UNIVERSITAT ROVIRA i VIRGILI

Novel approach to improve lipid prediction by ^{1}H-NMR spectroscopy in severe hypertriglyceridemia

Trabajo de Fin de Grado

Grado de Bioquímica y Biología Molecular

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Content

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Summary

The Liposcale® test uses proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy to calculate lipid concentration (triglycerides and cholesterol) and particle number of the three lipoprotein classes (VLDL, LDL and HDL). The predicted lipid concentrations highly correlate with the standard biochemical determination. However, in cases of hypertriglyceridemia (HTG) with triglycerides (TG) >300 mg/dl, the Liposcale test suffers some interferences, especially in the prediction of HDL-cholesterol (HDL-C). In order to mitigate these interferences a spike-in was performed in HTG samples with TG>600 mg/dl. 50 µl of the sample were added to 150 µl of a human serum standardized buffer, low on TG. The concentrations of TG, total cholesterol and HDL-C predicted after preforming the spike-in had a better correlation with the biochemical determinations than the concentrations predicted with the standard Liposcale® protocol. In addition, the range of values of the determination of HDL-C with the spike-in approach was closer to the range of values of the biochemical determination. Therefore, the spike-in approach improves lipid prediction by the Liposcale® test in HTG samples. A second aim of this project was to identify the presence of chylomicrons (CM) in serum with the Liposcale® test. A chylomicronemic sample was measured by Liposcale® and it was compared to samples with HTG. The sample with CM had an unusually high concentration of VLDL particles (VLDL-P) with an extreme average size of these particles (Z-VLDL). Additionally, ascendant volumes of a CM-rich fraction were added to a CM-free serum and to the human serum standardized buffer. Only the VLDL-P and TG were increased linearly, confirming that the presence of CM is detected as a rise of VLDL-P by the Liposcale® test. This parameter, along with Z-VLDL could be used as a diagnostic tool for chylomicronemia. In conclusion, this study further proves NMR spectroscopy as a powerful tool to assess disease risk and evaluate the patient's outcome.

Keywords

Liposcale

NMR spectroscopy

Chylomicrons

Hypertriglyceridemia

VLDL particles

Abbreviations

ALT: Advanced lipoprotein test

CM: Chylomicrons

CV: Cardiovascular

CVD: Cardiovascular disease

HDL: High-density lipoprotein

HDL-C: High-density lipoprotein cholesterol

HDL-P: High-density lipoprotein particles

HTG: Hypertriglyceridemia

IDL: Intermediate-density lipoprotein

LDL: Low-density lipoprotein

LDL-C: Low-density lipoprotein cholesterol

LDL-P: Low-density lipoprotein particles

LPL: Lipoprotein lipase

MS: Mass spectrometry

NRM: Nuclear magnetic resonance

PC: Principal component

PCA: Principal component analysis

PLS-DA: Partial leasts square- discriminant analysis

RF: Radiofrequency

TG: Triglycerides

TC: Total cholesterol

VLDL: Very low-density lipoprotein

VLDL-P: Very low-density lipoprotein particles

WHO: World Health Organization

Z-VLDL: Size of very low-density lipoprotein particles

1. Introduction

1.1. Biosfer Teslab

Biosfer Teslab is a spin off company of the Rovira i Virgili University (URV) and the Pere Virgili Health Research Institute (IISPV). It offers analytical services to study and monitor alterations in lipid metabolism and its associated cardiovascular (CV) risk using the Nuclear Magnetic Resonance (NMR) high-performance technology.

1.2. Lipoproteins

Cholesterol and triglycerides (TG) in blood are transported in association with proteins (forming lipoproteins) because they are insoluble in water. The function of these lipoproteins is to transport lipids from the liver to peripheral tissues and vice versa (1).

Lipoproteins are classified taking into account their size, lipid composition and the type of apolipoprotein they present in their surface. The main lipoprotein classes are chylomicrons (CM), very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL) (1,2).

Table 1. Classification of lipoproteins. Table adapted from: Mach F, Baigent C, Catapano AL, et al. 2019 ESC/EAS Guidelines for the management of dyslipidaemias: Lipid modification to reduce cardiovascular risk.

Lipoprotein	Density (g/ml)	Size (nm)	Major Lipids	TG (%)	Cholesteryl esters (%)	Cholesterol (%)	Apoproteins	
							Major	Others
Chylomicrons	<0.930	80-100	Triglycerides	90-95	2-4	1	Apo B-48,	Apo A-I, A-II, A-IV, A-V
VLDL	0.930-1.006	30-80	Triglycerides	50-65	8-14	4-7	Apo B-100	Apo E, C-II, C-III, E, A-V
IDL	1.006-1.019	25-30	Triglycerides and cholesterol	25-40	20-35	7-11	Apo B-100	Apo E, C-II, C-III
LDL	1.019-1.063	20-25	Cholesterol	4-6	34-35	6-15	Apo B-100	
HDL	1.063-1.210	8-13	Cholesterol	7	10-20	5	Apo A-I	Apo A-II, C-III, E

1.2.1. Structure and function of lipoproteins

VLDL particles are TG rich and each have one Apo B-100 molecule. When the TG are hydrolysed by the lipoprotein lipase (LPL) in the muscle and adipose tissue, IDL and LDL particles are formed (*Figure 1*), which are enriched in cholesterol, especially LDL particles (1).

The function of LDL particles is mostly to transport cholesterol (LDL-C) from the liver to peripheral tissues; they are the most numerous particles that carry cholesterol. These particles also have one Apo B-100 molecule in their surface that interacts with the LDL receptor in the membrane of cells. Numerous studies have demonstrated that high levels of LDL-C are associated with an elevated risk of cardiovascular disease (CVD) (3). On the other hand, HDL particles transport cholesterol (HDL-C) from peripheral tissues to the liver (reverse cholesterol transport) (*Figure 1*), which makes them anti-atherogenic. They also have anti-oxidant, anti-

inflamatory, anti-thrombotic and anti-apoptotic properties (2). Low levels of HDL-C are associated with a higher risk of CVD (4). *Figure 1* shows the metabolic pathways of lipoproteins. The exogenous pathway indicates the uptake of lipids from the intestine in CM and the endogenous pathway shows the transformation of lipoproteins since they exit the liver until they arrive to the tissues (5).

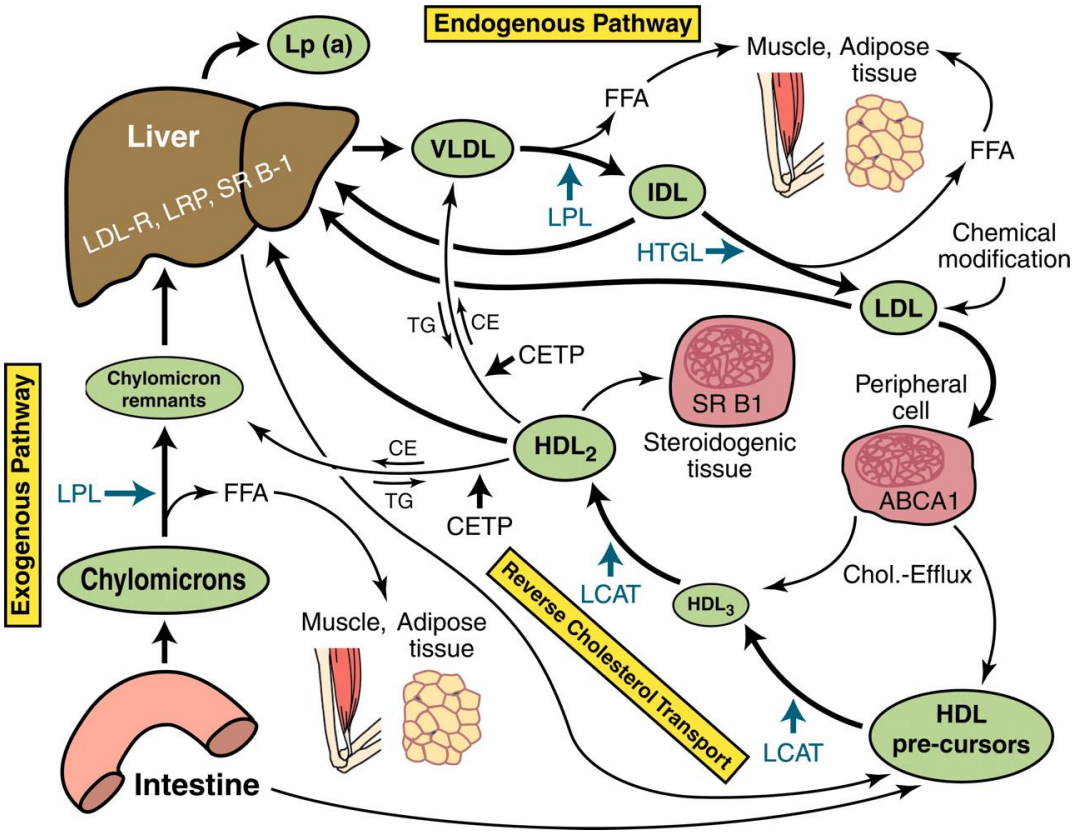

Figure 1. Lipoprotein metabolism. (5)

1.2.2. Lipoproteins and cardiovascular disease (CVD)

According to the World Health Organization (WHO), CVD is the leading cause of mortality in developed countries and it causes a loss of quality of life and a great economic cost to the health system. CVDs are responsible for over 4 million deaths in Europe each year (6). In Spain, they are the cause of death in 30% of the total deaths per year.(7) CVD prevention is very important, especially as the prevalence of some risk factors, like diabetes and obesity are increasing (3).

All ApoB-lipoproteins (VLDL, IDL and LDL) can cross the arterial wall and cause the initiation of an atheroma (8), which can lead to myocardial infarction or death. In Spain, 50% of the population has total cholesterol (TC) levels above 200 mg/dl; 45% has LCL-C>130 mg/dl; 25% has HDL-C levels below the recommended values (>40 mg/dl in males and >50 mg/dl in females), and 20% has high TG levels (TG>150 mg/dl). Traditionally, CV risk has been determined by several risk factors, such as smoking, high levels of total and LDL cholesterol, high blood pressure, obesity or type 2 diabetes (9). However, half of the individuals who develop a CV event have normal levels of LDL-C. One of the reasons of these events is atherogenic dyslipemia, characterized by low HDL-C, high TG and VLDL-C levels, but normal or slightly high LDL-C (10). Despite having normal LDL-C, it is carried primarily in small LDL particles (LDL-P), which are more

prone to enter the arterial wall and they are more susceptible to oxidation, so they can be more easily phagocytized by macrophages. Therefore, they are more atherogenic than the larger particles (2). In a standard lipid panel, these patients will go undetected, because it only gives information about the concentration of LDL-C but no information about the number and size of the particles carrying it. For this reason, as it is shown in *Figure 2*, two patients with the same concentration of LDL-C can have different LDL-P distributions and, therefore, a different level of risk of CVD (11). In the case of HDL, low HDL-C is associated with cardiovascular disease (CVD). However, some studies suggest that the concentration of HDL particles (HDL-P) is a more accurate indicator of CVD risk than HDL-C (12). In addition, another study has shown that individuals with fewer HDL-P may be more susceptible to developing atherosclerosis, even if they have normal or high levels of HDL-C (13). For this reason, it is best to use both HDL-C and HDL-P determinations together to assess the anti-atherogenic properties of HDL.

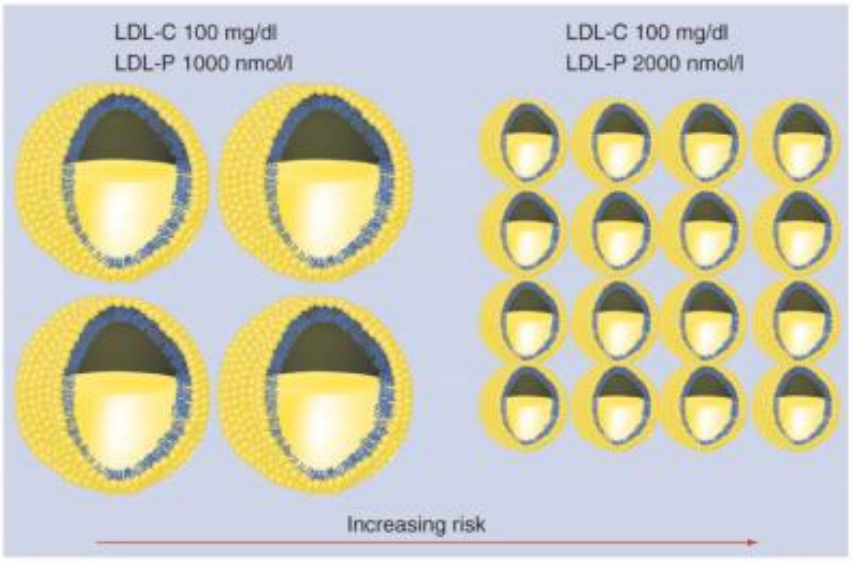

Figure 2. Variability in LDL particle composition despite equivalent LDL-C levels. (11)

The advanced lipoprotein tests (ALTs), including the Liposcale® test, are powerful tools that could provide a more accurate risk evaluation and help prevent CV events. The ALTs allow a complete characterization of lipoprotein particles, analysing serum or plasma samples using NMR spectroscopy. Therefore, they can have a relevant impact in public health, as they enable a better evaluation of a patient's CVD risk in several cases where traditional biomarkers are not enough (14).

1.3. Hypertriglyceridemia

Hypertriglyceridemia (HTG) is described as the presence of TG in blood serum in a concentration higher than 150 mg/dl. High levels of TG have been associated with CVD, obesity, metabolic syndrome and diabetes mellitus. CV risk is elevated because of an increase in TG-rich lipoproteins (VLDL and VLDL remnants), which are highly associated with atherosclerosis.(15) Additionally, high levels of TG are usually associated with low levels of HDL-C, which can also increase CV risk (16). A small fraction of patients with a concentration of TG > 880 mg/dl show presence of CM in serum (chylomicronemia) (17). TG are mainly transported in CM and VLDL-P and they are hydrolysed by the lipoprotein lipase (LPL). In healthy patients, CM are hydrolysed and cleared from the blood in 3 or 4 hours, so they are not found in fasting serum. However,

because of a mutation in the lipoprotein lipase (LPL) gene, some patients present a high concentration of CM and VLDL-P in serum. Another mechanism that can cause extreme TG accumulation is the saturation of TG clearance, which happens when the concentration of TG is higher than 700 mg/dl, approximately (18). Patients with chylomicronemia have a higher risk of suffering acute pancreatitis, due to the extremely high levels of TG (17).

In addition, a high concentration of TG in serum causes interference with analytical methods in clinical laboratories. Lipaemia increases the turbidity of the sample and it can also change the viscosity, making it harder to process the samples and to provide accurate results (19). One example is the Friedewal formula used to calculate LDL-C, which cannot be used when TG are higher than 400 mg/dl. High TG can also interfere with direct measures of HDL-C.(16) Therefore, in hypertriglyceridemic patients it is more difficult to make a precise assessment of disease risk and it can affect the patient's outcome (20).

1.4. Metabolomics

1.4.1. Introduction to metabolomics

Diseases, diet and pharmacotherapy, among others, can cause changes in the homeostasis of different biological components. Nowadays, to understand these disturbances, systems biology is applied, which includes the "omics" sciences. The omics sciences study the structure, function and dynamics of biological components (cell, tissue, organ and organism) to understand and predict their behaviour and the interactions between them. This field includes genomics, transcriptomics, proteomics and metabolomics. First, advancements in genomics led to the genome sequencing and then to the development of other disciplines like transcriptomics and proteomics, which determine the mRNA transcription level and the protein abundance in a specific moment, respectively. Finally, this led to the development of metabolomics, which measures low-molecular-weight metabolites in a cell, tissue organ or organism in a specific moment. The progression of the "omics" sciences development is shown in *Figure 3*.

The metabolite profile can be altered due to diet, a disease or because of taking a drug, among other factors. Metabolomic fingerprinting can detect these changes in the metabolome that reflect the situation of a cell, tissue or the whole organism. That is why the metabolome is considered a reflection of a phenotype. Metabolites are usually measured in blood, urine or tissue extracts. In some cases, changes in the metabolome can appear before the disease symptoms start. Therefore, metabolomics is a promising approach as a tool for diagnostics and for finding new disease biomarkers (21).

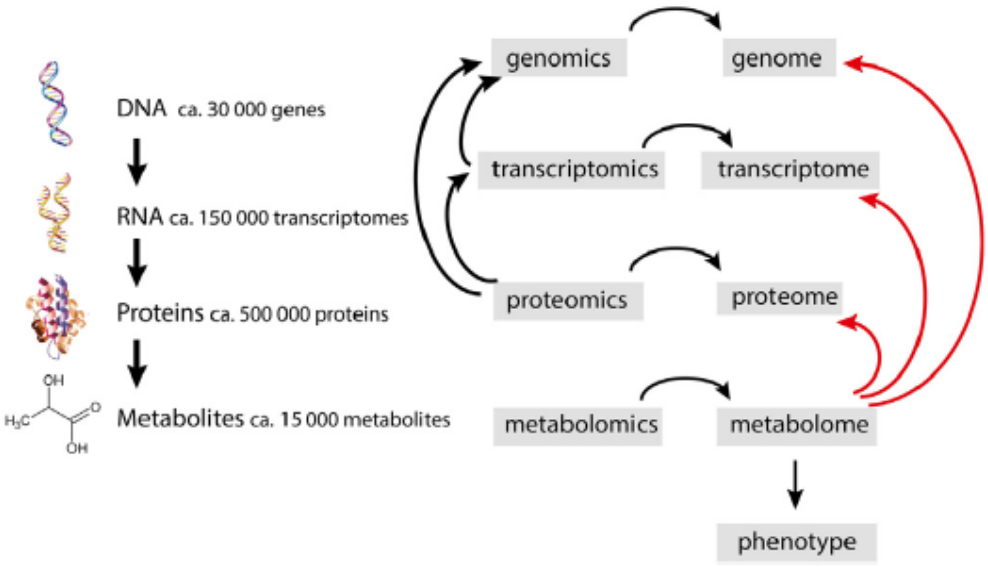

Figure 3. The omics cascade.(21)

1.4.2. NMR spectroscopy in metabolomics

The most used techniques to measure the metabolome are NMR and mass spectrometry (MS). Although MS is a more sensitive technique, NMR has some advantages that make it very useful in metabolomics. NMR is a faster, robust, unbiased technique, and it is non-destructive, which means that the sample can be stored for future analysis. In addition, NMR analysis requires very little sample pre-treatment, the whole sample is analysed at once and there is no need to separate different compounds. It can also be used to determine metabolites in solid matrices, like tissue or cells, when an extraction procedure is applied to them, and to successfully quantify concentrations of those metabolites (21).

NMR spectroscopy can be used to identify standard biomarkers in CVD (cholesterol, triglycerides) and it can also give a quantitative analysis of different classes of lipids and lipoproteins (22), as it is explained in section 1.6.

1.5. NMR spectroscopy

The phenomenon of nuclear magnetic resonance (NMR) is based on the interaction of the nuclei of some atoms with a magnetic field. Only the nucleus of an atom with an odd number of protons or neutrons can have a magnetic moment and a nuclear spin associated to it. When they are in the presence of a magnetic field, the nuclei can have two spin states: some will be in a higher energy, +½ state, and the rest will be in a lower energy, -½ state.

In NMR spectroscopy, a low-amplitude radiofrequency (RF) is applied, perpendicular to the magnetic field. This RF pulse can cause nuclei to change from one energy state to another, so the nuclei start resonating. When the RF is turned off, the nuclei return to their initial state and they emit RF waves which are collected as Free Induction Decays (FIDs). This signal is transformed into frequency with the Fourier Transformation, which gives off a spectrum of absorption (intensity) versus frequency. The spectrum can be one-dimensional (1D) or two-dimensional (2D) (*Figure 4*).

Nuclei of the same element absorb electromagnetic radiation of a given frequency in a magnetic field. However, depending on the chemical environment of the nucleus, the resonance shifts. This phenomenon is called chemical shift (δ) and it is expressed in parts per million (ppm) (23).

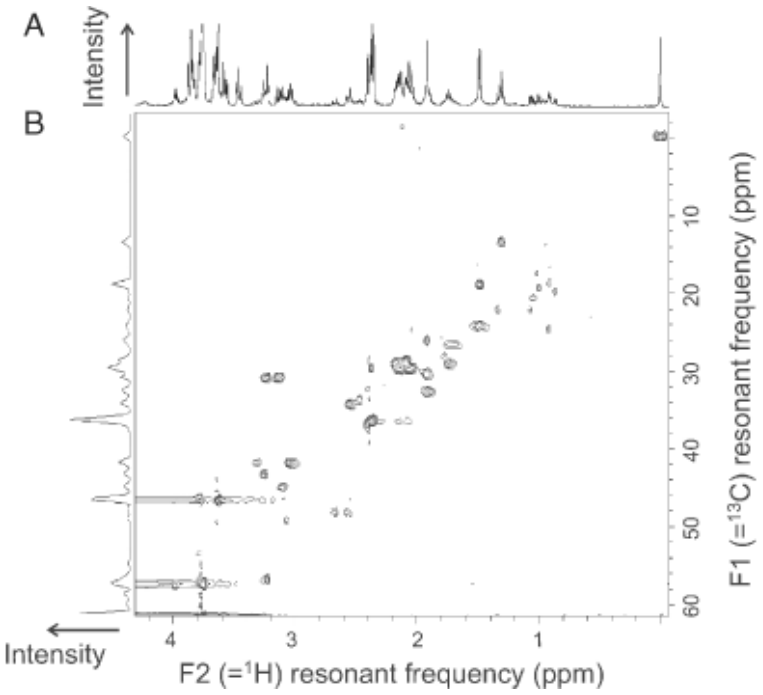

Figure 4. Example of a 1D (A) and a 2D (B) NMR spectra.(23)

1.6. Liposcale test

The analysis of lipoproteins by proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy is based on the fact that the methyl groups of the lipids that constitute lipoproteins resonate at slightly different frequencies depending on the size of the particle, with smaller particles resonating at lower frequencies. Therefore, lipoproteins can be quantified by decomposition of the ^{1}H-NMR signal of the methyl group of the lipids in individual signals. Lipid concentrations are directly proportional to the intensity of the signal, so this method can be used to estimate the concentration of lipids in blood serum. It also provides the concentration of the particles of the main lipoprotein classes (VLDL, LDL and HDL) and of their three subclasses (small, medium and large) (24,25).

The Liposcale® test is a new approach to this method because it uses 2D DOSY-NMR (Diffusion Ordered Nuclear Magnetic Resonance Spectroscopy). It allows the quantification of the concentration of cholesterol and triglycerides of the main lipoprotein classes (VLDL, IDL, LDL and HDL), the particle number and size of the VLDL, LDL and HDL classes, as well as the particle number of their different subclasses (*Figure 5*) (14). Each subclass of lipoprotein is associated to a diffusion coefficient and once they are calculated, the size of the different lipoprotein subclasses is directly estimated through the Stokes-Einstein equation. A larger diffusion coefficient represents a smaller lipoprotein particle. The lipoprotein particle number is calculated by dividing the spatial volume of total lipid molecules by the average size of the lipoprotein particles (14).

The complete characterization of blood lipoprotein profile allows a better estimation of the CVD risk and, therefore, it improves early detection of individuals with a high CVD risk.

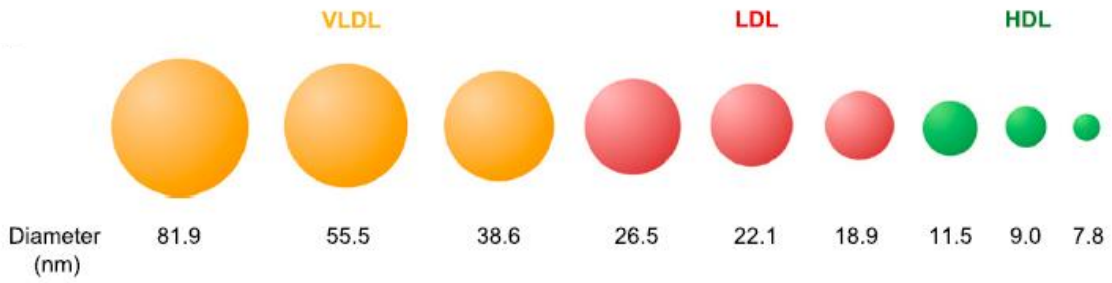

Figure 5. Nine lipoprotein subclasses – small, medium and large VLDL, LDL and HDL – determined out of the NMR spectra of the Liposcale® test. Figure modified from Mallol R, Amigó N, Rodríguez MA, et al. Liposcale: A novel advanced lipoprotein test based on 2D diffusion-ordered 1H NMR spectroscopy. *J Lipid Res*. 2015;56(3):737-746.

1.6.1 Limitations of the Liposcale test

The presence of a very high concentration of TG in blood serum affects the determination of some of the Liposcale® variables, like HDL-C. The Liposcale® test could be affected by an elevation of the viscosity caused by the high concentration of TG. Usually, high levels of TG are related to a decrease in the concentration of HDL-C (16). However, in a sample of HTG individuals (TG>300 mg/dl), some individuals show unusually high HDL-C measured by the Liposcale® test, while the standard biochemical determination is slightly low or normal in most patients (<40 mg/dl in males and <50 mg/dl in females) (26). Thus, there is a discrepancy between the Liposcale® test prediction of HDL-C and the enzymatic determination in some individuals with TG>300 mg/dl that could be improved.

2. Objectives

The aim of this study was to design an experimental method for samples with HTG in order to improve the prediction of HDL-C by Liposcale®. Samples with TG>600 were spiked with a human serum standardized buffer, low in TG, and then the prediction of HDL-C was compared with the measurement made by enzymatic methods, used as reference.

In addition, another aim of this study was to identify the presence of CM in serum with the Liposcale® test. Previous studies have been able to measure the concentration of triglycerides in CM with NMR (27). In this project, the goal was to detect the presence of CM as an increase of VLDL-P with the Liposcale® test.

3. Materials and methods

3.1. Samples

56 samples with HTG were selected from the Biosfer Teslab database of samples from the HUSJR (Hospital Universitari Sant Joan de Reus, Spain). Samples with TG>300 mg/dl were selected because high concentrations of TG interfere with the prediction of HDL-C.

A serum sample with a high concentration of chylomicrons (CM) was used (intact serum). The lipoprotein fractions were separated by high speed centrifugation for 15 minutes in a Heraeus Biofuge Primo micro-centrifuge (20). A separate fraction rich in chylomicrons (d<0.930 g/ml) and VLDL particles (d=0.930-1.006) was obtained, along with another fraction with the rest of the serum components (chylomicron-free, CM-free serum).

3.2. Sample processing

Liposcale. 200 µl of serum sample were diluted with 300 µl of PBS 50 mM (pH=7.4) and 50 µl of deuterated water (D_2O) were added. After that, a mix process was performed in order to achieve a full optimum homogenization of the sample. This process was carried out with the liquid handler Gilson robot.

Spike in experiments. 8 of the HTG samples with a concentration of TG>600 mg/dl were used. 50 µl of the sample were added to 150 µl of human serum standardized buffer with a concentration of TG of 57 mg/dl and concentration of HDL-C of 75 mg/dl. Then, 300 µl of PBS 50 mM (pH=7.4) and 50 µl of D_2O were added.

Sample with hyperchylomicronemia and chylomicron-rich fraction. The intact serum and the CM-free serum were processed with the Liposcale® protocol (14). Then, ascendant volumes of the CM-rich fraction were added (10, 20 and 40 µl) to the CM-free serum. The same methodology was used with a sample with a concentration of TG of 57 mg/dl and a concentration of HDL-C of 75 mg/dl. 300 µl of PBS 50 mM (pH=7.4) and 50 µl of D_2O were added to each of the samples.

3.3. Lipoprotein measurements

The lipoprotein profile of all samples was measured by NMR spectroscopy, using the Liposcale® test protocol (14). In the HTG samples from the HUSJ, total cholesterol (TC), LDL-C, HDL-C and TG were also quantified using standard enzymatic assays adapted to the Cobas-Mira-Plus autoanalyzer (SPINREACT S.A.U., Spain).

3.3.1. NMR spectroscopy

Serum samples were analysed by NMR spectroscopy using the Liposcale® test protocol. ^{1}H-NMR spectra were recorded on a BrukerAvance III 600 spectrometer, operating at a proton frequency of 600.20 MHz (14.1 T), at 305.9 K. A double stimulated echo (DSTE) pulse program with bipolar gradient pulses and a longitudinal eddy current delay (LED) was used. The relaxation delay was 2 s, the finite impulse decays were collected into 64,000 complex data points and 32 scans were acquired on each sample (14).

3.4. Liposcale test

The Liposcale® test uses ^{1}H-NMR spectroscopy to determine the size and concentration of the different lipoprotein subclasses. The NMR spectra is deconvoluted to obtain concentration of the main lipoprotein classes (VLDL, LDL and HDL) and of their subclasses (small, medium and large). The size of each lipoprotein class can also be calculated.

The concentration of TG and cholesterol in the lipoprotein fractions are predicted by a PLS regression model. The validation performance of the PLS models were assessed by venetian blinds cross-validation splitting the data 10 times. Coefficients of determination between the predicted and reference concentrations ranged from 0.79 to 0.98 in the calibration step and from 0.81 to 0.98 in the validation step (14).

3.5. Statistical analysis

A Partial Least Squares-Discriminant Analysis (PLS-DA) model was built to classify those HTG samples with an inaccurate prediction of HDL-C and those with an accurate prediction using the Liposcale® test results as predictors. A second PLS-DA was carried out using the HTG samples with a concentration of TG between 300 and 600 mg/dl (43 samples). Additionally, two Principal Components Analysis (PCA) were performed using a general population and the HTG samples (TG>300 mg/dl) along with a sample with CM.

All the analyses were performed with MATLAB version 7.10.0.499 R2010a (MathWorks). PLS and PCA models were built using the PLS Toolbox (Eigenvector Research).

4. Results

4.1. Classification of HTG samples with interferences in HDL-C prediction

A PLS-DA was performed to distinguish HTG samples with an inaccurate lipid prediction of HDL-C by the Liposcale® test from those with an accurate prediction (*Figure 6A*). The PLS-DA was able to discriminate both groups of samples. The separation was mainly observed among the first latent variable (LV1), which accumulates the 42.02% of the total variance. When analysing the loadings of the LV1 (*Figure 6B*), it was observed that TG had the highest contribution. Since TG mostly accumulate in VLDL particles, all the parameters related to this lipoprotein class, VLDL-TG and all its three subclasses, also present a high contribution in the model. A few variables - small LDL particles and triglycerides transported by HDL and IDL – showed an opposite contribution to the model than the rest of them. After analysing the amount of TG in both groups, a cut-off value of 600 mg/dl was observed to be capable of discriminating the samples with a less accurate prediction of HDL-C.

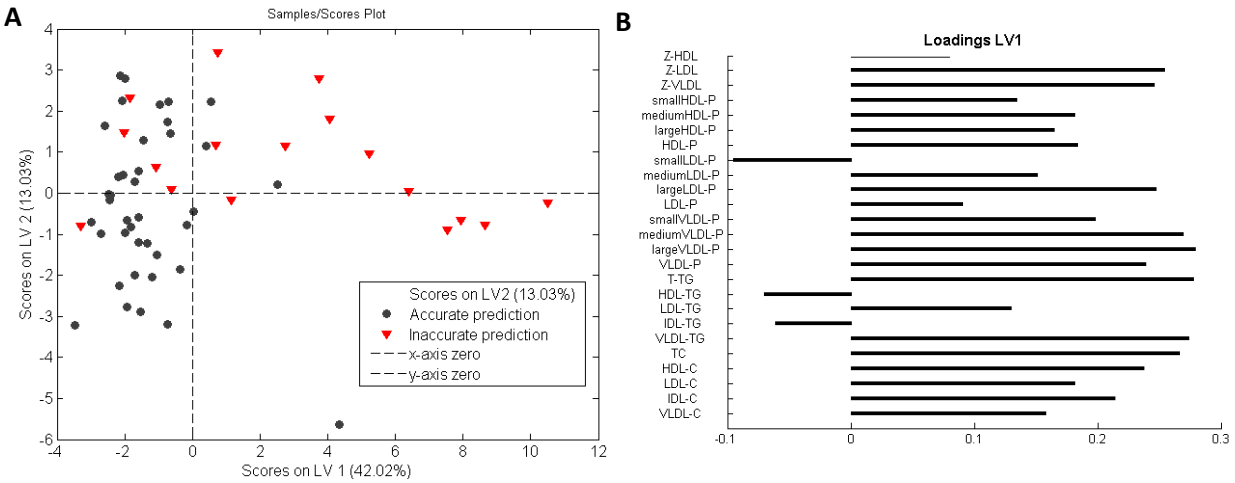

Figure 6. Scores plot (A) and loading plot (B) for PLS-DA model to discriminate inaccurately predicted samples (red) from the samples with an accurate prediction (black).

4.2. Improvement of HDL-C prediction

In order to improve lipid prediction from eight samples classified as suffering from some interference in HDL-C prediction by the PLS-DA, spike-in experiments were performed. All the samples presented severe hypertriglyceridemia (TG>600 mg/dl) and most of them presented unusually high values of HDL-C when measured with the standard Liposcale® protocol (*Table 2*). This effect could be a consequence of an increase in the serum viscosity due to the high TG concentration. The spike-in of the samples with a human serum standardized buffer lowers the viscosity and, thus, reduces the interference in HDL-C prediction. The concentration of HDL-C, TG and total cholesterol (TC) were calculated again with the Liposcale® test in the spiked samples (*Table 2*). Additionally, *Table 2* shows the standard biochemical determination of HDL-C, TG and TC performed with Cobas-Mira-Plus autoanalyzer. The spike-in approach showed HDL-C values more similar to those determined with enzymatic assays, used as a reference.

Table 2. Concentration of HDL-C, TG and TC in 8 HTG samples measured with the Liposcale® test (before and after the spike-in) and with the standard enzymatic assays.

	HDL-C (mg/dl)			Triglycerides (mg/dl)			Total cholesterol (mg/dl)		
	Standard protocol	**Spike-in**	**Enzymatic assays**	**Standard protocol**	**Spike-in**	**Enzymatic assays**	**Standard protocol**	**Spike-in**	**Enzymatic assays**
1	104.48	29.91	41	823.88	1047.11	987	546.99	358.03	273
2	81.77	28.75	27	907.44	1079.21	1097	474.62	307.01	300
3	16.44	27.28	31	883.17	1083.58	1045	261.74	337.35	281
4	37.24	26.36	25	867.34	960.19	1131	477.26	320.47	384
5	104.44	40.57	20	1195.61	1838.09	2229	593.66	397.46	382
6	64.21	20.96	26	645.16	842.50	812	354.75	271	190
7	91.13	38.53	23	931.65	1263.93	1346	382.61	358.89	341
8	81.27	24.37	31	575.19	835.91	701	335.53	269.24	220

Scatter plots of the concentrations of TG, TC and HDL-C measured with the standard Liposcale® protocol and the spike-in against the direct determination by the Cobas-Mira-Plus autoanalyzer were performed (*Figure 7*). The pearson coefficients (r) between the TG determined by the standard enzymatic assays and the TG predicted by the Liposcale® test was 0.93 when the standard protocol was used and 0.98 when the spike-in was performed. On the other hand, the correlation coefficient between the TC determined by the standard enzymatic assays and the TC predicted by Liposcale® was 0.56 with the standard protocol and 0.74 with the spike-in. Therefore, the predictions of TG and TC are improved with the spike-in protocol. In the case of the HDL-C determination, before performing the spike-in, the correlation coefficient was 0.52. With the spike-in approach, the correlation coefficient between the enzymatic assays and Liposcale was 0.55. The correlation coefficient for the HDL-C determination is not as high as the previous ones because the range of values is very small. Even still, the spike-in protocol shows an improvement compared to the standard protocol. *Figure 8* shows the concentration of HDL-C in each sample determined by the three previous methods. The range of values of the Liposcale® prediction of HDL-C was much wider when the standard protocol was used. For this reason, the correlation coefficient does not change much with the new protocol. However, with the spike-in protocol the predicted concentrations of HDL-C were in the range of the enzymatic determinations and the values were also more coherent with the reference values (*Figure 8*).

Thus, the spike-in protocol improves the prediction of HDL-C, TC and TG in HTG samples with TG>600 mg/dl.

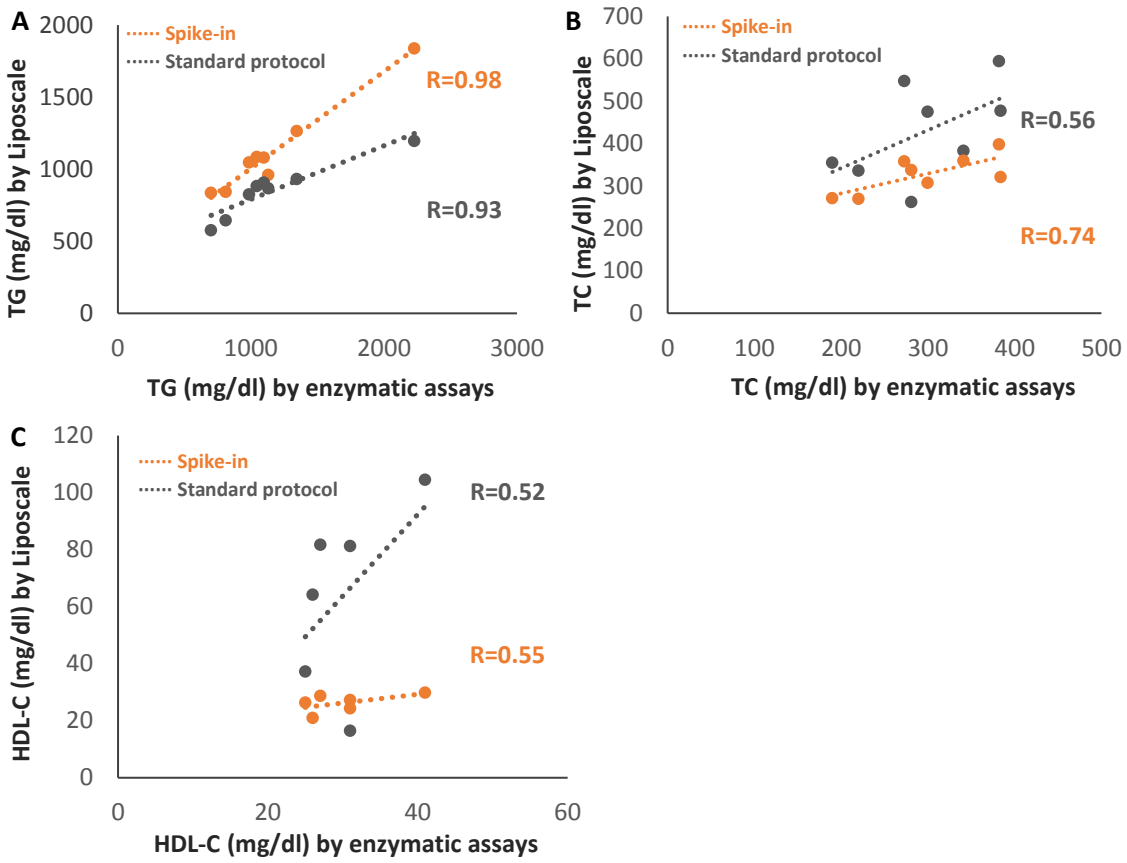

Figure 7. Regression plot of the concentration TG (A), TC (B) and HDL-C (C) obtained with the standard Liposcale® protocol and the spike-in protocol against the enzymatic determination. Two of the samples (5 and 7) had a concentration of HDL-C lower than the limit of detection, so they are not shown in the regression plot of HDL-C.

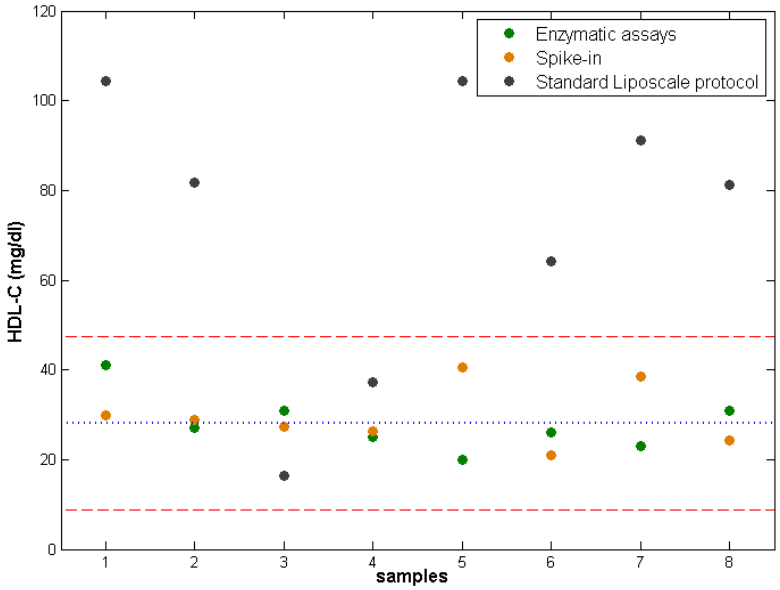

Figure 8. HDL-C values determined by enzymatic assays (green), spike-in (orange) and the standard Liposcale® protocol (black). Blue line: mean of the enzymatic determination of HDL-C; Red lines: mean ± 3 x standard deviation of the enzymatic determination of HDL-C.

Finally, ^{1}H-NMR spectra of the HTG samples before and after the spike-in were compared (*Figure 9*). Due to the high concentration of TG, the spectra had a different shape and a much higher intensity in the spectral regions that are used to predict lipid concentration compared to the spectra obtained from samples with a lower concentration of TG (*Figure 9 and Supplementary figure S1*). This change of shape in the spectra is probably what causes interference in the HDL-C prediction by the Liposcale® test. After spiking the 8 samples with a pool with low concentration of TG, the shape of the peaks is more similar to the spectra of a sample with normal TG values used as control (*Figure 9 and Supplementary figure S1*). Thus, the spike-in reduces the effect of the interference and allows a more accurate prediction of HDL-C.

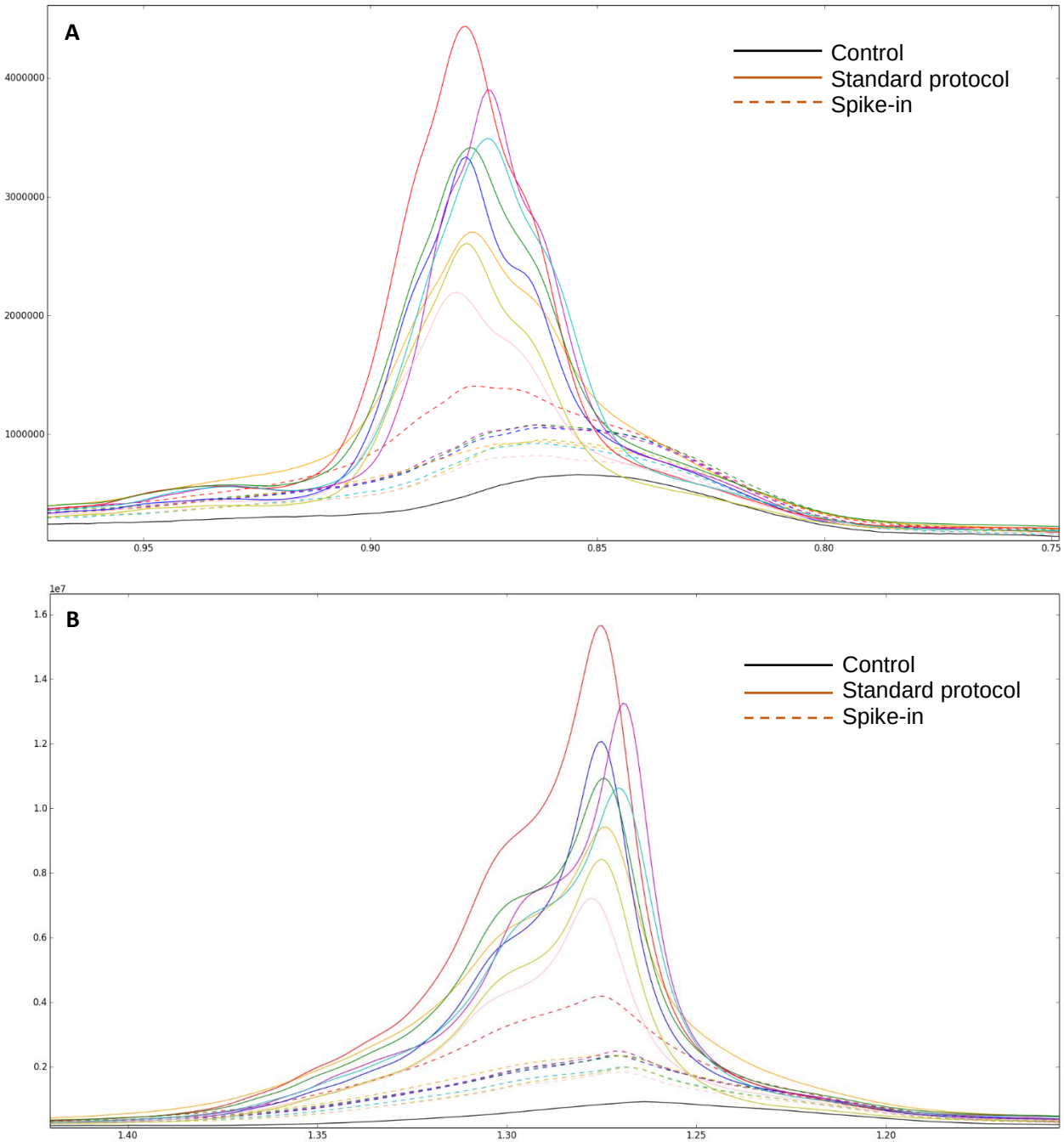

Figure 9. Spectral differences in the signal of the methyl (A) and methylene (B) groups before and after the spike-in. Each colour corresponds to one sample, before (continuous line) and after (dashed line) the spike-in. The spectra obtained after the spike-in have a lower intensity and a shape similar to the control sample (black line).

4.3. Detection of chylomicrons in serum samples

The intact serum and the CM-free serum from the sample with chylomicronemia were analysed with the Liposcale® test. The concentration of TG in the sample with CM was 2106 mg/dl, which is higher than the concentration found in the general population and in any of the HTG samples. The intact serum showed particularly large VLDL particles (VLDL-P), with an average size (Z-VLDL) of 44.97 nm. This value is really extreme in VLDL-P, which are usually between 41 and 43 nm of diameter. Z-VLDL of this sample was compared to a general population (*Figure 10*). The average size of VLDL-P in the sample with CM was bigger than the 99th percentile of the general population (42.78 nm). The number of VLDL-P was also higher in the sample with CM than in the general population (*Supplementary figure S2A*).

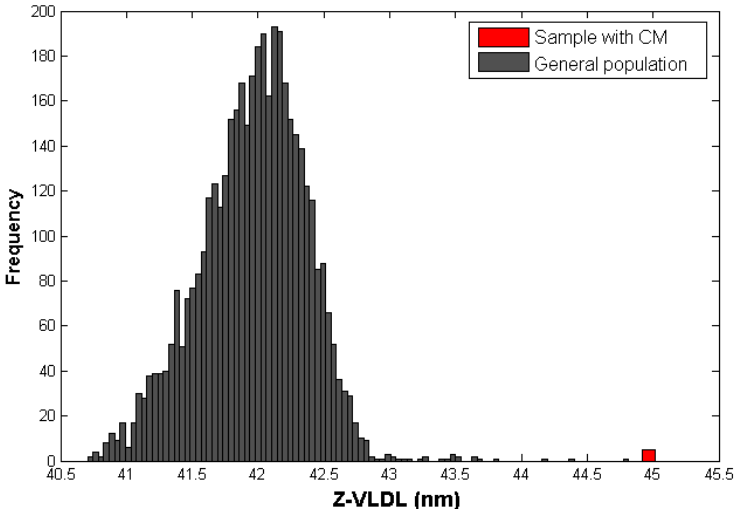

Figure 10. Size of VLDL particles (Z-VLDL) in a sample with chylomicrons (CM) compared to the general population.

A PCA was carried out to see if the chylomicronemic sample could be differentiated from the general population (*Figure 11*). The separation was mainly observed among the first principal component (PC1), which accumulates the 37.74% of the total variance. When analysing the loadings of the PC1 (*Figure 11B*), it was observed that TG had the highest contribution to the model, along with VLDL particles and its three subclasses. Since the sample with CM shows extreme values of these variables, this sample is located in the positive side of the PC1. The second principal component (PC2) shows a negative contribution of TG and VLDL-P and a positive contribution of LDL-C and LDL-P (*Supplementary figure S3A)*. Due to the presence of CM, the Liposcale® test suffers from interferences in the prediction of LDL-C and the predicted concentration of LDL-C is very low (5 mg/dl). This variable is very different from the general population so it contributes to the separation of the chylomicronemic sample. Thus, the PCA model is able to distinguish the sample with CM from the general population mainly according to the TG and VLDL-P concentration and also the low concentration of LDL-C.

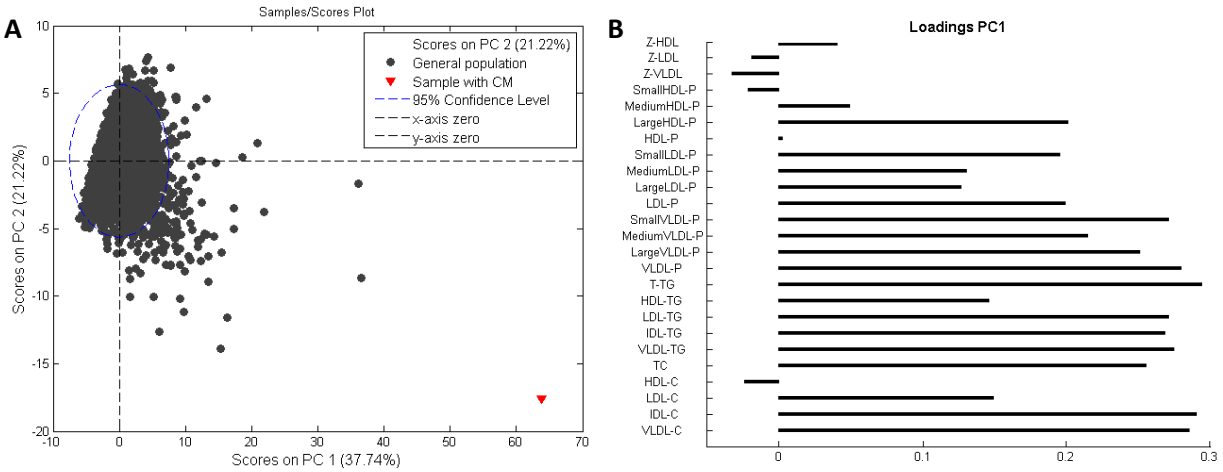

Figure 11. Scores plot (A) and loading plots plot of the PC1 (B) for PCA model of a general population (black) and one sample with chylomicrons (red).

Additionally, the sample with CM was compared to a subset of HTG individuals with TG>300 mg/dl to see if the presence of CM can be differentiated from a classical HTG. The Z-VLDL of the chylomicronemic sample was also higher than the 99th percentile of the HTG population (44.47 nm). In the HTG population only 3 individuals have Z-VLDL>44 nm (*Figure 12*). The bigger average size of the VLDL-P is mainly associated to an increase in the medium VLDL-P (and total VLDL-P) compared to the HTG samples with TG>300 mg/dl (*Supplementary figure S2B and S2C*).

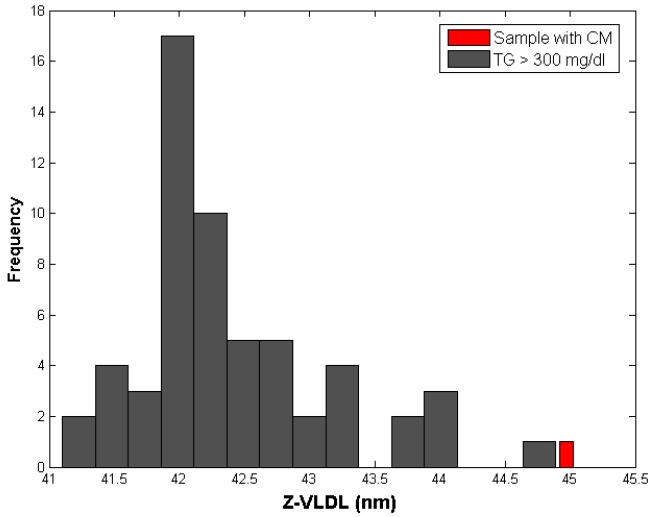

Figure 12. Size of VLDL particles in a sample with chylomicrons (CM) compared to a subset of HTG individuals.

Finally, a second PCA was performed with the subset of HTG individuals and the chylomicronemic sample (*Figure 13*). The separation was mainly observed among the first principal component (PC1), which accumulates the 43.62% of the total variance. When analysing the loadings of the PC1 (*Figure 13B*), it was observed that TG had the highest contribution to the model, along with VLDL particles and their size (Z-VLDL). As it can be seen in the PCA (*Figure 13A*), the sample with CM showed extreme values of these variables compared to the HTG population. Therefore, it confirms that the chylomicronemic sample can be distinguished from HTG samples by the concentration of VLDL-P and their size. The second principal component (PC2) shows a negative contribution of HDL-TG and medium HDL-P and a positive contribution of LDL-C and LDL-P (*Supplementary figure S3B*). The sample with CM has a higher proportion of TG in the HDL particles than the HTG samples. Taking into account the low concentration of LDL-

C due to the interferences caused by CM, the chylomicronemic sample shows great differences in these variables compared to the HTG samples so they also have a big contribution in the separation of the sample with CM (*Figure 13A*).

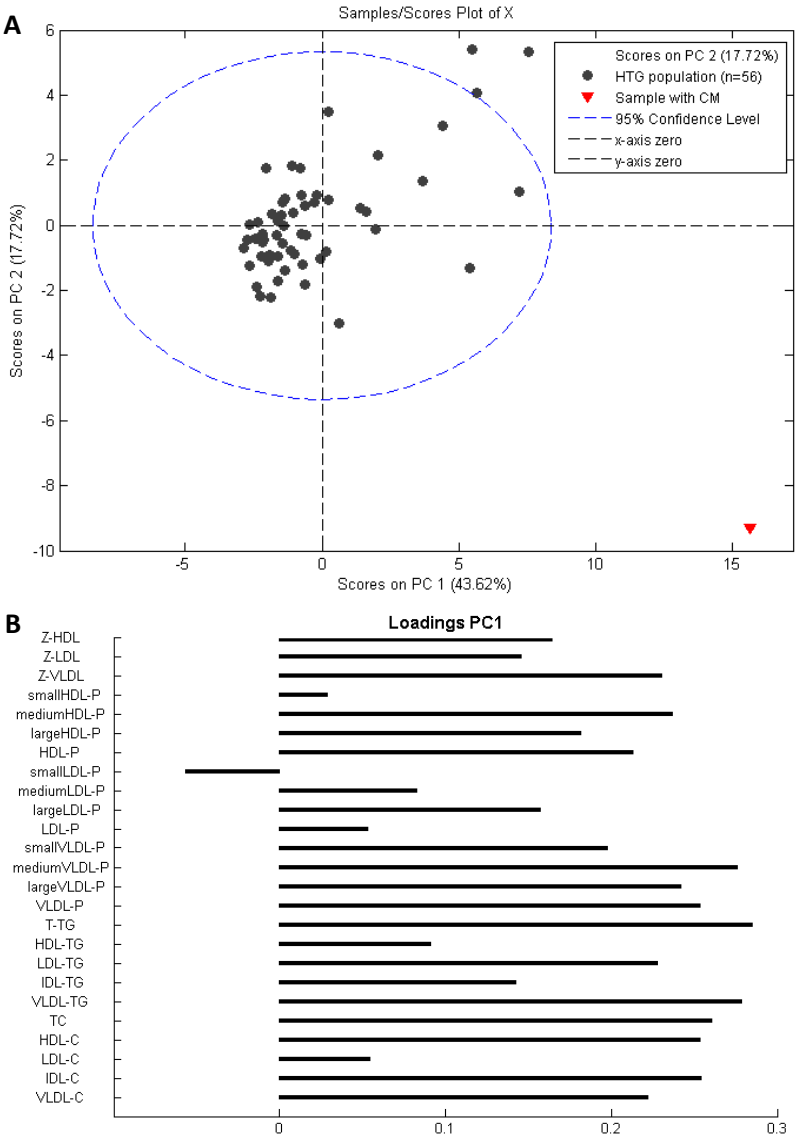

Figure 13. Scores plot (A) and loading plots plot of the PC1 (B) for PCA model with HTG samples (black) and one sample with chylomicrons (red).

To verify that the concentration of VLDL-P increased due to the presence of CM, ascendant volumes of the CM-rich fraction were added to the CM-free serum and to a human serum standardized buffer, low in TG. *Figure 14* shows the variation in the concentration of VLDL-P, largeVLDL-P, VLDL-TG and TG after adding CM to the CM-free serum and to the human serum standardized buffer. All of them present a linear increase. Therefore, it can be assured that the presence of CM is detected by the Liposcale® test as a rise in VLDL-P.

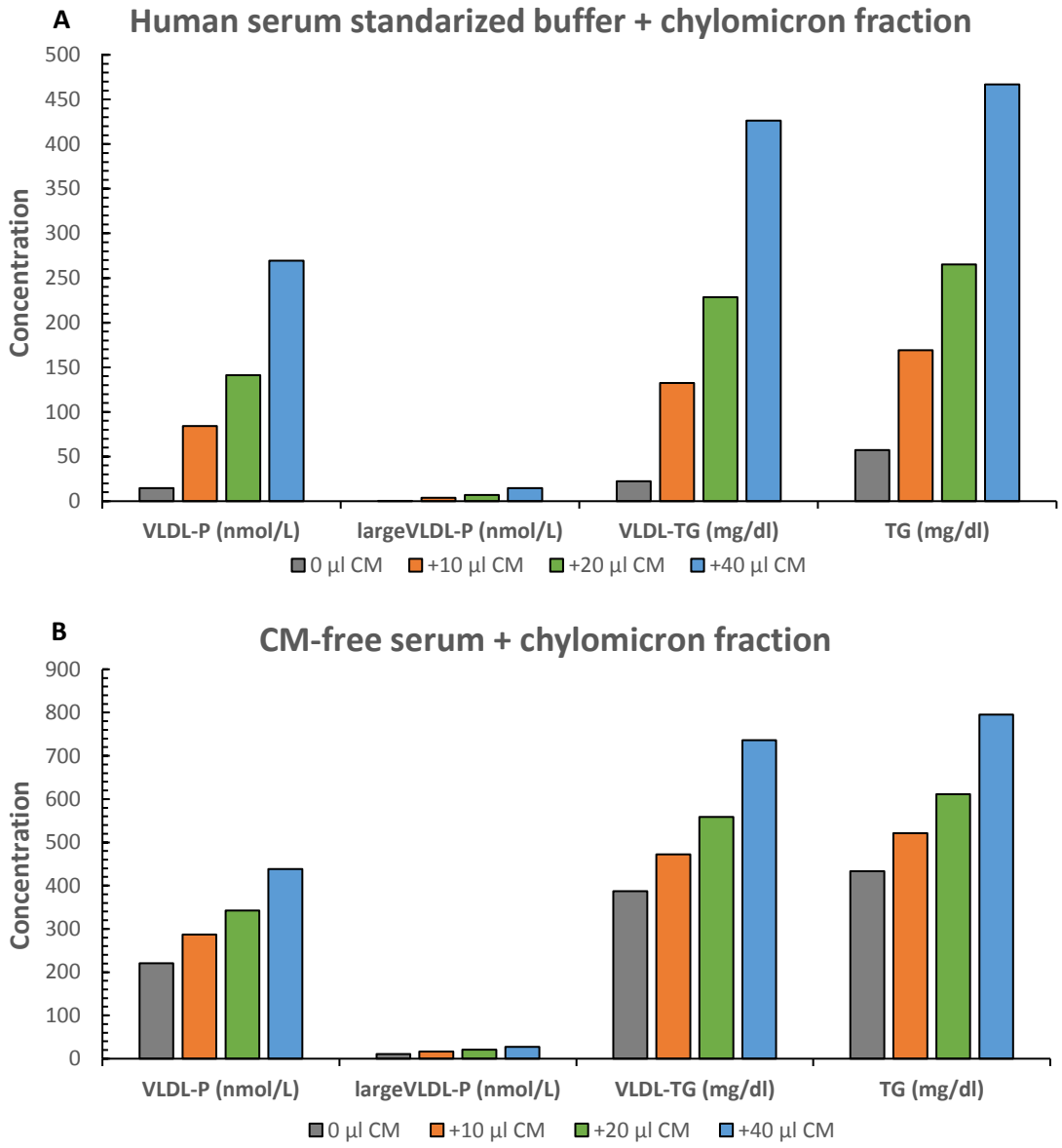

Figure 14. Increase of VLDL-P, largeVLDL-P, VLDL-TG and TG after adding ascendant volumes of a chylomicron-rich fraction to a human serum standardized buffer (A) and to the CM-free serum (B).

5. Discussion

The Liposcale® test allows the quantification of the particle number and size of the three main lipoprotein classes (VLDL, LDL and HDL) and their three subclasses (large, medium and small), in addition to the usual biomarkers (LDL-C, HDL-C, TC and TG) used in the clinical practice (14). This proves to be an advantage compared to the standard biochemical determination in the prediction of CV risk, particularly in patients with a discordance between LDL-C and LDL-P or HDL-C and HDL-P. In both cases, particle concentration is a more accurate indicator of CVD risk than the cholesterol concentration (11,12). However, a high concentration of TG causes interferences in the prediction of HDL-C by the Liposcale® test.

In order to determine a differential pattern between hypertriglyceridemic samples with an accurate prediction of HDL-C and those with an inaccurate prediction a PLS-DA model was created. The main difference found by the PLS-DA between both groups was the concentration

of TG. When the levels of TG are higher than 600 mg/dl most of the samples suffer interferences in the prediction of HDL-C. This threshold can be directly introduced in the Liposcale® report as warning in patients with TG>600 mg/dl. Although there could still be samples with a concentration of TG<600 mg/dl with an inaccurate prediction, those samples have a predicted HDL-C closer to the value determined by biochemical methods, so the difference is not very significant. Furthermore, it has to be taken into account that the reference method (direct determination) can also suffer from the interference caused by a high concentration of TG (28).

The aim of this project was to determine if the prediction of HDL-C was better when a spike-in approximation was performed, compared to the standard Liposcale protocol. When 50 µl of sample are added to 150 µl of a human serum standardized buffer (spike-in), the HTG sample is diluted 4 times and the concentration is within the linear interval of the Liposcale test so more concordant values are obtained. Due to the fact that the sample is diluted with a serum pool instead of water, the viscosity of the sample does not change, allowing a better NMR acquisition. The concentration of TG and TC obtained with the spike-in experiments showed a better correlation with the biochemical determinations than the standard Liposcale test protocol. In addition, the predicted values of HDL-C were within the same range as the enzymatic determination so the prediction was more accurate. Therefore, with this method, the determinations of TG, TC and HDL-C by the Liposcale® test are improved after the spike-in experiments. To fully optimize this method, further tests would be needed with more samples to obtain more robust results.

Additionally, the original spectra obtained with the standard Liposcale® protocol in the HTG samples showed great differences with the spectra of samples with a lower concentration of TG. In samples with TG>600 mg/dl, the spectral regions that are used to predict lipid concentration have a bigger intensity and different shape compared to those with lower levels of TG. For this reason, the Liposcale® test suffers interferences in the prediction of some variables, especially HDL-C. When the spike-in were performed, the spectra showed a similar shape to the control sample. This way, the Liposcale® test can make a better prediction of HDL-C in samples with TG>600 mg/dl. In the future, the spectra obtained in the samples without performing the spike-in can be used to build a new PLS regression model, similar as the one built for the Liposcale® test (29).

The second aim of the study was to establish a parameter that could identify chylomicrons in serum with the Liposcale® test. The first observation was that the average size of the VLDL-P in the sample with hyperchylomicronaemia was bigger (44.97 nm) than in most of the samples with HTG and the general population (41-43 nm). Additionally, the number of VLDL particles (particularly medium VLDL-P) was also significantly higher in the chylomicronemic sample than in HTG samples. The two PCAs show that the sample with CM can be differentiated from the HTG samples and from the general population by analysing the concentration of TG, VLDL-P, Z-VLDL and LDL-C. The low concentration of LDL-C is probably a consequence of the interference caused by the extreme concentration of TG but it is difficult to draw conclusions from only one sample. When ascendant volumes of a CM-rich fraction were added to the CM-serum and to a human serum standardized buffer, only VLDL-P, largeVLDL-P, VLDL-TG and total TG were increased linearly. Thus, the presence of CM is especially noticeable as an increase of VLDL-P. This increase happens because methyl protons in chylomicrons resonate at the same frequency as VLDL-P, so the Liposcale® test detects them as VLDL-P. The study of one sample with chylomicronemia serves as an initial approach to see if the size of the VLDL particles along with their concentration could potentially become a parameter to detect chylomicrons with the

Liposcale® test. The detection of chylomicrons in serum can be an indication of poor lipid clearance capacity, so NMR spectroscopy can be a powerful tool to assess disease risk in those patients (27).

6. Conclusions

^{1}H-NMR spectroscopy is a powerful tool to accurately predict lipid concentration and lipoprotein particle number and size. Therefore, it can provide a more precise risk assessment of CVD than a standard lipid panel (24). In this study, a new protocol was designed for samples with TG>600mg/dl, in order to obtain accurate values of HDL-C by Liposcale®. On the other hand, a preliminary study was carried out to establish a diagnostic parameter in cases of chylomicronemia. The presence of CM in serum is reflected as an increase of VLDL particle number and size, compared to other samples with HTG. These parameters could be used to detect chylomicrons in serum. However, to confirm this, a larger study must be carried out with more chylomicronemic samples to obtain statistically significant results.

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Supplementary material

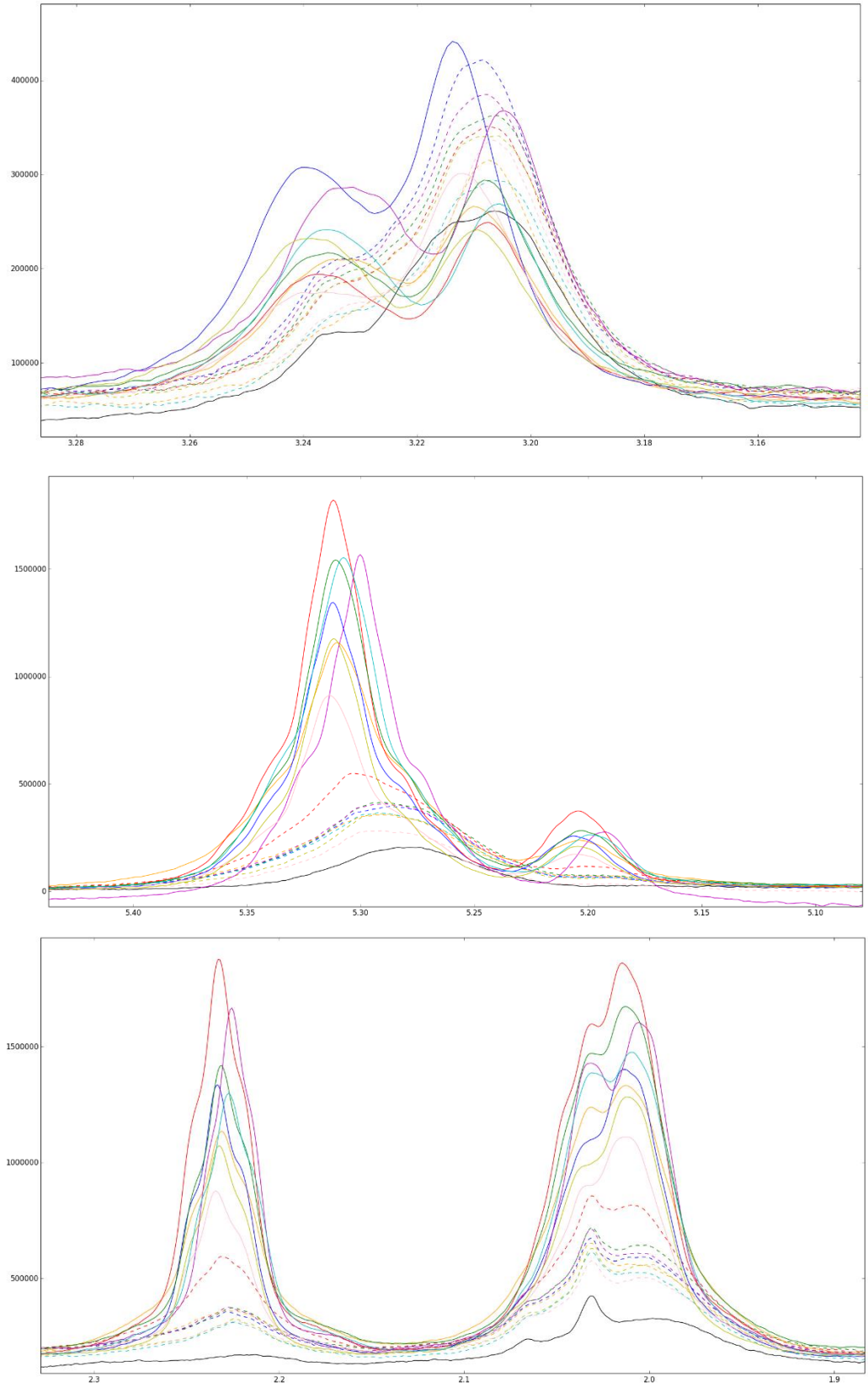

Figure S1. Spectral differences in the signal of choline (A), glucose (B) and glycoproteins (C) before and after the spike-in. Each colour corresponds to one sample, before (continuous line) and after (dashed line) the spike-in. The spectra obtained after the spike-in have a lower intensity and a shape similar to the control sample (black line).

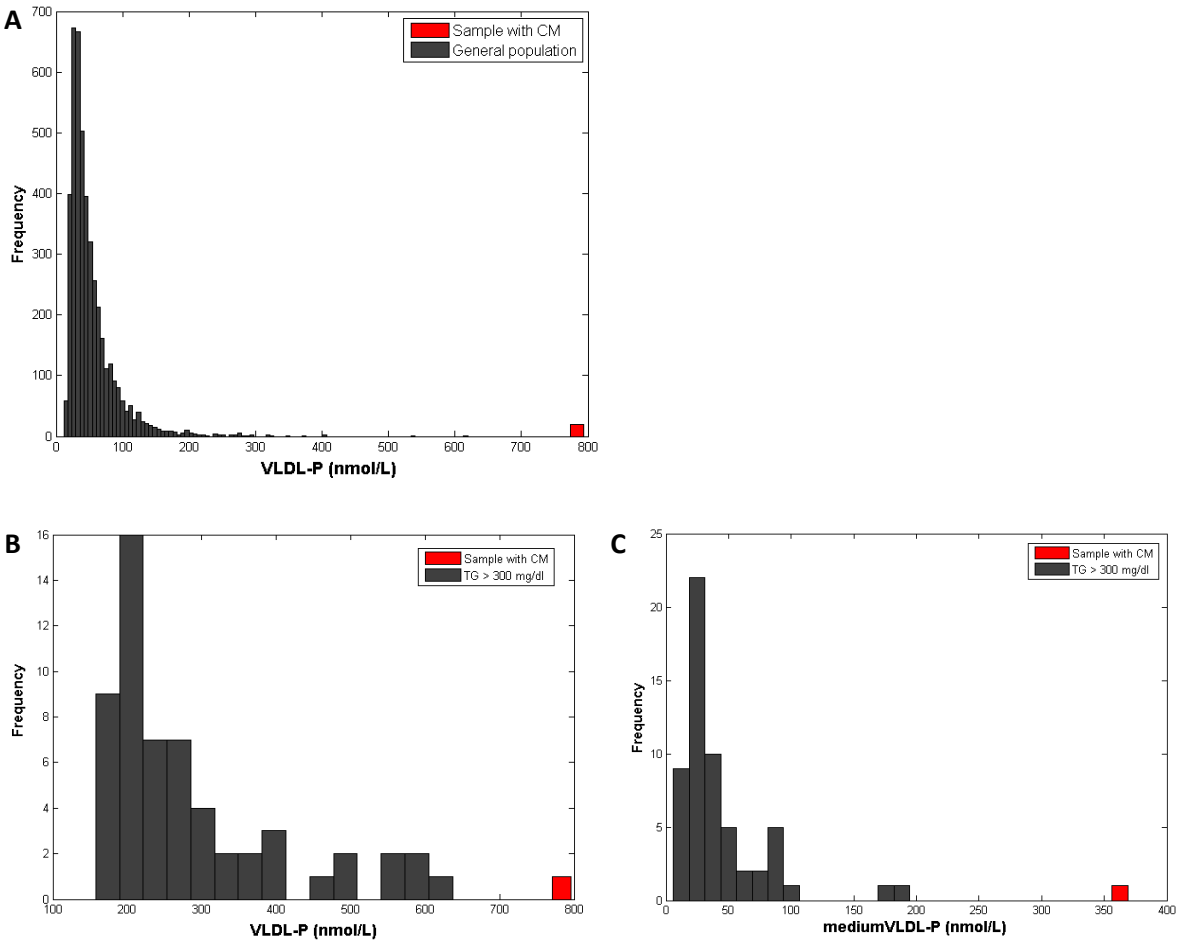

Figure S2. (A). Number of VLDL-P in a sample with CM compared to the general population. **(B) and (C).** VLDL-P and medium VLDL-P in a sample with CM compared to a subset of individuals with TG>300 mg/dl.

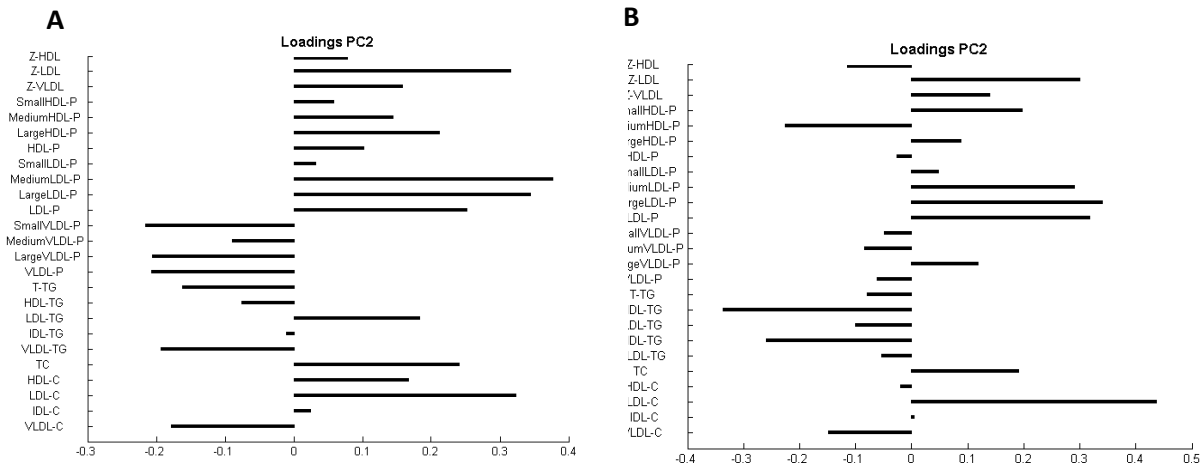

Figure S3. Loadings plot of PC2 of the PCA model of a general population (A) and HTG samples (B) with the sample with CM.