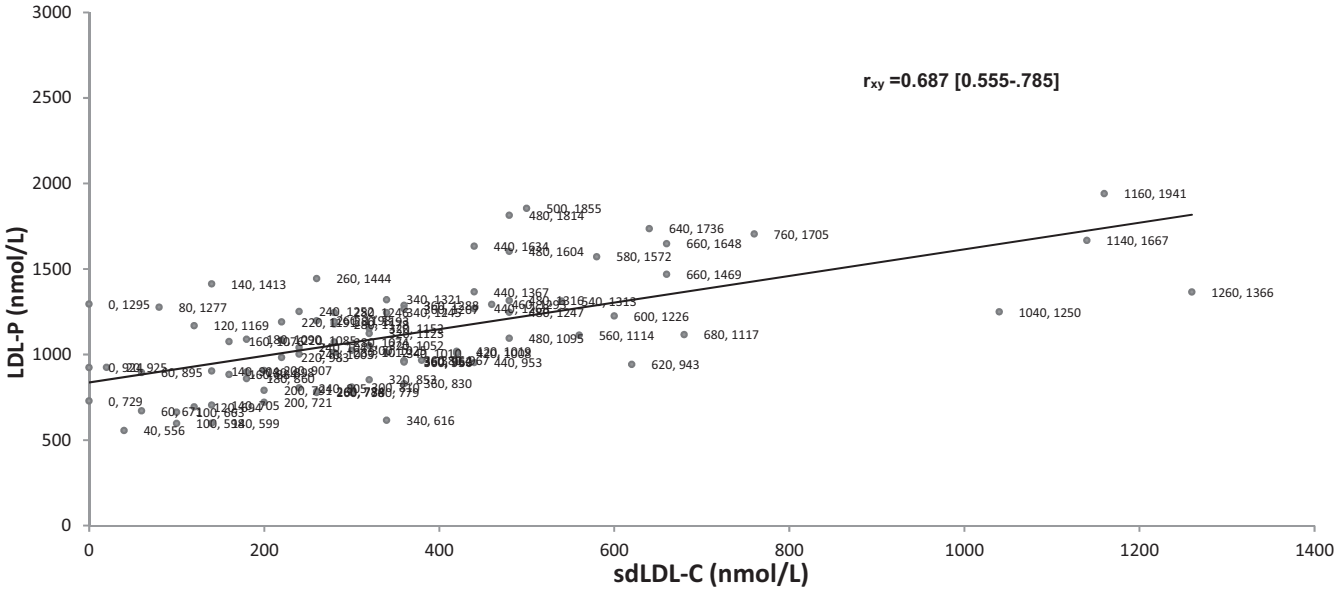

FIGURE 1 Association between sdLDL-C and LDL-P

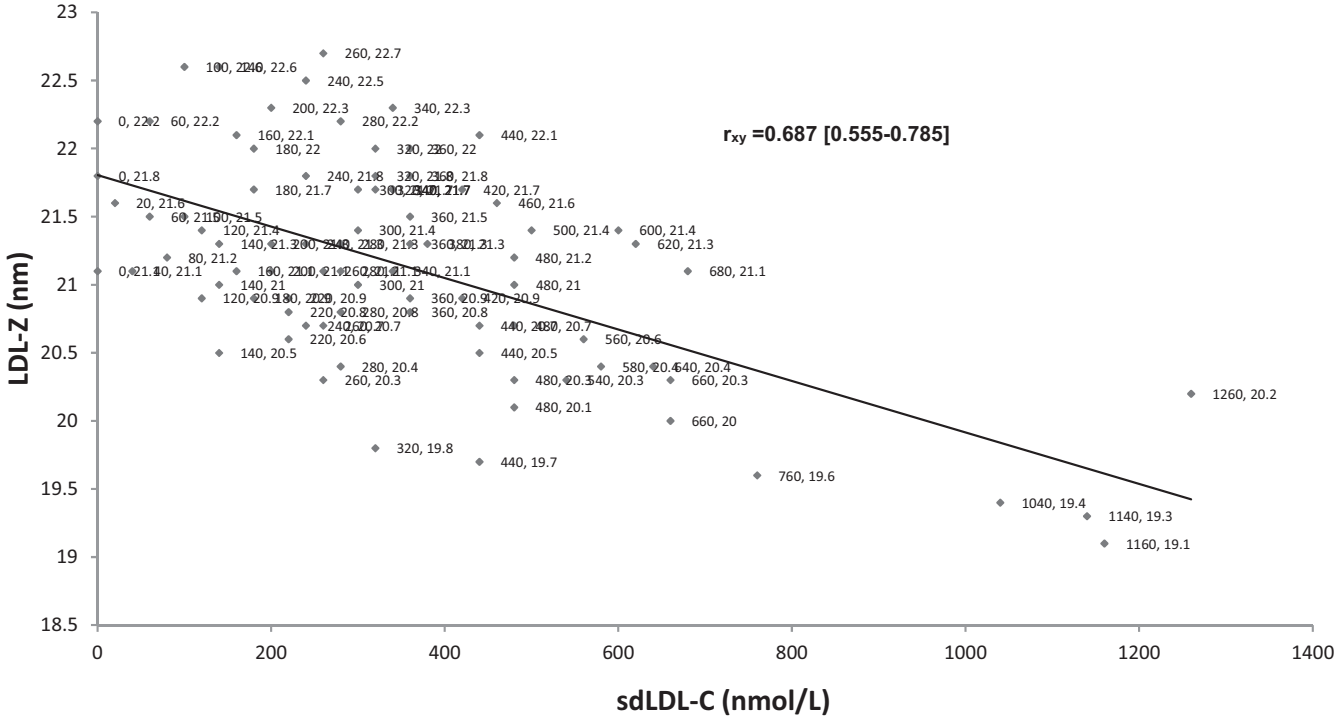

FIGURE 2 Association between sdLDL-C and LDL-Z

In Tables 1 and 2, some characteristic of population studied are shown. In table 1, means and ranges of each variable included in VITAGE study can be seen. Means and ranges from each variable are representative of general population.

With these data, the principal aim of this study was to investigate the relationship between all these characteristics and the concentration of sdLDL-C obtained by precipitation.

Firstly, a correlation study was carried out as a tool to assess the validity of the precipitation method As shown in Figures 1 and 2, there was a direct linear association between sdLDL-C and LDL-P (r_{xy} = 0.687 [0.555-0.785]; $P = 2.1 \times 10^{-7}$). Besides, there was an inverse association between sdLDL-C and LDL-Z (r_{xy} = −0.603 [−0.723-0.447]; $P = 1.6 \times 10^{-12}$). Both were statistically significant associations.

The strong positive association between sdLDL-C and LDL-P proves that the cholesterol isolated by the precipitation method represents a great part of the cholesterol contained in sdLDL particles as determined by NMR. Therefore, it could be that by

TABLE 2 Univariate regression analyses results

Variable	B	*P*
Age (y)	0.18	.174
BMI (kg/cm^2)	23.30	.04
Systolic pressure (mm Hg)	−1.350	.755
Diastolic pressure (mm Hg)	2.859	.571
Alcohol (g/d)	0.62	.8
Diet (kcal/d)	0.073	.925
ALT (μkat/L)	323.063	.021
AST (μkat/L)	414.234	.087
GPT (μkat/L)	210.727	.167
Creatinine (μmol/L)	2.248	.500
CRP (mg/L)	1.167	.648
Albumin (mg/L)	1.163	.942
TTR (g/L)	1.614	.070
Glucose (mmol/L)	4.585	.487
Fib (ng/L)	23.233	.063
Vit A (μmol/L)	−21.507	.062
Vit E (μmol/L)	−15.525	.001
Cholesterol (mmol/L)	113.001	.059
Tg (mmol/L)	374.575	5.43×10^{-13}
LDL-C (mmol/L)	135.64	.01
HDL-C (mmol/L)	−445.004	$.06 \times 10^{-6}$
IDL-C (mmol/L)	414.234	5.63×10^{-5}
VLDL-C (mmol/L)	688.12	1.02×10^{-9}
Apo A (mg/dL)	−5.036	0.002
Apo B (mg/dL)	10.55	2.83×10^{-8}
Apo E (mg/dL)	77.65	.04
Apo CIII (mg/dL)	28.064	.001
Lpa (mg/L)	1.538	.376
FFAs (ng/mL)	0.077	6.26×10^{-9}
PUFAs (ng/mL)	2.498	.988
MUFAs (ng/mL)	0.598	.476
IA	240.718	.078
LDL-Z (nm)	−185.137	1.02×10^{-9}

Abbreviations: AI = c-LDL/c-HDL, atherogenic index; ALT, alanine amino transferase; ApoA-I, apolipoprotein A; ApoB, apolipoprotein B; ApoCIII, apolipoprotein CIII; ApoE, apolipoprotein E; AST, aspartate aminotransferase; B, regression coefficient; BMI, body mass index; c-HDL, high-density lipoprotein cholesterol; c-IDL, intermediate-density lipoprotein cholesterol; c-LDL, low-density lipoprotein cholesterol; CRP, c-reactive protein; c-VLDL, very low-density lipoprotein; FFA, free fatty acids; Fib, fibulin; GPT, glutamate-pyruvate transaminase; LDL cholesterolTg, triglycerides; LDL-P, particle concentration; LDL-Z, LDL diameter; Lpa, lipoprotein A; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; sdLDL-C, small dense; TTR, transthyretin; Vit A, vitamin A; Vit E, vitamin E.

TABLE 3 Regression coefficients for the variables included in the multivariate model

Variable	B [95% CI]	β	*P*
Age (y)	0.169 [−2.627 a 2.966]	0.011	.904
BMI (kg/cm^2)	0.016 [0.032 a 0.114]	0.175	.048
ALT (μkat/L)	78.172 [−95.288 a 251.631]	0.068	.372
Vit E (μmol/L)	−8.780 [−20.717 a 3.158]	-0.230	.147
Tg (mmol/L)	0.182 [0.391 a 0.325]	0.352	.013
HDL-C (mmol/L)	-10.388 [−248.315 a 227.539]	-0.013	.931
IDL-C (mmol/L)	245.684 [−769.679 a 278.312]	0.080	.353
Apo B (mg/dL)	0.071 [0.025 a 0.116]	0.415	.003
Apo E (mg/dL)	3.997 [−70.137 a 62.142]	−0.011	.904
Apo CIII (mg/dL)	10.638 [−27.980 a 6.702]	0.132	.225
FFAs (ng/mL)	0.001 [−0.004 a 0.061]	0.264	.084
LDL-Z (nm)	−126 [−212 a −396]	-0.394	.005

Abbreviations: ALT, alanine aminotransferase; ApoB, apolipoprotein B; ApoCIII, apolipoprotein CIII; ApoE, apolipoprotein E; B, regression coefficient at a 95% confidence interval or CI 95%; BMI, body mass index; c-HDL, high-density lipoprotein cholesterol; c-IDL, intermediate-density lipoprotein; FFAs, free fatty acids; LDL-Z, LDL diameter; Tg, triglycerides; Vit E, vitamin E; β, standardized regression coefficient.

measuring sdLDL-C, LDL-P can be estimated. On the other hand, the negative association between in sdLDL-C concentration and LDL size indicates that people that have smaller particles have more cholesterol transported by them. To summarize, these first results confirm that there is an association between LDL characteristics obtained by NMR and cholesterol measured after isolating sdLDL by precipitation.

Taking into account these results, it would be interesting to study whether it is possible to estimate the presence of particles of small size and its number by measuring sdLDL-C.

According to recent articles, it seems that the presence of an elevated number of sdLDL particles is a clear risk. In fact, the Hortega-Liposcale Follow-up study [23] found an association between the presence of lipoproteins with small and medium size and the increased cardiovascular risk, especially of coronary heart disease.

This is particularly important due to the fact that discordance between LDL-C and LDL-P has been described in some cases.[20,21] The discordance is due to the fact that cholesterol concentration in these particles varies depending on the size of the different LDL subclasses.[20-22] Especially relevant is the MESA study.[22] This study has demonstrated the discordance between LDL-C and LDL-P and its relationship with the development of atherosclerosis and coronary events.

In another study (clinical trial), it has been found that in women with discordant LDL-related measures, coronary risk could be overestimated or underestimated when LDL-C concentration was considered alone.[20] Recently, Tsai MY et al have demonstrated that sdLDL-C concentration measured by Denka Seiken automated assay is associated with CHD events independent of LDL-C concentration. Clinically, they found that sdLDL-C assessment would be more beneficial in patients with intermediate CHD risk, since they could be benefit from intensive treatments.[28]

On account of these studies, LDL-C concentration is not enough to estimate the cardiovascular risk in patients in which LDL-C goals are achieved but elevated concentration of sdLDL particles is found. Keeping in mind this fact, it would be really interesting to know this information through a simple, fast, and inexpensive method. In a recent study, it was demonstrated that in patients with elevated LDL-P the risk of a CHD event increased, and patients undergoing LDL-P measurements were more likely to receive more aggressive lipid-lowering therapy and had a lower cardiovascular risk than a cohort in which just LDL-C was measured.[28,29]

Considering the results from this first study, a regression analysis was performed. The main objective of this second part is to assess the impact of a set of clinical and biochemical variables on sdLDL concentrations. In Table 2, all univariate regressions between sdLDL-C concentration and each variable are shown. Only those significant variables ($P < .05$) were included in the multivariate analysis: age, BMI, ALT, vitamin E, and concentrations of HDL, IDL, ApoB, Apo E, Apo CIII, FFAs, and LDL-Z. Once the variables were included in the multivariate analysis, only TG, LDL-Z, FFA and ApoB concentration continued to be significant (see Table 3).

The final multivariate model is summarized in Table 3. It explains a 57% of sdLDL-C variation. The major contributors in the model are ApoB and LDL-Z. This is a coherent result because sdLDL particles are formed by cholesterol and a single ApoB molecule. However, LDL-C was not finally included in the last model due to its collinearity with apoB and minor significance. Extrapolating to clinical practice, these results agree with literature, since they imply that there are individuals with a normal LDL-C and a high sdLDL-C and vice versa.[20] The discordance phenomenon could be present in people with low LDL-C concentration, even in people who achieve LDL-C goals.[20,21]

Regarding the relation between sdLDL-C concentration variation and LDL-Z, we found that an increase in the diameter of LDL particles implies a decrease in sdLDL-C concentration. Importantly, taking into account the multivariate regression, an increment of 1 nm in LDL size leads to a 126 nmol/L reduction in sdLDL-C concentration. As a consequence, smaller LDL particles contain a higher concentration of cholesterol. Due to its composition, smaller LDL particles would support the formation and progression of the atheroma plaques in higher degree than larger ones. Considering the relationship between sdLDL particles and the development of atherosclerosis, sdLDL-C should be thoroughly investigated as a novel predictor of premature ischemic events. Therefore, when NMR is not available, measuring sdLDL-C concentration could be a reliable tool for diagnosis risk stratification instead of LDL-C measurement. Implementing this method in clinical laboratories is easy since it does not require the acquisition of new equipment nor does it require specialized training. It could be especially important in cases as premature cardiovascular disease, or in patients under lipid-lowering therapy with high cardiovascular CVR who achieve LDL-C goals.

Finally, it is also important to consider the contribution of significant variables as Tg, BMI, and FFAs in the multivariate model. All of these are linked to atherogenic dyslipidemia, which is well-related to the presence of sdLDL particles. Individuals with a high BMI have an excess of FFA, which in turn leads to a high concentration of Tg. An elevated concentration of Tg leads to increased formation of sdLDL particles. Consequently, the concentration of Tg influences sdLDL-C more that it does LDL-C; reviewing the most relevant articles, we could deduce that the LDL-C reducing therapies should not be the only therapeutic strategy for preventing atherothrombosis events. Other factors should be considered, such as Tg concentration, which implies sdLDL particles formation.

Only significant variables from the univariate analyses were used to construct the multivariate model.

Vitamin E concentration is relevant even though it was not included in the multivariate model. However, Vit E contribution to variations in sdLDL-C was more significant than other lipid variables directly related to the formation of sdLDL particles such as IDL-C. Vit E is an antioxidant primarily transported by LDL particles. The oxidation resistance of these particles will depend on their carrying antioxidants such as Vit E.[30] We have verified that there is an inverse relationship between Vit E and sdLDL-C. However, a study should be carried out to determine whether a Vit E deficiency favors the formation of sdLDL particles. In addition, we should find out whether Vit E deficiency is the reason why sdLDL particles have a greater tendency to undergo oxidation and give rise to more unstable atheromas, which increase the risk of ischemic events.[31]

To summarize, it is important to emphasize the fact that the number of articles that show the lack of concordance between the LDL-C and the number of sdLDL particles is increasing, and it must be took into account to explore residual cardiovascular risk. In cases when NMR is not available, to determine the sdLDL-C concentration could be a good strategy to carry out.

5 | CONCLUSION

This study demonstrates that the cholesterol contained in sdLDL measured by an easy and inexpensive method is related to the particle size determined by NMR independently of a great number of variables. Taking into account the necessity of improving risk stratification in cases when LDL-C determination is not enough, when NMR is not available, to quantify the cholesterol concentration in sdLDL particles can be a reliable alternative to estimate residual cardiovascular risk.

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AUTHOR CONTRIBUTIONS

BFC and BCE involved in design of the study, experimental part, and writing and revising full article; JR and ER contributed to select

population, experimental part, and revising full article; MGG, APM, and PA involved in revising full article; NA involved in experimental part and revising full article; XPS involved in writing and revising full article.

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