DEVELOPMENT AND EVALUATION OF A NOVEL ADVANCED LIPOPROTEIN TEST BASED ON 2D DIFFUSION-ORDERED 1H NMR SPECTROSCOPY

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Doctoral Thesis

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DOCTORAL THESIS

Supervised by Prof. Xavier Correig Blanchar

Department of Electronic, Electric and Automatic Engineering

UNIVERSITAT ROVIRA I VIRGILI

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I CERTIFY that the Doctoral Thesis entitled: **"DEVELOPMENT AND EVALUATION OF A NOVEL ADVANCED LIPOPROTEIN TEST BASED ON 2D DIFFUSION-ORDERED ^{1}H NMR SPECTROSCOPY"**, presented by **ROGER MALLOL PARERA** to obtain the title of Doctor, has been performed under my supervision in the Department of Electronic Engineering at the Rovira i Virgili University and meets the requierements to qualify for International Mention.

Tarragona, June 2014

Xavier Correig Blanchar

"If I have seen further it is by standing on the shoulders of giants"

Isaac Newton

15th February, 1676

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I sempre hi ha un primer dia. Tot té un origen. Així que també voldria agrair al Jesús haver-me donat la oportunitat de dur a terme la meva tesi doctoral al grup SIPOMICS. Moltes gràcies també al Nico i a tots els doctorands que pugeu molt fort. Rubén, Dídac, Xavi i Pere, molta sort!

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estada de doctorat al seu grup. Va ser una de les experiències més enriquidores de la meva vida.

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Per la part personal, durant aquests més de cinc anys de doctorat he passat moments personals de tots colors. Per això ara més que mai puc dir que el doctorat és una autèntica experiència vital. Als meus pares els hi vull agrair tot. Tot, tot i tot. Sense ells res hagués estat possible. No sé com us podré tornar tot el que m'heu donat. Crec, que l'única cosa que puc dir és que quan sigui gran vull ser com vosaltres. I l'única cosa que puc fer, és cuidar els meus fills com vosaltres m'heu cuidat a mi. Moltes gràcies.

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Al Joan i a l'Àlex per compartir amb mi una fantàstica casa durant l'equador d'aquesta tesi i haver-me visitat a Manchester. Sens dubte Cals Marquesos forma part

d'aquesta tesi. També agrair a la resta d'amics amb els que hem compartit tantes i tantes anècdotes, viatges i experiències durant aquests darrers anys.

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Roger

ABSTRACT

Cardiovascular disease (CVD) is the leading cause of death globally. There will be an estimated 23.6 million deaths by 2030 due to some form of CVD. Moreover, CVD costs more than any other diagnostic group: coronary heart disease alone costs the US $108.9 billion each year. Finally, the most vulnerable populations to suffer CVD are the populations with diabetes and other metabolic disorders. At least 65% of diabetics die from some form of CVD.

The classic cardiovascular risk factors include age, smoking, diabetes, blood pressure and dyslipidemia. Some forms of dyslipidemia include elevated total or low-density lipoprotein (LDL) cholesterol levels, low levels of high-density lipoprotein (HDL) cholesterol, and high levels of triglycerides. Although the use of statins is well established into the clinical guidelines for primary prevention of CVD, its use is controversial. Indeed, 50% of people who have a cardiovascular event have normal cholesterol levels. Thus, we are currently experiencing a paradigm shift in cardiovascular risk assessment based on the blood lipid profile.

This lack of benefit on the use of statins to reduce cardiovascular events might be explained partly due to the fact that two individuals with the same LDL cholesterol concentration can show different distributions of LDL particles. The individual with a preponderance of smaller-denser LDL particles will be at increasing risk, because smaller-denser LDL particles are more susceptible to oxidation and to enter the intima media of arterial walls leading to atherosclerosis. This preponderance of smaller and denser LDL particles is the result of an increase in triglycerides concentration, which also leads to a decrease in HDL cholesterol. This triad of lipid and lipoprotein abnormalities is known as atherogenic dyslipidemia and it is characteristic of diabetic patients, being also known as

diabetic dyslipidemia.

For this reason, they have appeared recently different analytical approaches to characterize lipoproteins aimed at providing complementary information on these macromolecular complexes beyond its lipid charge. In particular, the size and particle number of the different classes and subclasses of lipoprotein particles may improve cardiovascular risk assessment and management in those individuals at high risk of CVD, such as patients with diabetes and other metabolic disorders.

One of the analytical techniques that is emerging to the clinical arena is nuclear magnetic resonance (NMR). NMR is a highly reproducible, quantitative and versatile tool for the study of physical, chemical and biological properties of matter. Moreover, NMR can be used as a high-throughput analytical technique because sample manipulation is minimal. For these reasons, NMR has found a myriad number of applications in several areas of science and now is being introduced into the clinical practice thanks to the development and commercialization by Liposcience Inc. of The LipoProfile Test, an advanced lipoprotein test which measures the concentration of LDL particles among other lipoprotein parameters.

The doctoral thesis presented in this document is the result of the research conducted in the Department of Electronic, Electrical and Automation Engineering at the Rovira i Virgili University (URV). Other research institutions have been involved in this research, such as the Metabolomics Platform of the same university, the Research Unit of Lipids and Atherosclerosis (URLA) of Sant Joan University Hospital from Reus (HUSJR) and the CIBER of Diabetes and Metabolic Diseases (CIBERDEM). All these groups are part of the Institut d'Investigació Sanitària Pere Virgili (IISPV).

Thanks to this collaboration it has been possible to develop the LipoScale test, an

advanced lipoprotein test based on NMR spectroscopy. This test is aimed at obtaining a better characterization of plasma lipoproteins, i.e. lipid content, size and particle number, in order to improve cardiovascular risk prediction. The main difference between our approach and the commercial test is that they use standard 1D NMR methods, while our test is based on the use of magnetic gradients, which generates 2D spectra with direct and objective information on lipoprotein particle sizes.

This thesis has generated different scientific publications which will be detailed through the next sections, as well as a patent that has been filled to the European Patent Office (EPO) and a spin-off company which will commercialize the LipoScale test.

RESUM

Les malalties cardiovasculars (MCV) són la principal causa de mort a tot el món. S'estima que hi hauran 23.6 milions de defuncions el 2030 degudes a les MCV. A més, les MCV costen més que qualsevol altre grup diagnòstic: només les malalties coronàries costen als EEUU 108.9 bilions de dòlars cada any. Finalment, les poblacions més vulnerables a les MCV són les poblacions amb diabetis i altres desordres metabòlics. Almenys el 65% dels diabètics moren d'alguna forma de MCV.

Els factors de risc clàssics són l'edat, el tabaquisme, la diabetis, la pressió arterial i les dislipidèmies. Algunes formes de dislipidèmia inclouen concentracions altes de colesterol total o de les lipoproteïnes de baixa densitat (LDL), concentracions altes de triglicèrides, o concentracions baixes de les lipoproteïnes d'alta densitat (HDL). Encara que l'ús d'estatines està ben establert a les guies clíniques per a la prevenció primària de les MCV, el seu ús és controvertit. Verdaderament, el 50% de les persones que pateixen un event cardiovascular tenen nivells normals de colesterol. Per això, actualment estem vivint un canvi de paradigma pel que fa a l'avaluació del risk cardiovascular basada en un perfil bàsic de les concentracions de lípids en sang.

Aquesta falta de benefici en l'ús de les estatines per reduir el nombre d'events cardiovasculars pot ser explicada en part pel fet que dos individus amb la mateix concentració de colesterol LDL poden mostrar distribucions diferents d'aquestes partícules. L'individu amb més partícules LDL petites tindrà associat un risc cardiovascular més alt, ja que les partícules LDL petites són més susceptibles d'oxidar-se i d'entrar a les artèries, començant el procés conegut com arteriosclerosi. Aquests nivells més elevats de partícules LDL petites són deguts a un increment de les concentracions de triglicèrids, que també provoquen una baixada de les concentracions de colesterol HDL. Aquesta triada

d'anormalitats lipídiques i lipoproteiques és coneguda com dislipidèmia aterògena i és característica de pacients diabètics, essent coneguda també com dislipidèmia diabètica.

Per aquesta raó, han aparegut durant els últims anys diferents aproximacions analítiques per caracteritzar les lipoproteïnes de manera que es pugui determinar informació complementària d'aquests complexes moleculars, més enllà de la seva càrrega lipídica. Concretament, la mida i el nombre de partícules de les diferents classes i subclasses de lipoproteïnes milloren l'avaluació del risc cardiovascular en aquells individus d'alt risc de patir MCV, com pacients amb diabetis o desordres metabòlics.

Una de les tècniques analítiques que està emergent a l'escena clínica és la ressonància magnètica nuclear (RMN). La RMN és una eina altament reproduïble, quantitativa i versàtil per a l'estudi de les propietats físiques, químiques i biològiques de la matèria. A més a més, la RMN pot ser utilitzada com una tècnica analítica d'alt rendiment ja que la manipulació de les mostres és mínima. Per totes aquestes raons, la RMN ha trobat infinitat d'aplicacions en moltes àrees de la ciència i ara està essent introduïda a la pràctica clínica gràcies al desenvolupament i la comercialització per part de Liposcience Inc. del LipoPorfile Test, un test avançat de lipoproteïnes que determina el nombre de partícules LDL entre d'altres paràmetres.

La tesi doctoral presentada en aquest document sorgeix com a resultat de la investigació realitzada en el Departament d'Enginyeria Electrònica, Elèctrica i Automàtica de la Universitat Rovira i Virgili (URV). En la investigació han col·laborat altres institucions, com la Plataforma Metabolòmica de la mateixa universitat, la Unitat de Recerca en Lípids i Arteriosclerosi (URLA) de l'Hospital Universitari Sant Joan de Reus (HUSJR) i el CIBER de Diabetis i Malalties Metabòliques (CIBERDEM). Tots els grups formen part de l'Institut d'Investigació Sanitària Pere Virgili (IISPV).

Gràcies a aquesta col·laboració s'ha pogut desenvolupar The LipoScale Test, un test avançat de lipoproteïnes basat en RMN. Amb aquest test es pretén obtenir una millor caracterització de les lipoproteïnes plasmàtiques, tant el seu contingut lipídic com la seva mida i nombre de partícules, de manera que amb ell s'aconsegueixi una millor predicció del risc cardiovascular. La principal característica entre la nostra aproximació i la del test comercial és que Liposcience Inc. utilitza mètodes de RMN 1D estàndards, mentre que el nostre test està basat en l'ús de gradients de camps magnètic, els quals generen espectres 2D amb els que es pot obtenir informació directa i objectiva de la mida de les partícules lipoproteiques.

Aquesta tesi a generat diferents publicacions científiques que seran detallades al llarg del text, així com també s'ha fet la sol·licitud d'una patent europea i s'ha creat una spin-off per comercialitzar el test.

LIST OF ABREVIATIONS

ACQ	acquisition
AIDS	acquired immunodeficiency syndrome
ALT	advanced lipoprotein tests
ANN	artificial neural network
APO	apolipoprotein
APOA	apolipoprotein A
APOB	apolipoprotein B
ATP	adult treatment panel
BPP	bipolar pulse pairs
C	cholesterol
CAD	coronary artery disease
CE	cholesterol ester
CETP	cholesterol ester transport protein
CHD	cardiovascular heart disease
CIBERDEM	Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas
cIMT	carotid intima-media thickness
CMR	cardiometabolic risk
CM	chylomicrons
CVD	cardiovascular disease
CPMG	Carr–Purcell–Meiboom–Gill
DETOCSY	diffusion-edited total correlation spectroscopy
DIRE	diffusion and relaxation-edited
DKD	diabetic kidney disease
DLS	dynamic light scattering
DM	diabetic metabolism
DOSY	diffusion ordered NMR spectroscopy
DSS	4,4-dimethyl-4-silapentane-1-sulfonic acid
DSTE	double stimulated echo
EDTA	ethylene diamine tetraacetic acid
ERETIC	electronic reference to access in vivo concentrations
FDA	food and drug administration
FID	finite induction decay
FRS	Framingham risk score
GGE	gradient gel electrophoresis
GTM	generative topographic mapping
HCA	hierarchical cluster analysis
HDL	high density lipoprotein
HDL-C	HDL cholesterol
HDL-P	HDL particle number
HIV	human immunodeficiency virus
IDL	intermediate density lipoproteins
IDL-C	IDL cholesterol
IISPV	Institut d'Investigació Sanitària Pere Virgili
IM	ion mobility
KNN	Kohonen neural networks
LDL	low density lipoprotein

LDL-C	LDL cholesterol
LDL-P	LDL particle number
LED	longitudinal eddy-current delay
LS	light scattering
MCMC	Markov chain Monte Carlo
MP	metabolic pathway
N	normal
NCEP	national cholesterol education program
NMR	nuclear magnetic resonance
NOESY	nuclear overhauser effect spectroscopy
NRMSE	normalized root mean square error
PCA	principal component analysis
PE	percentage error
PFG	pulse field gradient
PFGSE	PFG spin-echo
PFGSTE	PFG stimulated echo
PL	phospholipids
PLS	partial least squares
RD	relaxation delay
RF	radiofrequency
RMS	root mean square
RMSPE	root mean squared percentage error
S-DOSY	statistical diffusion-ordered spectroscopy
sdLDL	small-dense LDL
SE	standard error
SNR	signal-to-noise ratio
SOM	self organized maps
STE–LED	stimulated echo and longitudinal eddy-current delay
STOCSY	statistical total correlation spectroscopy
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TC	total cholesterol
TEM	transmission electron microscopy
TG	triglycerides
TOCSY	total correlation spectroscopy
TSP	3-(trimethylsilyl) 2,2,3,3-tetradeutero-propionic acid
VAP	vertical auto profile
VLDL	very low density lipoproteins
VLDL-P	VLDL particle number

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LIST OF CONGRESSES

- R. Mallol, A Cabré, N. Amigó, M. Heras, L. Masana, X. Correig. Characterisation of Atherogenic Dyslipidemia using a novel NMR-based advanced lipoprotein test in type 2 diabetic subjects. XVII Congreso Nacional de la Sociedad Española de Arteriosclerosis, Barcelona (Spain) February 20-21. 2014. ****This poster won the 3rd award for the best communication at the Conference**
- N. Amigó, R. Mallol, M. Heras, A Cabré, N. Plana, L. Masana, X. Correig. Smaller and "Herniated" HDL in patients with type 2 diabetes mellitus. XVII Congreso Nacional de la Sociedad Española de Arteriosclerosis, Barcelona (Spain) February 20-21. 2014.
- R. Mallol, A Cabré, M. Rodríguez, N. Amigó, M. Vinaixa, N. Plana, M. Heras, L. Masana, X. Correig. Characterisation of Atherogenic Dyslipidemia using a novel NMR-based advanced lipoprotein test in type 2 diabetic subjects. 81st European Atherosclerosis Society Congress, Lyon (France) June 2-5. 2013.
- R. Mallol, N. Amigó, M. Rodríguez, M. Heras, N. Plana, L. Masana, X. Correig. Advanced Lipoprotein Testing Using Diffusion-edited 1H NMR Spectroscopy. 25 Congreso Nacional de la Sociedad Española de Arteriosclerosis, Reus (Spain) June 6-8. 2012.
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LIST OF PATENTS

- METHOD FOR THE CHARACTERIZATION OF LIPOPROTEINS, EP13382478.9, 27 November 2013
- METHODS FOR DETERMINING THE LIPID DISTRIBUTION BETWEEN THE CORE AND THE SHELL OF A LIPOPROTEIN PARTICLE, EP13382477.1, 27 November 2013

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To whom it may concern

This is to certify that Sr Roger Mallol Parera spent a period of 3 months as a research visitor in the School of Chemistry of the University of Manchester between March and June 2011. His stay in Manchester was highly productive, resulting in an improved understanding of the relationship between diffusion coefficients as measured by NMR, particle sizes, and polydispersity in blood lipoprotein samples.

Yours truly

Prof GA Morris

Gareth A Morris MA DPhil CChem MRSC Professor of Physical Chemistry

SPECIAL RESEARCH MENTION

FUNDACIÓN ESPAÑOLA DE ARTERIOSCLEROSIS
SOCIEDAD ESPAÑOLA DE ARTERIOSCLEROSIS

El **Comité Científico de la Sociedad Española de Arteriosclerosis**
y el **Comité Organizador del XXVII Congreso Nacional**
han decidido otorgar, por su calidad científica,

3er PREMIO MENCIÓN ESPECIAL 2014
A LA COMUNICACIÓN PRESENTADA
EN EL XXVII CONGRESO NACIONAL S.E.A.
BARCELONA 2014

a la comunicación póster:

CHARACTERIZATION OF ATHEROGENIC DYSLIPIDEMIA USING A NOVEL NMR-BASED ADVANCED LIPOPROTEIN TEST IN TYPE 2 DIABETIC SUBJECTS

presentada por los autores:

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1. INTRODUCTION

1.1. Background

1.1.1. Cardiovascular disease

According to the World Heath Organization (WHO), cardiovascular disease (CVD) is the leading cause of death globally. Indeed, an estimated 17.3 million of people died from CVD in 2008, representing 30% of all global deaths. An estimated 7.3 million, out of these deaths, were due to coronary heart disease and 6.2 million of them were due to stroke. There will be an estimated 23.6 million deaths by 2030 due to some form of CVD, mainly from heart disease and stroke. Moreover, CVD costs more than any other diagnostic group: coronary heart disease alone costs the US $108.9 billion each year. Also importantly, CVD is one of the most misdiagnosed and mistreated conditions in medicine.

CVD risk is most frequently the result of multiple interacting risk factors **[1]**. The classic cardiovascular risk factors include age, smoking, diabetes, blood pressure and dyslipidemia. Among the above-mentionned risk factors, this text is focused on dyslipidemias (i.e., abnormal levels of lipids in the blood) and lipoproteins (spherical macromolecular complexes that transport lipids). Briefly, lipoproteins have been historically classified according to their size and density, arising five main lipoprotein classes: Chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL), and high density lipoproteins (HDL). These macromolecular complexes transport lipid molecules from the liver or intestines to peripheral tissues through the blood stream for structural and energy functions, among others. Thus, cholesterol should not be taken as “good” or “bad”, but it should be thought as a vital molecule for life.

The role of lipoproteins in the metabolism of triglycerides and cholesterol in relation

to cardiovascular disease is highly complex. The cholesterol molecules transported by LDL lipoproteins are susceptible to be deposited within the intima media of arterial walls, triggering the development of atherosclerosis and subsequent cardiovascular events. In fact, there are a number of epidemiological studies that show that high concentrations of LDL cholesterol (LDL-C) are associated with increased severity of cardiovascular disease. Therefore, LDL-C is the primary target for the therapeutic treatment of adults with high cholesterol in the blood. On the other hand, HDL lipoproteins play the role of reverse cholesterol transport: they remove the cholesterol from the arterial walls to transport them back to the liver for excretion purposes **[2, 3]**. Thus, low concentrations of HDL cholesterol (HDL-C) are associated with an increased risk of atherosclerosis. However, HDL-C levels do not always predict cardiovascular risk. For example, high HDL-C levels caused by some genetic disorders may not protect against cardiovascular disease and low HDL-C levels caused by some genetic disorders may not increase the risk of cardiovascular disorders. Although HDL levels predict cardiovascular risk in the general population, the increased risk may be due to other factors such as accompanying lipid and metabolic abnormalities, rather than the HDL-C level itself. High levels of triglycerides (TG) are also a risk factor for cardiovascular disease because there is evidence suggesting that TGs are a marker of high atherogenic lipoprotein remnants.

Altogether, dyslipidemia can be defined as the presence of elevated plasma cholesterol, triglycerides, or both, or a low level of high-density lipoprotein that contributes to the development of atherosclerosis. The causes of these abnormal lipid levels may be primary (genetic) or secondary (lifestyle and other). Dyslipidemia is diagnosed by measuring serum lipids. Routine measurements (lipid profile) include total cholesterol (TC), TG, HDL-C and LDL-C. TC, TG and HDL-C are measured directly. The values TC and TG reflect the cholesterol and TG in all circulating lipoproteins, including chylomicrons, VLDL, intermediate

density lipoprotein (IDL), LDL and HDL. The values of LDL-C are calculated usually as the amount of cholesterol not contained in HDL and VLDL **[4]**. VLDL is estimated by TG/5 because the concentration of cholesterol in VLDL particles is generally one-fifth of the total lipid particle. Therefore,

$$LDL\text{-}C = TC - HDL\text{-}C - TG/5$$

This calculation is valid only when TGs are < 400 mg/dL and patients are fasting, because eating increases TGs. The calculated LDL-C value incorporates measures of all non-HDL, nonchylomicron cholesterol, including that in IDL and lipoprotein (a) [Lp(a)]. LDL-C can also be measured directly using plasma ultracentrifugation, which separates chylomicrons and VLDL fractions from HDL and LDL, and by an immunoassay method. Direct measurement may be useful in some patients with elevated TGs, but these direct measurements are not routinely necessary **[5]**.

The treatment of dyslipidemia includes changes in diet, exercise, and lipid-lowering drugs. Treatment is indicated for all patients with cardiovascular disease (secondary prevention) and for some without (primary prevention). The National Institutes of Health's National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATPIII) guidelines are the most common reference for deciding which adults should be treated **[6]**. The guidelines focus primarily on reducing elevated LDL-C levels and secondarily on treating high TGs, low HDL-C, and metabolic syndrome.

Although the use of statins is well established into the clinical guidelines for primary prevention of CVD, its use is controversial. Indeed, 50% of people who have a cardiovascular event have normal cholesterol levels. One explanation for this undesired clinical outcomes

may be as follows. Both LDL and HDL lipoprotein classes comprise a continuum distribution of lipoprotein particles in terms of their size, density, and composition. Due to the limitations of the current characterization techniques, main LDL and HDL lipoprotein fractions can be divided into small and large lipoprotein subclasses as a simplification, though some analytical techniques can provide even seven and five lipoprotein subclasses for the LDL and HDL main fractions, respectively. In terms of their association to cardiovascular risk, the smaller-denser LDL subclass is more susceptible to enter the intima media than the larger one and thus individuals with a preponderance of this subclass have been associated with an increase of cardiovascular risk. On the other hand, the presence of a larger HDL subclass is indicative of an efficient reverse cholesterol transport process, so individuals with a preponderance of larger HDL particles have been associated with a decrease of cardiovascular risk.

1.1.2. Advanced lipoprotein testing

The scenario presented above opened the door for shifting the cardiovascular risk prediction protocols based on lipid levels to a new paradigm of cardiovascular risk prediction based on the size and particle number of lipoprotein particles **[7, 8]**. A particular case in which this new paradigm can overcome the limitation of current protocols for cardiovascular risk prediction is in patients with a constellation of lipid and lipoprotein abnormalities known as atherogenic dyslipidemia: increased triglyceride levels, decreased HDL levels, and a preponderance of small LDL particles **[9]**. The atherogenic dyslipidemia is characteristic of diabetic patients, and thus it is also known as diabetic dyslipidemia. The atherogenic dyslipidemia may be a consequence of obesity, poorly controlled diabetes, or both, which can increase circulating free fatty acids (FFA), which leads to increased hepatic

VLDL production. TG-rich VLDL then transfers TG and cholesterol to LDL and HDL, promoting the formation of TG-rich, small and dense LDL and clearance of TG-rich HDL. Atherogenic dyslipidemia is often exacerbated by increased caloric intake and sedentary lifestyles that characterizes some patients with type 2 diabetes. Patients with other metabolic disorders such as patients with obesity or metabolic syndrome can also present this kind of dyslipidemia. All these populations are one of the most vulnerable populations to suffer CVD since at least 65% of diabetics die from some form of CVD and women with diabetes may be especially at risk of heart disease of this form.

The rationale for characterising such a dyslipidemia is the fact that it usually shows normal LDL cholesterol levels. However, cardiovascular risk is still high due to the presence of higher concentrations of small LDL particles. The reason for this is that two subjects with the same levels of LDL cholesterol can have different distributions of LDL particles in terms of their size **[10]**. Thus, a subject with smaller particles will need more particles in order to transport the same amount of cholesterol. As stated before, smaller LDL particles have an inherent increased risk for atherosclerosis, but determining only the concentration of LDL cholesterol will not uncover such risk. This is known as Residual Cardiovascular Risk.

This is why recent studies have stressed the importance of adding other parameters when analysing lipoproteins such ApoB concentration or the number of lipoprotein particles and their size **[11, 12]**. The role of apo B testing is under study because values reflect all non-HDL cholesterol (in VLDL, VLDL remnants, IDL, and LDL) and may be more predictive of CAD risk than LDL cholesterol **[13, 14]**. In addition, non-HDL cholesterol (TC - HDL cholesterol) may also be more predictive of CAD risk than LDL cholesterol. In this regard, the National Lipid Association Expert Panel has recently advised testing on LDL-P to assess and manage cardiovascular risk. Although ApoB and LDL-P are highly correlated, the latter

parameter is more accurate for the estimation of CVD risk because it does not include the particle numbers of VLDL and IDL fractions, less atherogenic than LDL fractions **[15]**. As has been mentioned before, diabetic individuals characterized by a dense LDL fraction (the so called Pattern B phenotype) as well as high level of triglycerides and a low level of HDL-C are a target group well-suited for an LDL-P measurement.

The set of analytical techniques that provide more detailed information on lipoprotein particles are known as advanced lipoprotein tests (ALT). Some limitations of ALT are the lack of standardization and the disagreement between different ALT approaches. Unfortunately, the number and nomenclature of lipoprotein subfractions are not uniform across the different techniques and have not been standardized, making it difficult to compare results from various tests. Moreover, some of these tests can only be performed at the company that markets the test, limiting the ability to obtain independent information on test performance. Thus, although it is unclear if these methods are ready for clinical use, it is necessary to perform more exhaustive and detailed analysis of (i) the utility of progressive changes in lipoproteins as therapeutic targets and (ii) whether certain subgroups of individuals may benefit from these analyses. Moreover, these methods may be important for advancing research and understanding the pathophysiology of CVD.

1.1.3. Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is a tool highly reproducible, quantitative and versatile for the study of the physical, chemical and biological properties of matter. Its physical principle relies on the fact that spins within a sample are excited by means of a magnetic field followed by a radio frequency pulse which aligns the spins in a manner that they can be measured. Once the radio frequency pulse is switched off, spins

recover their initial state at a rate that depends on their molecular weight. This recovering step is known as relaxation and the signal obtained between the alignment and the total recovery of the spins is called finite impulse decay (FID). The FID shows a decaying sinusoid containing all the signals from all the molecules within the sample. Fourier transformation of the FID yields the NMR spectrum of a given sample and it provides information of both the environment of the molecule moieties (structure elucidation) and the abundance of a given molecule (quantification).

NMR can be used as an analytical technique of high performance because sample handling is minimal **[16]**. For this reason, NMR has found a myriad of applications in many different areas of science. In particular, NMR has been shown to be a robust and reproducible tool to analyse lipoproteins for cardiovascular risk prediction **[17]**. The NMR spectrum of lipoproteins shows signals across a wide range of NMR frequencies **[18]**. The most studied signal is the signal coming from the methyl moiety of the lipids contained in a particular lipoprotein **[19-21]**. Because the methyl NMR signals of lipoproteins are broad and highly overlap, different strategies can be used to characterize the different classes and subclasses. However, current methods for lipoprotein characterization using NMR spectroscopy are indirect, so information regarding lipoprotein particles are based either on calibration using NMR spectra of lipoprotein fractions which have been calibrated with other techniques such as transmission electron microscope or gradient gel electrophoresis, or using regression methods such as partial least squares (PLS) which relate the information contained in the NMR envelope of arbitrary regions containing the NMR signals arising from the different lipid moieties to lipid and lipoprotein values **[17, 22]**. Another limiting aspect for translating NMR methods to clinical practice is the lack of standardization **[10]**. Current NMR methods for lipoprotein analysis do not match in terms of the number and type of parameters being provided. To overcome this, diffusion-ordered NMR spectroscopy (DOSY)

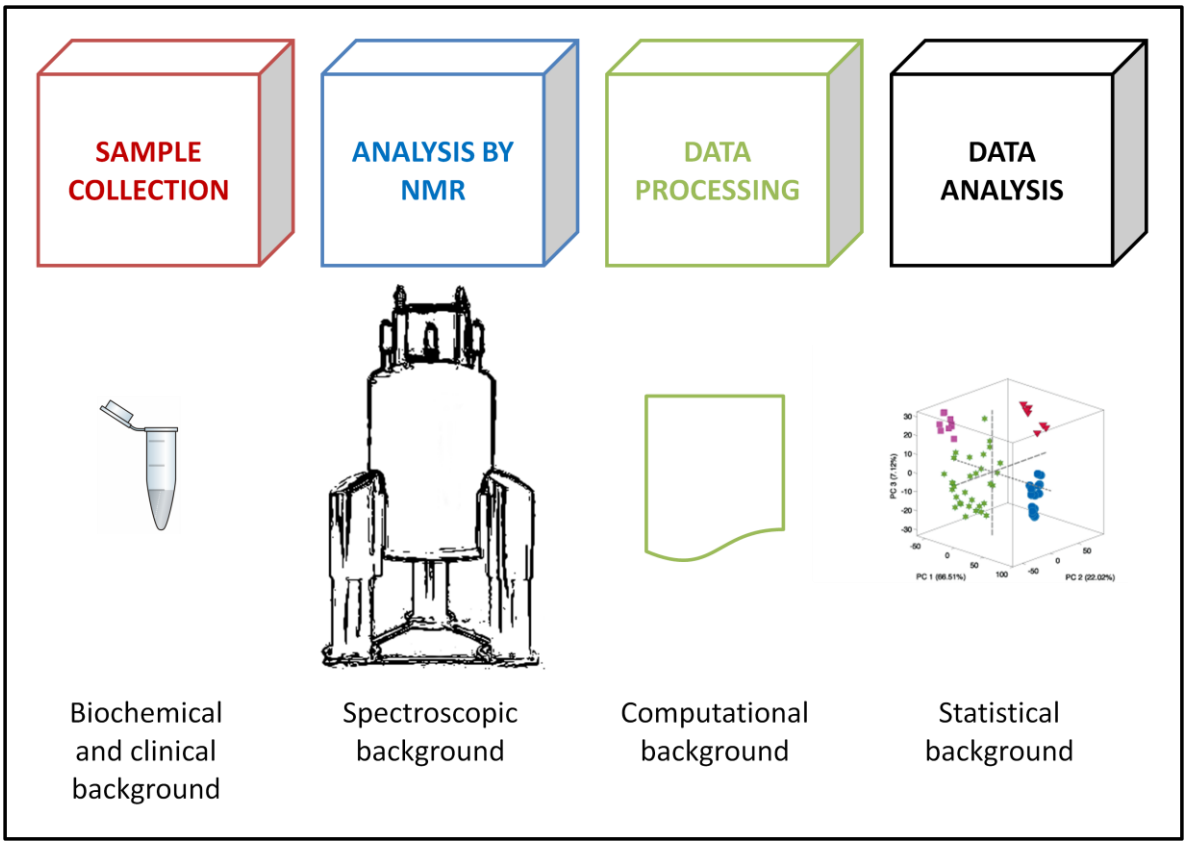
provide a direct measure of lipoprotein particle sizes, neither without prior calibration with other techniques nor using a library of defined lipoprotein fractions previously characterized using NMR **[23]**.

1.2 Motivation

Our aim was to develop a new methodology based on NMR to determine other parameters of lipoprotein particles beyond of their lipid content, such as size and particle number of several subclasses, thought to be better predictors of cardiovascular risk in these individuals whose classical risk factors do not unmask their real cardiovascular risk. These individuals include subjects with metabolic disorders, such as patients with obesity, diabetes and/or metabolic syndrome, among others.

In order to let the reader to get the sufficient knowledge for the critical judgement of this thesis and the scientific articles within it, Chapter 2 includes a description of the different theoretical aspects involving this very multi-disciplinar thesis, including theory on lipid and lipoprotein metabolism, nuclear magnetic resonance spectroscopy and mathematical tools for the treatment of NMR data (See **Scheme 1**).

The doctoral thesis presented in this document is the result of the research conducted in the Department of Electronic, Electrical and Automation Engineering at the Rovira i Virgili University (URV). Other research institutions have been involved in this research, such as the Metabolomics Platform owned by the same university and the CIBER of Diabetes and Metabolic Diseases (CIBERDEM), the Research Unit of Lipids and Atherosclerosis (URLA) of Sant Joan University Hospital from Reus (HUSJR). All these groups are part of the Institut d'Investigació Sanitària Pere Virgili (IISPV).



Scheme 1. Building blocks of the theoretical knowledge included within the framework of this thesis.

Thanks to this collaboration it has been possible to develop The LipoScale Test, an advanced lipoprotein test based on difussion NMR spectroscopy. This test is aimed at obtaining a better characterization of plasma lipoproteins, i.e. lipid content, size and particle number, in order to improve cardiovascular risk prediction. The main difference between our approach and the commercial test is that they use standard 1D NMR methods, while our test is based on the use of magnetic gradients, which generates 2D spectra with direct and objective information on lipoprotein particle sizes.

This thesis has generated different scientific publications which will be detailed

through the next sections, as well as a patent that has been filled to the European Patent Office (EPO) and a spin-off which will commercialize the test.

Future challenges that advanced lipoprotein tests must overcome will be the quest for the target population who will benefit such advanced tests, standardization of the different analytical techniques and the translation of these tests into clinical practice.

1.3. Objectives

The aim of this thesis has been **to develop and evaluate a novel advanced lipoprotein test, the Liposcale test, based on 2D diffusion-ordered ^{1}H NMR spectroscopy to measure the size and particle number of lipoproteins**. This new test has been developed either for measuring human plasma/serum samples as well as isolated lipoprotein fractions. Therefore, the goals related to this purpose are the following:

- Optimization of the measurement conditions of human plasma/serum samples by 2D diffusion-ordered ^{1}H NMR spectroscopy.
- Set up of the Lipsocale test, a deconvolution algorithm to obtain the size and particle number of several lipoprotein subclasses lipoprotein-related information from 2D diffusion- ordered ^{1}H NMR spectra.
- Application of the Lipsocale test to the study of dyslipidemias (e.g. atherogenic dyslipidemia).

1.4. Organization of the text

This chapter has provided a general overview of the background and motivation for the realization of this thesis. As has been highlighted previously, the need for better predictors of cardiovascular disease is increasing due to the pandemic levels that metabolic disorders are achieving. One of the main areas where new biomarkers can be looked for is in the area of lipids and lipoproteins. For this purpose, NMR spectroscopy appears to be a suitable analytical technique to develop new methods which will aid in the assessment and management of cardiovascular events.

Chapter 2 presents an state of the art of the current applications of lipoprotein analysis methods by NMR spectroscopy and their applications to study diabetes and related disorders. The contents of this chapter have been published recently as a review article in the journal *Progress in Nuclear Magnetic Resonance Spectroscopy*. The first part of the review describes the main concepts on lipid metabolism and one of the most important lipid abnormalities: the diabetic dyslipidemia. It is in this sort of dyslipidemia where the advanced lipoprotein tests can take an advantage over other common lipid tests due to the fact that this dyslipidemia is characterized, among others, by showing normal LDL cholesterol levels but a preponderance of small LDL particles, resulting in an increase in total LDL particle concentrations. The second part of the review focuses on the practical aspects of human plasma/serum analysis by NMR, covering issues such as sample handling and conservation, common NMR measurements of plasma/serum samples and diffusion NMR measurements to obtain optimum information on lipoprotein particles.

Chapter 3 contains three scientific articles published during the realization of this thesis. These scientific articles deal with the different phases involved in the development

and evaluation of a novel NMR-based advanced lipoprotein test either in plasma/serum or lipoprotein fractions. Our first results show the development of a new methodology to measure the size of lipoprotein fractions previously isolated using ultracentrifugation. We evaluated this methodology with the results obtained from the transmission electron microscope (TEM). These results have been published in the journal *Analytical and Bioanalytical Chemistry*.

The second scientific article presented in **Chapter 3** describes our first attempt to analyze a number of human plasma samples using 2D diffusion-edited ^{1}H NMR spectroscopy. We also wrote the necessary MATLAB code to optimize the different lorentzian parameters to characterize an arbitrary number of lipoprotein subclasses. The NMR areas obtained through this optimization process were used to classify the different samples according their associated dyslipidemias using the unsupervised Principal Component Analysis (PCA). These results were published in the journal *METABOLOMICS*. The above-described methodology was the origin for a further improvement both in the NMR experiments and the NMR data processing, leading to the development of the final advanced lipoprotein test. Overall, our advanced lipoprotein tests provides the lipid concentration (cholesterol and triglycerides) and size for the main lipoprotein fractions and the particle concentration of the main lipoprotein fractions as well as nine subclasses. To mention, partial least squares (PLS) regression models were built to obtain the lipid concentrations. The development and evaluation of this NMR-based advanced lipoprotein test have been submitted to the *Journal of Lipid Research*.

The advantage of this novel advanced lipoprotein test is based on the use of diffusion measurements, which yields the size of the main lipopotein particles directly, without the need for external libraries or calibrations with a reference technique. This novel

advanced lipoprotein test was evaluated through the validation of the LDL particle number parameter, the most important risk factor for cardiovascular disease in patients with diabetic dyslipidemia. Moreover, the LDL particle number is being a matter of debate in the scientific community in order to incorporate it to the clinical guidelines for screening and managing of dyslipidemias and their associated cardiovascular risk. Thus, the accurate validation of this parameter is of most value in order to translate its use into clinical practice. In this regard, we obtained high accuracy and precision values of this parameter compared with standard measures of LDL apolipoprotein B, which is a surrogate of the number of LDL particles.

The **Annexes** contains three scientific studies showing the usefulness of the NMR-based lipoprotein analysis. The first one deals with the monitorization of a pharmacological intervention on women with polycystic ovary syndrome. The second one studies the effect of a nutritional intervention on the lipoprotein profile. And the latter studies the effect of different protocols to extract information on lipoprotein particles usign DOSY.

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2. STATE OF THE ART: "HUMAN SERUM/PLASMA LIPOPROTEIN ANALYSIS BY NMR: APPLICATION TO THE STUDY OF DIABETIC DYSLIPIDEMIA"

2.1. Introduction

NMR spectroscopy is an analytical technique used in the profiling of blood. Depending on the sample preparation method, it is used to examine either serum or plasma. With this technique it is possible to obtain the spectra of the most abundant chemical components such as lipoproteins, aminoacids, organic acids, carbohydrates and albumin with minimum sample preparation **[1,2]**. NMR has proven to be a straightforward and useful technique for the qualitative and quantitative measurement of lipoproteins, giving the cholesterol and triglyceride concentration **[3,4]**, as well as the number of subfraction particles and their size **[5]**. This is a great achievement because even though the behavior of the different lipoprotein subfractions is modulated by their size, the overlap between them in the spectra is severe, and sophisticated deconvolution algorithms are required for data analysis **[6]**.

The importance of NMR spectroscopy as an analytical tool for metabolomic studies with biological fluids, especially for serum and plasma samples, can be clearly assessed by considering the relevant literature during the past few years. Since the year 2000, the ISI Web of Science lists more than 500 papers on the subject (and more than 130 in 2011). These papers deal with NMR spectroscopy, metabolomics and serum/plasma. Human serum/plasma NMR measurements have been applied, for example, to monitor changes in the organization of lipids within particle density subclasses **[7]**, to assess diabetes studies **[8–10]**, to study the effects of exercise on lipoprotein profiles **[11]**, to monitor changes in lipids induced by diet therapies **[12,13]**, to assess cholesterol-lowering drugs **[14,15]** and to investigate dyslipidemias associated with HIV/AIDS therapy **[16–18]**, among others. The serum/plasma NMR measurement is also a highly valued tool in scientific experiments on animal models (rats, mice, etc.) that aim to assess the effects of drug/diet intervention **[12,13]**. However, in most of these publications, the information extracted from spectra

about lipoproteins is rather qualitative **[1]**.

The Adult Treatment Panel Guidelines of the National Cholesterol Education Program and, more recently, the European Societies of Cardiology and Atherosclerosis have established lowdensity lipoprotein cholesterol (LDL-C) treatment goals, and secondary non-high-density lipoprotein (HDL-C) treatment goals for people with hypertriglyceridemia, but these risk factors are unable to predict cardiovascular events in individuals with ‘‘apparently’’ normal lipid levels. These events are frequently found in individuals with such metabolic disorders as obesity and diabetes mellitus **[19]**. This means that lipoprotein characterization needs to be more sophisticated, and justifies the advent of the advanced lipoprotein tests (ALT), a set of techniques that enable the lipoprotein to be subfractionated and the lipids from the different subfractions to be measured. In this context, Liposcience Inc. has recently received 510(k) clearance from the U.S. Food and Drug Administration to market the Vantera(R) Clinical Analyzer. It is the first NMR-based diagnostic platform that is commercially available for in vitro diagnostic use, consisting in the determination of the low density lipoprotein particle number (LDL-P). If cleared, it would become the first 121 NMR-based diagnostic platform to be commercially available for in vitro diagnostic use. If this trend continues, NMR spectrometry will become the reference technique for the determination of such cardiovascular risk factors associated with lipids as low-density lipoprotein particle numbers (in particular LDL-P). There is still some controversy about the clinical utility of the ALT (i.e. LDL and HDL subfractionation) **[20,21]**, one of the greatest impediments being the lack of standardization in the characterization techniques. Ala-Korpela’s group, very active in the area since the mid 90s, reported in 2009 an NMR-based highthroughput analytical method for serum profiling, which can quantify up to 130 metabolites, lipids and 14 subfraction lipoproteins **[2]**. This analytical platform has turned out to be very useful in epidemiological cohort studies **[22–24]**, and has found associations in wide-genome studies.

Recently, some articles have been published and patents filed that directly relate to the use of diffusion NMR spectroscopy for the characterization of lipoproteins **[25,26]**. The application of magnetic gradients in the z-direction attenuates the diffusion of the smaller lipoproteins more strongly. Therefore, for low gradients all the lipoprotein response is present, but for high gradients only those of the larger lipoproteins are observed. Using this technique, the lipoprotein diffusion coefficients can be determined experimentally and, according to the Einstein–Stokes law, these values can give a direct estimation of the lipoprotein radii. However, at the moment the approach has not been perfected and cannot give reliable and meaningful results for clinical applications.

The aim of this review is to help researchers interested in serum/plasma-based NMR analysis of lipoproteins for clinical diagnostics, metabolomic studies, etc. We provide comprehensive information about all the steps needed to characterize lipoproteins by NMR spectroscopy. In **Section 2.2.1** there is an introduction to lipoproteins and their role in metabolism and the clinical importance of lipoprotein characterization for assessing risk factors important for cardiovascular diseases (CVD). Because diabetic patients are one of the target groups for ALT, **Section 2.2.2** gives an explanation about diabetic dyslipidemia. **Section 2.2.3** discusses the clinical utility of the ‘‘new biomarkers’’ measured by ALT. The state of the art of these techniques is reviewed in the same section. **Section 2.3** describes all the NMR measurement procedures used for serum/plasma, giving information about blood collection and conservation procedures and also about the pulses used. Diffusion-edited NMR pulses will be reviewed in detail because they enhance the NMR signal from lipoproteins and eliminate the signal from small metabolites. The reader will find a detailed discussion of the advantages and disadvantages of the various pulses used in diffusion NMR. As has been mentioned above, extracting the information about the lipoproteins from the lipid peaks in NMR spectra (i.e. methyl or methylene) is not straightforward, so we will make a detailed review of the various signal processing strategies. We group them in

three main approaches: (a) curve fitting methods, which are based on a mathematical deconvolution of the peak to extract new features from the lipid peak(s) that will then be used as inputs for multivariate analysis; (b) correlation statistical methods, where the points of the original lipid peak envelop are considered to be input features for further processing algorithms since these methods do not deconvolute the peak; and (c) diffusion NMR methods. In this subsection, we will consider all the studies made of 2D diffusion NMR, regardless of whether there has been a deconvolution process or only a statistical correlation used in the analysis. Finally, in **Section 2.5**, the application of NMR lipoprotein analysis in diabetes will be reviewed in detail and **Section 2.6** will draw the final conclusions and discuss the future outlook.

2.2. Clinical interest of lipoprotein analysis

2.2.1. Lipoproteins and the lipoprotein metabolism

Lipids are key molecules for life **[27]**. For instance, triacylglycerols provide energy for metabolic processes, and fatty acids, cholesterol and phospholipids are part of cell membranes that, as well as having a role as precursors of hormone molecules are also constituents of the central nervous system and genetic signal modulators. However, their hydrophobic chemical structure makes them incompatible with an aqueous medium like blood. In order for lipids to circulate throughout the blood stream and reach peripheral tissues, they are packaged into macromolecular complexes together with protein molecules. This gives rise to lipoproteins. The general structure of lipoprotein molecules is spherical and they are synthesized within the liver and intestines. Non-polar lipids (triacylglycerols and cholesterol esters) are found within the core, while polar lipids (phospholipids and free cholesterol) are distributed through a surface monolayer (**Figure 1**).

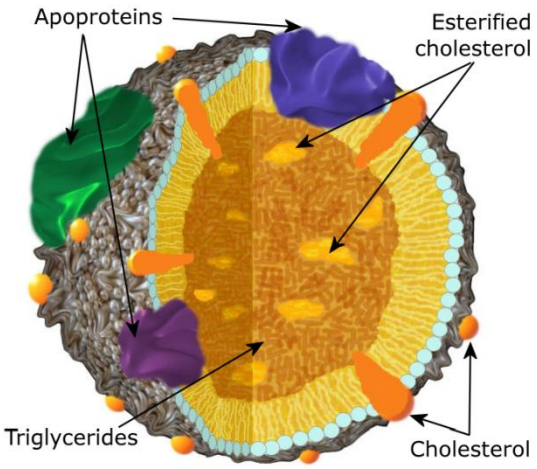

Figure 1. An schematic diagram of a typical lipoprotein, with its lipid content (taken from **[136]**).

The protein components of lipoproteins are called apolipoproteins (or apoproteins), a group of proteins of immense structural diversity, which are located on the surface together with polar lipids. They define the structure, metabolism and functionality of a given lipoprotein class, as well as how it interacts with receptor molecules or different enzymes in the liver and peripheral tissues. Lipoproteins have historically been defined according to their density and are divided into five main classes **[28,29]**: chylomicrons; very low density lipoproteins (VLDL); intermediate density lipoproteins (IDL); low density lipoproteins (LDL), and high density lipoproteins (HDL) **[29]**. Each of these lipoprotein classes can be further divided into several subclasses, but the number and nomenclature is method-dependent (see **Section 2.2.3.2**), since the density of lipoproteins is really a continuum, not discrete values corresponding to a finite number of classes.

Chylomicrons transport triglycerides and esterified cholesterol formed by dietary lipids (such as free fatty acids, monoglycerides and free cholesterol) being processed. In addition to cholesterol absorbed from the diet, they may also receive new cholesterol synthesized in the gut and cholesterol transferred from other lipoproteins. Chylomicrons are rich in triglycerides. However, while tissues take up fatty acids and glycerol from

released and hydrolyzed core triglycerides, as the circulating chylomicrons become progressively smaller, their triglyceride content decreases and they become relatively richer in cholesterol and protein. They become what is known as a chylomicron remnant.

VLDLs are secreted by the liver to supply triglycerides to tissues in the fasting or postprandial state. VLDL particles are somewhat smaller than chylomicrons and they undergo exactly the same lipid release process described for chylomicrons. The loss of triglycerides converts some VLDL into IDL and LDL, which are very rich in cholesterol.

HDL are the most diverse lipoproteins. They are synthesized in the liver and small intestine as protein-rich, disc-shaped particles containing ApoA1 and phospholipids. These particles mature by the acquisition of cholesterol from peripheral tissues and the action of several sterifying enzymes (LCAT) **[30]**. The HDL particles make it possible for the cholesterol to flow out of the cells, esterify in plasma, transfer to other lipoproteins and return to the liver for excretion. The HDL mediation of the transfer of lipids and apolipoproteins between lipoprotein classes also plays an important role in the overall lipoprotein metabolism.

2.2.2. Diabetic dyslipidemias and CVD risk

Diabetic dyslipidemia is defined as a characteristic set of lipid and lipoprotein abnormalities with a high prevalence in diabetic subjects **[31]**. In these subjects there is a low HDL concentration, a high triglyceride concentration and a clear preponderance of small, dense (SD) LDLs **[32–34]**. Disturbances in the production and clearance of plasma lipoproteins are characteristic of the metabolic abnormalities of diabetic patients. Therein brief, these abnormalities are the slower clearance of chylomicrons, increased production of VLDL, and the incomplete conversion of VLDL to LDL **[35,36]**.

On the other hand, this overproduction of VLDL increases the exchange of triglycerides from VLDL to the cholesteryl esters in HDL and LDL and thus decreases the concentration of cholesterol in LDL and HDL particles, leading to small and denser LDL and HDL. Small HDL particles are rapidly catabolized, which contributes to the low HDL cholesterol levels observed in this situation **[37]**. Moreover, small dense LDL is found in most hypertriglyceri- demia states, including diabetes. The potential role of this phenotype in atherosclerosis is based on the physicochemical properties of these particles. For instance, sdLDL can be oxidized more easily, the particles do not interact well with LDL receptors, and they may associate more readily with proteoglycans on the surface of cells or in the matrix. Thus, it is thought that SD LDLs have an important role in diabetic dyslipidemia along with reduced HDLs and increased triglyceride levels **[38]**.

Several studies support the view that each of these abnormalities increases the risk of suffering cardiovascular heart disease (CHD) **[39,40]**. CHD is the main cause of death of diabetic patients **[41]**. Diabetic subjects are two-to-four times more likely to suffer a cardiovascular event **[42]**. Moreover, development of dyslipidemia may be a precursor of diabetes. To assess cardiovascular risk, clinicians use a standard lipid panel, which includes the concentration of plasma triglycerides (TG), total cholesterol (TC), LDL cholesterol (LDL-C) and HDL cholesterol (HDL-C). All these parameters are experimentally measured except for the LDL-C, which is estimated using the Friedewald formula in clinical settings even though it can be measured directly **[43]**. A critical limitation of this formula is that it cannot be used when the concentration of plasma triglycerides exceeds 400 mg/dL, which is common in diabetic dyslipidemia. In addition, the resulting LDL-C value may include the concentration of cholesterol from IDL (IDL-C), making the use of this parameter for the clinical interpretation of a pathological state even more inaccurate.

2.2.3. Advanced lipoprotein tests

2.2.3.1. New biomarkers for cardiovascular risk assessment

New insights into the relationship between lipoproteins and atherosclerosis have highlighted the limitations of reporting the total amount of cholesterol in each major lipoprotein fraction. For instance, individuals receiving statin therapy to reduce LDL-C may still be at significant risk of developing CHD. This residual risk is higher in patients with metabolic syndrome, diabetes mellitus and/or obesity disorder, which are experiencing a significant increase in prevalence in recent decades. LDL-C, then, has less predictive power in these populations. An explanation for the abovementioned paradox regarding LDL-C and cardiovascular risk is that two individuals can possess the same total LDL-C, but if the amount of cholesterol that LDL particles carry is not constant, for the same amount of LDL-C, different subjects will have different LDL particle (LDL-P) numbers **[19]**. Nevertheless, smaller LDL particles have less cholesterol **[44]** than bigger ones, so denser LDL lipoproteins will be indicative of higher LDL-P and, thus, a higher risk of CVD. This is why recent studies have stressed the importance of adding other parameters when analyzing lipoproteins such as the Apo B concentration or the number of lipoprotein particles and their size. In this regard, the National Lipid Association Expert Panel has recently advised testing of LDL-P to assess and manage cardiovascular risk **[45]**. Although Apo B and LDL-P are highly correlated, the latter parameter is more accurate for estimating CVD risk because it does not include the particle numbers of VLDL and IDL fractions, which are less atherogenic than LDL fractions **[45]**. As has been mentioned above, diabetic individuals characterized by a dense LDL fraction (the so called Pattern B phenotype **[46]**) and a high level of triglycerides and a low level of HDL-C are a target group that is well-suited for an LDL-P measurement.

NMR is the most common technique used (see **Sections 2.2.3.2** and **2.4.1**) for measuring LDL-P **[6]**. Liposcience, Inc. has performed more than 6.5 million tests using its NMR LipoProfile® test. At the same time, there is some controversy about whether the LDL

and HDL subfraction parameters improve cardiovascular risk prediction in comparison with the standard lipid panel guidelines. It has been reported that the relationship between LDL particle size and cardiovascular risk is not inherent to this parameter but related to the fact that smaller particles lead to an increase in the particle number of this type of LDL **[45]**.

The set of analytical techniques that provides the most detailed information on lipoprotein particles is known as advanced lipoprotein testing (ALT) **[20]**.

In summary, although advanced lipoprotein testing and fractionation is not yet ready for routine clinical use, there is a need to account for a complete lipoprotein subclass composition, size and functionality analysis. This will be useful for developing and monitoring novel diet and drug therapies and getting insight into the pathophysiology of atherosclerosis.

Because of their high cost, advanced lipoprotein tests cannot be applied to the general population, but in the context of personalized medicine, they could be used in selected groups of patients who have been identified as having some risk factors (i.e. patients with diabetic dyslipidemia).

Some of the limitations of ALT are the lack of standardization and the varying approaches. Unfortunately, the number of lipoprotein subfractions and their nomenclature are not uniform across the techniques, which makes it difficult to compare results from different tests (**Table 1**) **[47]**. Moreover, some of the tests can only be performed by the company that markets the test, so the possibility of obtaining independent information on test performance is severely limited **[21]**.

Table 1. Comparison of reported analytical techniques for advanced lipoprotein tests.

Advanced lipoprotein testing (ALT)	Subfractions	Cholesterol	Triglycerides	Particle Number	Size	References
Vertical Auto Profile (VAP) II	3 VLDL, 1 IDL, 5 LDL, 5 HDL	√	–	–	–	**[51]**
Gradient Gel Electrophoresis (GGE)	7 LDL, 5 HDL	–	–	–	√	**[52]**
Dynamic Light Scattering (DLS)	CM+VLDL+IDL, 2 LDL, HDL*	–	–	–	√	**[53]**
High Performance Liquid Chromatography (HPLC)	2 CM, 5 VLDL, 6 LDL, 7 HDL	√	√	–	√	**[54]**
Ion Mobility Analysis (IM)	3 VLDL, 2 IDL, 4 LDL, 2 HDL	–	–	√	√	**[55]**
Diffusion-Ordered NMR Spectroscopy of lipoprotein fractions	any fraction[a]	-	-	-	√	**[56]**

[a]Subfractions previously isolated by density gradient ultracentrifugation

A recent study was aimed at evaluating the degree of heterogeneity among four ALT methods that assess LDL particle size. Complete agreement among these methods was only 8% **[48]**. Another review brought together all the results of studies comparing different ALT methods **[49]** and found that the agreement between methods for LDL subfraction determination varied widely (7–94%).

Although it is unclear if these methods are ready for clinical use, more exhaustive and detailed analyses need to be made to determine whether (i) progressive changes in lipoproteins are useful as therapeutic targets and (ii) certain subgroups of individuals can benefit from these analyses. These methods may also be important for advancing research and understanding the pathophysiology of CVD **[50]**. Moreover, many of these tests are of limited accessibility and it sometimes is difficult to identify the groups that might benefit from them.

2.2.3.2. Reported analytical techniques

As stated above, there are a variety of ALT techniques. The techniques based on serum/plasma NMR spectroscopy will be reviewed in **Section 2.4**. Here, we briefly describe and discuss the most important methods.

– *Density Gradient Ultracentrifugation*: The Vertical Auto Profile (VAP) II test is a rapid and sensitive ultracentrifugation method which measures the relative cholesterol distribution for different lipoprotein subfractions **[51]**. With this technique it is possible to obtain 3 VLDL fractions, 1 IDL fraction, 5 LDL fractions, and 5 HDL fractions. However, it does not provide either the concentration of triglycerides, or the numbers and sizes of the lipoprotein particles.

– *Gradient Gel Electrophoresis*: Gradient gel electrophoresis can fractionate LDL and

HDL subfractions directly from plasma according to their size. With this method, the size and relative (%) distribution of 7 LDL and 5 HDL subfractions can be determined **[52]**. It often requires custom-made gels and strict attention to laboratory quality control because small variations in gel quality and laboratory conditions may affect accuracy. The GGE method (available from Berkeley Heart Lab, Inc.) delivers the distribution of LDL size phenotype and refers to a preponderance of larger particles as Pattern A and a preponderance of smaller particles as Pattern B **[46]**.

– *Dynamic Light Scattering*: Dynamic Light Scattering (DLS) can assess lipoprotein particle sizes by measuring the intensity of scattered light **[53]**. This technique has been used to determine the mean particle size of CM + VLDL + IDL, large LDL, smalldense LDL, and HDL fractions previously fractionated using sequential ultracentrifugation.

– *High Performance Liquid Chromatography*: A simple and fully automated HPLC method commercialized by LipoSearch® can measure the cholesterol and triglyceride content as well as the size of the major lipoproteins and their subclasses (2 CM, 5 VLDL, 6 LDL and 7 HDL) **[54]**.

– *Ion Mobility Analysis*: This method relies on differences in electrophoretic mobility of gas-phased lipoprotein particles **[55]**. After an ultracentrifugation step to remove albumin, it can measure the size and concentration of 3 VLDL, 2 IDL, 5 LDL, and 2 HDL.

– *Diffusion-Ordered NMR Spectroscopy of lipoprotein fractions*. This technique uses the methyl peak of isolated lipoproteins to calculate the diffusion coefficient of the lipoproteins and, from this value, to estimate their size **[56]**.

2.3. Serum/plasma lipoprotein analysis by ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy

2.3.1. Main characteristics of serum/plasma NMR analysis

As has been shown in the sections above, measuring lipids in plasma and serum is of great interest since it enables abnormal metabolic levels, and unusual cholesterol and triglyceride transport mechanisms to be highlighted, and it provides valuable clues for clinical diagnosis. Detailed information about the lipids in biological fluids can be obtained by rapid total lipid ^{1}H NMR profiling without the need to apply complicated sample preparation procedures. However, the results will be rendered worthless if samples are not collected, stored, and prepared correctly. For this reason, protocols for handling biofluids, such as plasma and serum, have recently been proposed **[57]**.

When the analytical measurement is made, the operational NMR equipment must remain stable and any analytical drift must be identified, quantified and compensated for in order to reduce systematic differences over time. Bias should also be minimized so that measurements between different labs are comparable (this is one of the key advantages of the NMR approach). Fortunately, the coefficient of variation associated with inter- and intra-laboratory spectroscopic NMR experimentation is typically very low and, generally speaking, biological differences between individuals are far greater than the variations induced by experimental factors. Moreover, Lenz and co-workers have demonstrated high variability between subjects but low variability within subjects and study days in ^{1}H NMR measurements in plasma **[58]**.

Whenever possible, the analyst should be involved throughout the design and decision-taking-process of all protocols, from the experimental design and sample collection to final data results. This involvement is becoming more and more important in serum and plasma analysis, since it can lead to good inter-laboratory reproducibility. Complex biofluid matrices can be easily affected by contamination and external factors,

which are visible in the NMR spectra, so samples should be prepared and measured carefully if the results are to be of any biological import.

2.3.2. Serum/plasma handling and conservation

2.3.2.1. Introduction

Blood plasma and serum are considered to be the most representative biofluids of the homeostatic state of the body. Because most of the tissues and organs in the body are profusely irrigated, the serum/plasma composition reflects even minimal changes in the whole body metabolism. This is why the collection of blood plasma or serum is the first step in most clinical, metabolic and nutritional studies. Nevertheless, untreated blood is not a suitable biofluid for lipoprotein NMR measurements. Although some studies with raw blood have been performed with NMR **[59–61]**, lipoproteins are not present in erythrocytes and, even for the measurement of low molecular weight metabolites, the presence of cells and their sedimentation gives rise to problems of lack of homogeneity in the magnetic field and line broadening.

Plasma and serum contain high levels of proteins and many endogenous metabolites, some of which have yet to be identified. They also contain significant amounts of active enzymes and exogenous microorganisms that will change the concentration of the metabolites or generate new ones after a short period at physiological temperature. Therefore, improper storage and handling of plasma or serum samples may significantly influence accuracy and final results compared to the analysis of fresh samples. In addition, there are specific protocols for different analytes and sparse information about specific storage conditions and handling protocols.

2.3.2.2. Collection procedures

In order to obtain serum or plasma, blood should be collected in clean tubes and allowed to clot or be centrifuged. Collection of plasma requires an appropriate anticoagulant to prevent clotting before the cellular components are removed by centrifugation. Some commonly used anticoagulants include heparin, ethylene diamine tetraacetic acid (EDTA), citrate, and oxalate salts of sodium, potassium, or lithium. EDTA, oxalate, and citrate prevent clotting by complexing divalent calcium (Ca^{2+}), which is required for coagulation. Heparin interferes with the formation of fibrin **[62]**.

For the NMR analysis of plasma, Li-heparin is preferred because other anticoagulants such as EDTA, sodium citrate or oxalate generate intrusive signals in the NMR spectrum **[63]**. Moreover, citrate and oxalate tubes contain an aqueous solution that dilutes the collected sample. Also, in order to prevent clotting, the high concentration of these anticoagulants changes the osmolarity of plasma, induces a shift of water from erythrocytes and adds an additional factor of dilution to lipoproteins by 10% or more. EDTA (especially K_3-EDTA) can cause less severe, but still noticeable (3–5%), dilution. Tubes with protease inhibitor cocktails, routinely used in proteomic studies, should also be avoided for the same reason.

2.3.2.3. Serum vs. plasma

For some NMR applications serum may be preferred to plasma. Anticoagulant problems such as fibrinogen precipitation on freezing and thawing are avoided if serum is used. Serum is prepared by allowing the blood sample to coagulate. This can be done by collecting the blood in a clean glass tube and allowing it to stand at room temperature until it clots. Serum contains all of the original blood components except the cellular elements and protein components that constitute the clot. This approach makes the analysis of

lipoproteins easier than the analysis of plasma. However, the delay introduced by clot formation allows some biological processes to take place in the blood at room temperature. Therefore, the binding of lipoproteins to the clot must be taken into account. Our experience tells us that clotting at low temperature (4 ºC) yields more serum and clot variability than clotting after half an hour at room temperature.

Thus, it is recommended that the clot be formed at room temperature and the supernatant serum collected in another suitable storage container. A separator gel in the tubes could be used to facilitate collection, but some gels are not suitable for NMR measurements because they can introduce interfering compounds that are NMR-visible. Clot sedimentation for obtaining serum could produce hemolysis of the blood sample, a reaction that has an interfering effect in most of the classic clinical assays. However, the lipoprotein profile by NMR has been demonstrated to be virtually unaffected by gross hemolysis **[6]**.

2.3.2.4. Cryopreservation

When the total amount of blood fraction is ready, the aliquots of blood plasma or serum samples, if needed, should be created prior to freezing and storage so that multiple freezing/thawing operations are not required.

For lipoprotein and metabolite analysis of plasma/serum by NMR, periods of up to several days at 2–4 ºC are acceptable. Refrigerated plasma or serum specimens can be stored for up to 7 days without affecting NMR results if they are kept below 4 ºC **[6]**. Samples can generally be successfully stored at -20 ºC for moderate periods (up to 1–2 months), but some enzymes, such as plasma esterase, may still be active at this temperature. Thus, for long storage periods (years) cooling at -70 ºC to -80 ºC (or even

lower) is required **[9]**.

The only specimens for which freezing may adversely affect NMR lipoprotein results are postprandial samples or hypertriglyceridemic samples with fasting triglyceride values greater than 300 mg/dL **[64]**.

Before the cryopreserved samples are measured, they should be thawed on ice, in a cold-water bath or at room temperature, not by heating. Correct homogenization of samples after thawing is mandatory. Exposing samples to multiple freeze–thaw cycles is more likely to alter NMR results, so this procedure should be avoided **[6]**.

2.3.3. ^{1}H NMR measurement of serum/plasma

2.3.3.1. ^{1}H NMR sample referencing

One major advantage of using NMR spectroscopy to study plasma and serum is that measurements can often be made with minimal sample preparation (usually with only the addition of 5–10% D_2O for locking, and a suitable standard in the sample or in an inner capillary for referencing or quantification purposes). Moreover, a holistic analytical profile can be obtained on the whole biological sample.

The water-soluble compound 3-(trimethylsilyl) 2,2,3,3-tetradeutero-propionic acid (TSP) has lately been used as the preferred reference for calibrating and quantifying aqueous NMR samples. Unlike 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS), which has signals arising at 3.1 ppm (a triplet), 1.9 ppm (a pentet), and 0.8 ppm (another triplet) all of which correspond to methylene protons of the alkylic chain, TSP does not add any other signals apart from the sharp methyl resonance at 0 ppm. However, for serum or plasma, TSP must not be used because it interacts with proteins **[65]**. In the work of Kriat et al. **[66]** although formate is present in serum in small amounts, this compound is added as the

internal standard for referencing (at 8.47 ppm) and for the quantification of some metabolites in plasma **[67]**. However, it probably alters the pH of the sample and it has not been used for lipoprotein measurement in any published report.

One general way to use TSP as a reference substance is to introduce a co-axial inner capillary tube with the compound dissolved in deuterated water. This system prevents the TSP from interacting with the blood proteins and prevents the sample from being modified by deuterated solvents. The double tube system can also be used for quantification after the nine methylsilyl protons have been fitted around 0 ppm.

Because of the stability of modern NMR spectrometers, 'Digital ERETIC' (Digital Electronic REference To access In vivo Concentrations, Bruker®) is an interesting method for quantification in serum. It does not involve any reference substance but adds a digital signal at a chosen frequency to the final corrected spectrum **[68]**. This signal is previously calibrated against a reference sample and corrected taking into account the variation of the 90º pulse length, the number of scans and the receiver gain, always after the tuning and matching have been automatically adjusted **[69]**.

2.3.3.2. The Nuclear Overhauser Effect Spectroscopy (NOESY)-presaturation sequence

In our experience, and in accordance with published protocols **[57,2]**, the best way to make a quick, quantitative ^{1}H NMR measurement of plasma is to use a one-dimensional Nuclear Overhauser Effect Spectroscopy (NOESY)-presaturation sequence that employs the first increment of a 2D NOESY pulse sequence with water irradiation during the relaxation delay (RD) and the mixing time (τ_m) (**Figure 2**). This has the form (RD–90º–τ_1–90º–τ_m–90º AQ) and in standard experiments the τ_m (mixing time) is around 100–150 ms, although this parameter is not critical. The 90º pulse length is calibrated for each sample **[70]**. In a 600

MHz Avance III spectrometer equipped with a 5 mm CPTCI cryoprobe (Bruker®), for example, it varies from 9 µs to 13 µs depending on whether the blood sample is plasma or serum. The spectral width is set to 30 ppm to facilitate phasing and baseline processing in an automated way, and 64–128 transients are collected into 64-128 k data points for each ^{1}H spectrum.

It should be pointed out that in spectrometers in a thermostatted room the application of automatic routines in all the steps of acquisition (sample temperature equilibration, tuning, matching, shimming, 90º pulse calibration, fixed receiver gain and number of scans) yields an analytical variation of less than 1% (with the digital ERETIC® signal) in blood plasma and serum samples **[68]**, far less than is normally encountered in biological processes.

2.3.3.3. NMR editing techniques

The single pulse spectrum of human blood plasma is very complex. The highly overlapped resonances from metabolites, proteins, lipids and lipoproteins in the narrow chemical shift range of 1–13 ppm make it very difficult to successfully identify components even at a high magnetic field ^{1}H observation **[1]**. Therefore, throughout these studies, different pulse sequences were used so that results would be more accurate and reliable.

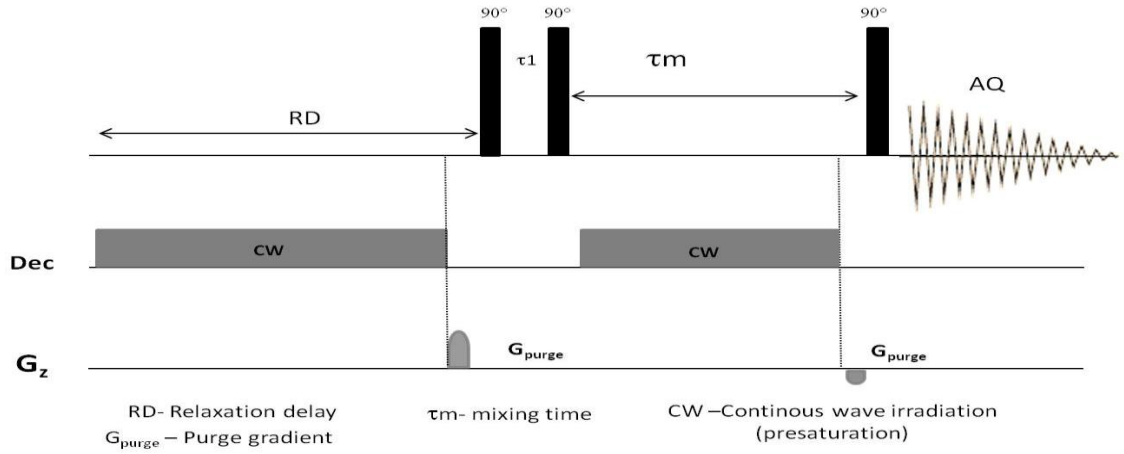

Figure 2. Noesy-presat 1D pulse sequence with water presaturation during recycling delay and mixing time.

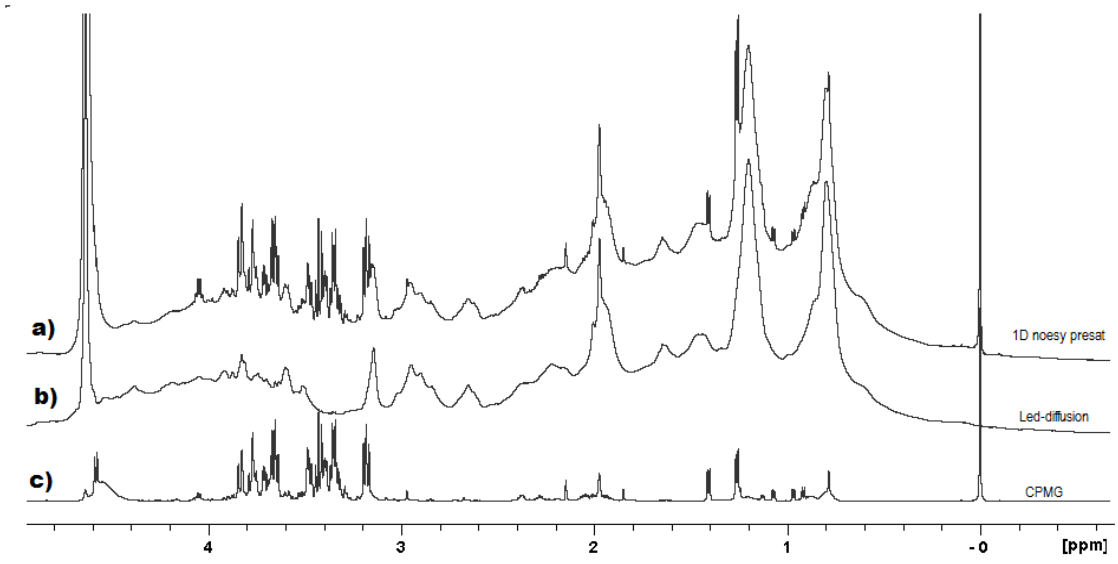

Figure 3. Complete ^{1}H NMR profiling of serum which includes: a) A nuclear Overhauser Effect SpectroscopY (NOESY)-presaturation sequence (RD*-90°–τ_1–90°–τ_m*–90° ACQ) to acquire a quantitative serum spectra suppressing the water peak, b) A CPMG sequence (spin-spin T_2 relaxation filter; RD–90°–[τ_2–180°–τ_2]$_n$–ACQ) to acquire low molecular weight metabolites and c) A diffusion-edited pulse sequence with bipolar gradients and the Longitudinal Eddy-current Delay (LED) (RD-90°-G_E-180°-(-G_E)-90°-G_s-Δ-90°-G_D-180°-(-G_D)-90°-G_s-τ_{led}-90° ACQ). Legend: * Presaturation of water signal, RD Recycling Delay, 90° or 180° radiofrequency (rf) pulse, τ_1 interpulse delay, τ_m mixing time, τ_2 half spin-echo time, G_E half bipolar encoding gradient, Δ Diffusion time, G_D half bipolar decoding gradient, G_s spoil gradient, τ_{led} longitudinal eddy-current delay time, ACQ acquisition.

A 1D NMR profiling of a sample of serum **[71]** usually includes a set of ^{1}H NMR spectra with a standard 1D spectrum (such as 1D-NOESY presaturation or WET pulse), a transversal relaxation (T_2) weighted filtration sequence (such as the Carr–Purcell–Meiboom–Gill spin-echo pulse **[72,73]**, which removes the lipoprotein background), and a diffusion-edited spectrum that removes signals from low molecular weight molecules (see **Section 2.3.4**). **Figure 3** shows that the application of these three NMR pulses gives three different spectra from a single sample of serum.

A wide variety of useful spectroscopic techniques can be used to edit the NMR-detectable metabolite information in body fluids. For example, a combination of T_2 editing and diffusion in a single experiment (DIRE) has been used by Lindon et al. as a proof of concept with human plasma **[74]**. Their experiment allowed a variable attenuation of low and high molecular components of plasma but has not been generally applied in a clinical or metabolic study. Most NMR pulses and editing techniques are based on taking proper account of NMR relaxation times (T_1, spin-lattice or longitudinal relaxation and T_2, spin–spin or transversal relaxation) so that they can be used to efficiently reduce the dominant protein background in blood plasma and serum samples **[75]**. They enable mobile low molecular weight metabolites to be detected and quantified **[76]**, but signals from lipoproteins are practically removed so they do not fall within the scope of this review. On the other hand, a diffusion-edited NMR spectrum enhances the NMR signal of lipoproteins and removes the signal of small metabolites. Thus, the composition and structure of the constituent lipids of lipoproteins can be assigned and analyzed (**Figure 4**).

2.3.3.4. ^{1}H NMR in the analysis of lipoproteins

In the sections above, it has been clearly stated that different ^{1}H one-dimensional edited spectra can provide spectroscopic data from the different components of interest of

a biofluid (and in general any mixture) with no previous physical or chemical separation **[77]**. In addition NMR provides invaluable information for the early diagnosis and assessment of health **[78,79]**. In general, except for T_2 edited experiments, such as the CPMG sequence, the ^{1}H NMR spectrum of serum strongly reflects the lipoprotein subclass profile of the sample.

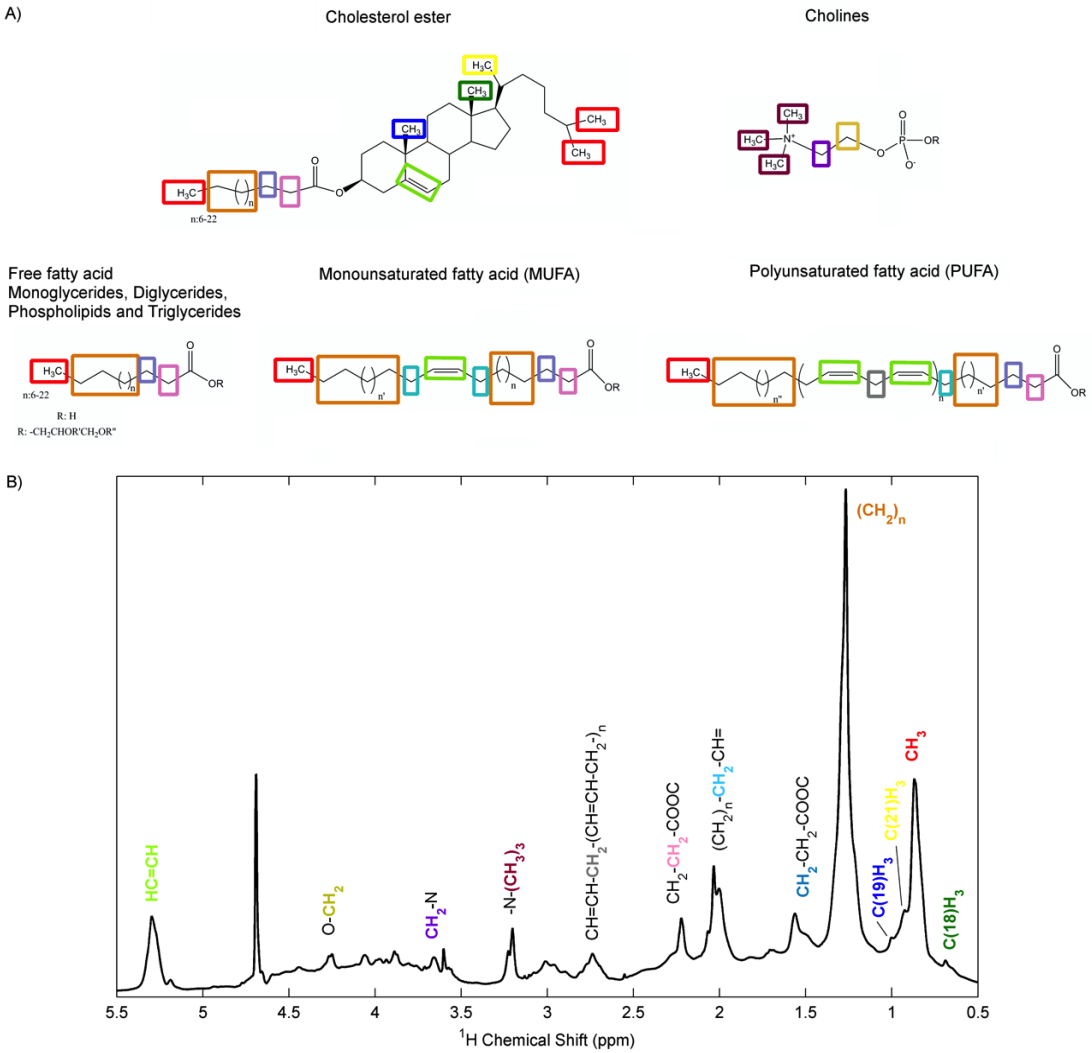

Figure 4. (A) Moieties for the principal lipids found in lipoproteins and (B) Correspondence between moieties and their NMR spectra signals.

The most important reason for using NMR in the analysis of lipoproteins is to avoid their tedious physical isolation from plasma. This approach also enables direct measurements to be made with little sample preparation or handling. Another important capability of NMR is that it can directly measure the number of lipoprotein particles, a new CVD risk factor (see **Section 2.2.2**). This is why NMR has been widely used for lipoprotein profile analysis (see the discussion in **Section 2.2.3.1**). This is possible because the three terminal methyl moieties of triglycerides and the esterified cholesterol of lipoproteins give the same amount of ^{1}H signal. Moreover, the resonance frequency is related to composition and size **[80]** of the lipoprotein which carries these lipid structures, with larger and smaller lipoproteins resonating at lower and higher frequencies, respectively (**Figure 7**).

The concept of using proton NMR spectroscopy to measure plasma lipoprotein concentrations efficiently and without reagents was introduced in the early 1990s by researchers from North Carolina State University **[64]** (see **Section 2.4**). Since then, ^{1}H NMR spectroscopy has received wide clinical interest as a method for quantifying lipoprotein subclasses. This methodology was first developed in practice independently by the groups of Otvos et al. **[81]** and Ala-Korpela et al. **[82,83]**. Thanks to their work the number of clinical studies to which it has been applied over the years has increased significantly **[6,79]**.

Ala-Korpela and co-workers developed a method for the absolute quantitation of human lipoproteins based on ^{1}H NMR spectroscopy and sophisticated line-shape fitting techniques **[82]**. In their earlier studies, they took NMR measurements of 66 blood EDTA-plasma samples and 20 VLDL, IDL, LDL and HDL isolated subfractions at the stabilized temperature of 37 ºC using a 400 MHz spectrometer. The water signal was suppressed by a standard binomial 1–1 pulse sequence. A double tube system (5 mm O.D. capillary filled with D2O solution of TSP inside a 10 mm O.D. NMR tube) was used for purposes of referencing and quantification.

In more recent works by the same group **[84,85]**, NMR measurements were performed with a single pulse in a more sensitive 600 MHz spectrometer using a 5 mm double tube system (2 mm O.D. D_2O capillary inside a 5 mm O.D. NMR tube) without any water suppression.

Although some lipoprotein NMR measurements have been performed below physiological temperature (at 298 K **[26]** or 303 K **[86]**), at these temperatures the core cholesterol esters of lipoprotein particles considerably diminish their NMR visibility **[64]**. Therefore, in most NMR measurements the temperature is set at physiological conditions **[2]**, and even in the case of the NMR LipoProfile test® **[5]**, plasma samples are heated to 318 K to facilitate the deconvolution of highly overlapped lipoprotein subclass signals **[6,9]**. The resonance frequency of NMR signals of lipoprotein fractions depends on their size and composition, but the neighboring NMR signals of each class differ only slightly in chemical shift, thus limiting the reproducibility and accuracy of lipoprotein quantification in blood plasma by NMR line-shape fitting approaches. Therefore, to overcome the problem of extensive lipoprotein signal overlap in the analysis of ^{1}H NMR spectra of plasma, another physical independent measurement, such as diffusivity, could be desirable in addition of ^{1}H spectra.

There is a considerable amount of literature on NMR molecular diffusion measurements by pulse field gradient stimulated echo (PGSTE) **[87]**, ranging from basic theory **[88]** to practical aspects **[89,90]** and applications **[91]**. However, the aim of this review is to describe lipoprotein analysis by NMR so only diffusion measurements on lipoproteins will be discussed.

2.3.4. ^{1}H NMR diffusion spectroscopy

2.3.4.1. Basis of NMR diffusion measurement

Since the initial work by Stejskal and Tanner **[92]**, the pulse field gradient spin-echo (PFGSE) NMR measurement has become the most commonly used method for studying molecular motion properties such as diffusion. The basic sequence consists of one 90º pulse to generate transverse magnetization and a 180º pulse flanked by two gradient pulses to generate the spin-echo (**Figure 5**). The measurement of diffusion by NMR and its processing steps have led to the so-called diffusion ordered spectroscopy (DOSY) **[91]**, an interesting and powerful technique for extracting the translational diffusion coefficients of the components of a mixture.

After the initial 90º pulse, the generated transverse magnetization starts to dephase due to chemical shift, and the evolution of hetero- and homonuclear coupling. The first pulsed linear field gradient adds a strong dephasing of the generated magnetization with a phase angle proportional to the length (δ) and the amplitude (G) of the gradient. This process is called gradient encoding or defocusing. Then, the intermediate 180º pulse is able to refocus the magnetization generating the echo. However, due to the gradient previously applied, the phase varies linearly along the Z-axis and only the spins within a narrow slice of the sample have phase angle coherence (i.e. the same phase angle). At this point the spins are dephased and no NMR signal can be detected from the whole sample. The second gradient pulse reverses the respective phase variation of the first gradient (decoding/refocusing) and now the spin-echo can be recorded in the usual way (**Figure 5**).

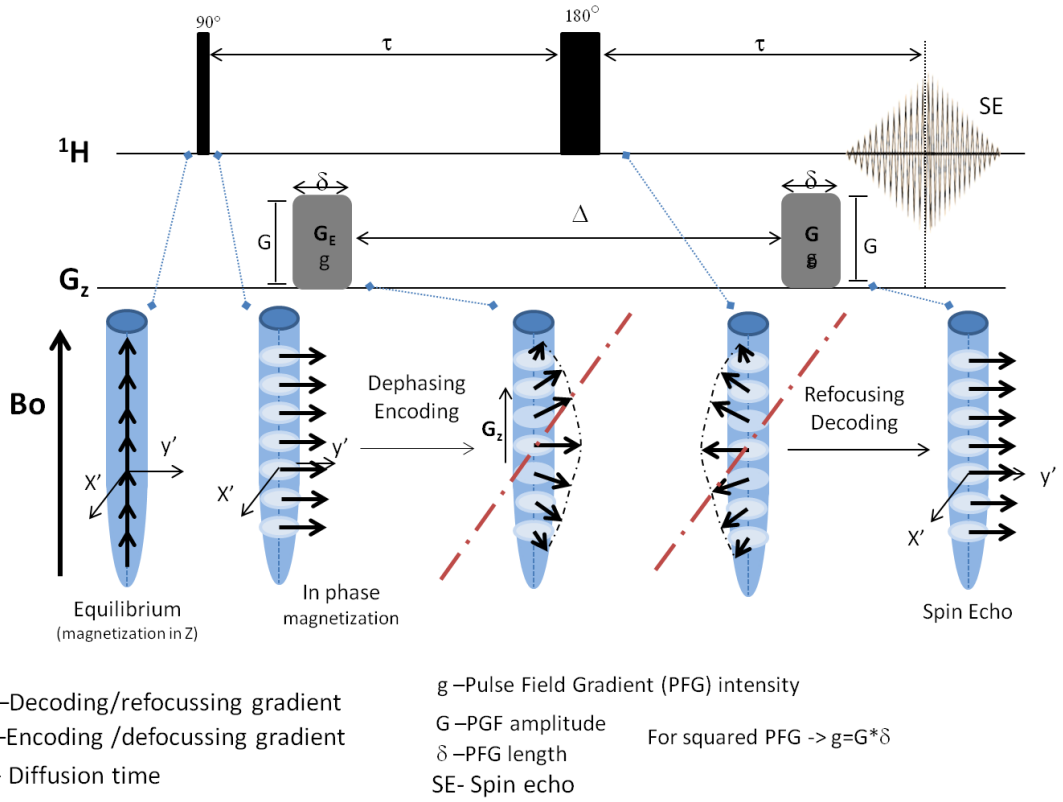

Figure 5. Basic pulse field gradient spin echo and evolution of magnetization **[92]**.

Thus, theoretically, the encoding and decoding gradients have no effect on the spin-echo. However, while they are being applied some of the spins move out of their slice as a result of Brownian motion (self-diffusion). These spins are not refocused by the second gradient and this leads to an attenuation of the echo amplitude, which is proportional to the average displacement of the molecules. Moreover, the transversal (T_2) NMR relaxation also reduces the echo amplitude. Thus, the attenuation of the echo signal intensity (I) is given by:

$$\boldsymbol{I = I_0 e^{\frac{-\tau_1}{T_1}} e^{\frac{-\tau_2}{T_2}} e^{-\gamma^2 g^2 \Delta D}} \quad \textbf{(1)}$$

where τ = period that the magnetization is under the effect of the transversal relaxation process (component of the XY plane); T_2 -= characteristic transversal relaxation time; I_0 = initial intensity of the NMR signal; γ = gyromagnetic constant of the nucleus (commonly ^{1}H); g = intensity of applied gradient (dependent on its duration, shape and strength (G)); D =

diffusion coefficient (related to the hydrodynamic radius of the molecule and the viscosity and temperature of the sample); and Δ = diffusion time, the period from encoding to decoding echo.

So that the longitudinal and transversal relaxation contributions remain constant, the usual way to conduct the diffusion experiment is to set the diffusion time (Δ) and vary the magnetic field gradient (g), usually changing the current intensity in the gradient coil.

If the diffusion time is constant, the relaxation terms become constant and can be incorporated into the initial intensity (I_0), thus leading to a reduced expression:

$$\boldsymbol{I = I'_0 e^{-\gamma^2 g^2 D}} \quad \textbf{(2)}$$

where I'_0 is the initial intensity $I'_0 = I_0 \exp(-2\tau/T_2)$ reduced by relaxation. Therefore, applying different gradient intensities, the diffusion coefficient (D) can be calculated.

In order to illustrate the basis of a diffusion NMR experiment, we choose a VLDL fraction, rather than a serum/plasma sample, because the lipoprotein size dispersion is much lower. **Figure 6a** shows a set of attenuated spectra of a VLDL fraction and **Figure 6b** shows the calculation of the diffusion coefficient from them, using **Equation 2**.

Once the diffusion coefficient has been calculated, the Stokes–Einstein equation can be applied to obtain the hydrodynamic radius (r_H) **[91]**:

$$\boldsymbol{D = \frac{kT}{6\pi\eta r_H}} \quad \textbf{(3)}$$

where k is the Boltzmann constant, T is the absolute temperature, g is the system viscosity and r_H is the hydrodynamic radius. This equation assumes equilibrium of the system, infinite dilution conditions, a continuum solvent and spherical molecules **[93]**.

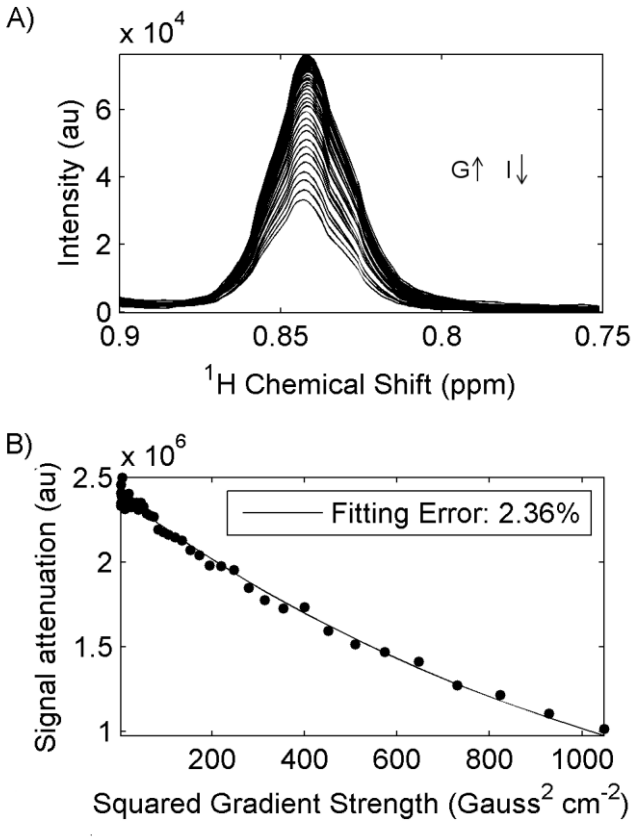

Figure 6. (A) Attenuated spectra of a VLDL fraction with increasing field strength, *G*. (B), extraction of the diffusion coefficient, fitting **Equation 2**.

This methodology makes it possible to use the additional dimension of diffusion and is called diffusion-ordered spectroscopy (DOSY) **[77]**. As an application, **Figure 7** illustrates a ^{1}H schematic DOSY peak map from the methyl peak of six lipoprotein fractions isolated by sequential ultracentrifugation **[56]**. The x-axis shows the ^{1}H spectra fractions, while the second dimension plots the *D* associated to each fraction, calculated according to **Equation 2**. It can be seen that larger fractions have higher chemical shifts. It is interesting to note that the resolution provided by the *D* dimension will play an important role in the analysis of lipoproteins, because it improves the separation between them. Once the *Ds* have been obtained, the application of **Equation 3** will determine their associated radii.

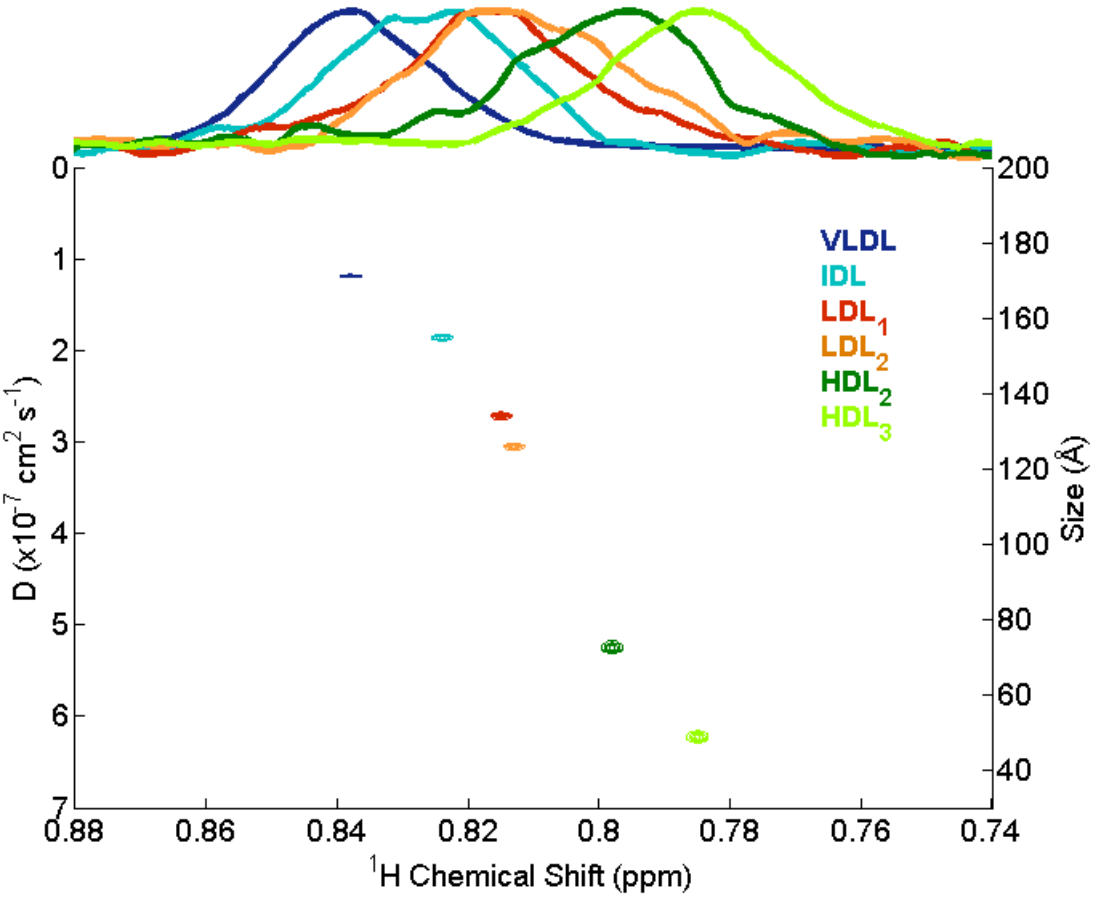

Figure 7. Schematic DOSY peak map of VLDL, IDL, LDL1, LDL2, HDL2 and HDL3, showing that the resonance of the fractions depends on their size (the larger the lipoproteins, the higher the shifts in ^{1}H-NMR spectra). The second dimension plots the diffusion coefficient *D* (10^{-7} cm^2s^{-1}) or size (Å) [68], after **Equation 3** has been applied. Spectra were acquired at 310 K and viscosity was measured for each sample.

2.3.4.2. ^{1}H NMR diffusion measurements

Lipoproteins are measured with 1D NMR methods mainly using line-shape fitting methods or multivariate approaches (see **Section 2.4**) across such specific areas of the spectra as the methyl and methylene peaks. However, because the signals of lipoprotein fractions overlap, the inherent accuracy of the method is limited, especially in medium-sized particles such as IDL or LDL **[94]**. Therefore, by giving additional information on lipoprotein sizes DOSY and other diffusion techniques open up a new dimension in lipoprotein analysis. Moreover, if the gradient ranges are changed to higher values, the

interfering signals from smaller molecular weight metabolites in serum can be removed from NMR spectra with most of the signal intensity from the lipoprotein molecules being retained.

Since the work by Liu et al. **[74]**, it has been suggested that diffusion-edited ^{1}H NMR spectroscopy could be used to improve the experimental signal of lipoproteins and hence the resolution of the lipoprotein subclass quantification.

First of all, to avoid errors in the calculation of the diffusion coefficient, it is important that the gradient strength be properly calibrated. The most common calibration procedure is to use a pulse field gradient (PFG) experiment on a substance with a known diffusion coefficient, such as water or ethanol, at a temperature of interest **[95]**. Subsequently, other experimental settings can be applied to improve the acquired data **[87]**.

2.3.4.2.1. Spin echo (PFGSE) replaced by stimulated echo (PFGSTE). During pulse-field gradient spin-echo (PFGSE), the transversal (T_2) NMR relaxation reduces the echo amplitude. Thus, when measuring the diffusion of the large, slow-moving lipoproteins, the diffusion time needed to observe the attenuation is too long and the transversal relaxation (T_2) can completely reduce the NMR signal. The gradient stimulated echo (PFGSTE) sequence proposed by Tanner **[96]** (**Figure 8**) is a simple improvement that is useful for studying compounds such as macromolecules and lipoproteins, in which T_1 is much larger than T_2. This sequence splits the 180º pulse of the spin echo into two 90º pulses: the first stores half of the transverse magnetization along the z-axis and, therefore, only the slower longitudinal relaxation (T_1) is the effective relaxation path during diffusion time (D). The second 90º pulse restores the transverse magnetization just before the decoding gradient. This pulse significantly reduces the attenuation of the NMR signal in molecules with short

transverse relaxation (T_2) (initial intensity is $\boldsymbol{I_0' = \frac{1}{2} I_0 e^{\frac{-\tau_1}{T_1}}}$ instead of $\boldsymbol{I_0' = I_0 e^{\frac{-\tau_1}{T_1}} e^{\frac{-\tau_2}{T_2}}}$, see **Equations 1** and **2**) and yields a better signal/noise ratio although the amplitude of the stimulated echo is half that of the corresponding spin echo. In conclusion, the stimulated echo is the pulse sequence of choice for lipoproteins that have a longer T_1 than T_2 values and slow diffusion.

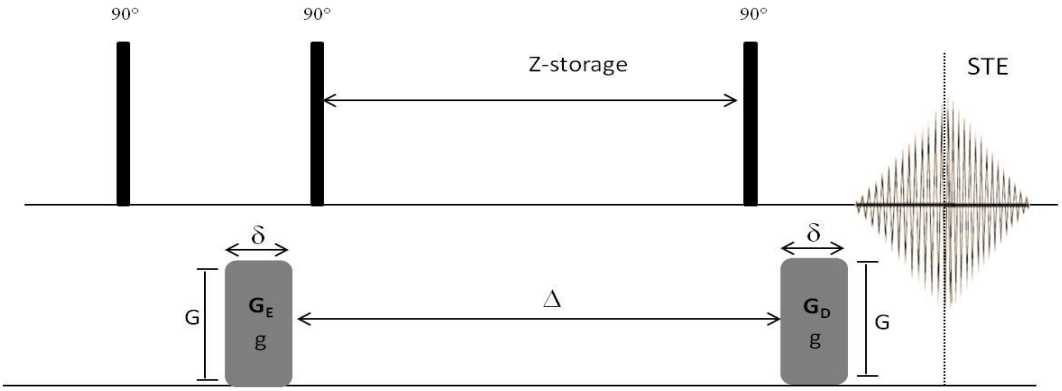

Figure 8. Pulse field gradient stimulated echo with z-storage that avoids T_2 relaxation **[96]**.

2.3.4.2.2. Eddy currents. During the application of a PFG experiment, the magnetic field is switched on and off very quickly, and the changes in the magnetic flux induce current field transients in the gradient coils. These currents are known as eddy currents and can introduce baseline, line-shape, phase and amplitude distortions and loss of coherence in the acquired spectra, especially when large magnetic field gradients are suddenly switched on and off. These distortions invalidate the information obtained from a 1D NMR spectrum (**Figure 9**).

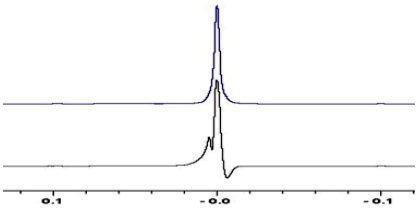

Figure 9. TSP signal of diffusion spectra with PFG affected by eddy currents (lower spectra,

acquired with rectangular pulse, no longitudinal eddy current delay (LED)) and without the effect of eddy currents (upper spectra, acquired with shine-shaped pulse and LED).

The potential error due to eddy currents is larger for unshielded probes. Fortunately, since the early 1990s, probes equipped with actively shielded magnetic field gradients have become widespread **[97]**. There are also three additional experimental strategies, which can be applied simultaneously to overcome eddy current distortion problems:

(a) A shaped gradient pulse, with slower rise and fall times, can be effective at reducing eddy currents **[98]**. Moreover, shaped gradients are more reproducible and less likely to produce vibrations in the sample or probe. In this way, PFGSTE experiments are usually performed using trapezoidal or sine-shaped pulse gradients.

(b) Another approach is to increase the delay time from the end of the gradient pulse to the start of the acquisition so that the spin-echo is recorded in a negligible eddy current field. This strategy is known as the ‘‘longitudinal eddy current delay’’ (LED) pulse sequence **[99]** (**Figure 10a**).

(c) One other possibility is to use preparatory gradient pulses with different polarities. The most common preparatory gradients are two pairs of magnetic field gradients with inverse polarities separated by 180º RF pulses **[100]**. The replacement of the single PFG by a pair of matched gradient pulses with inverted polarities separated by a 180º RF pulse has a self-compensating effect on eddy currents and a cumulative dephasing effect on sample magnetization encoding/decoding. This type of alternating-gradient sequence is called bipolar pulse pairs (BPP). It considerably reduces eddy currents and improves the performance of the diffusion experiment (**Figure 10b**) Thus, a single PFG of say, 2 ms, could be replaced with a BPP of 1 ms each and reduce eddy currents almost completely.

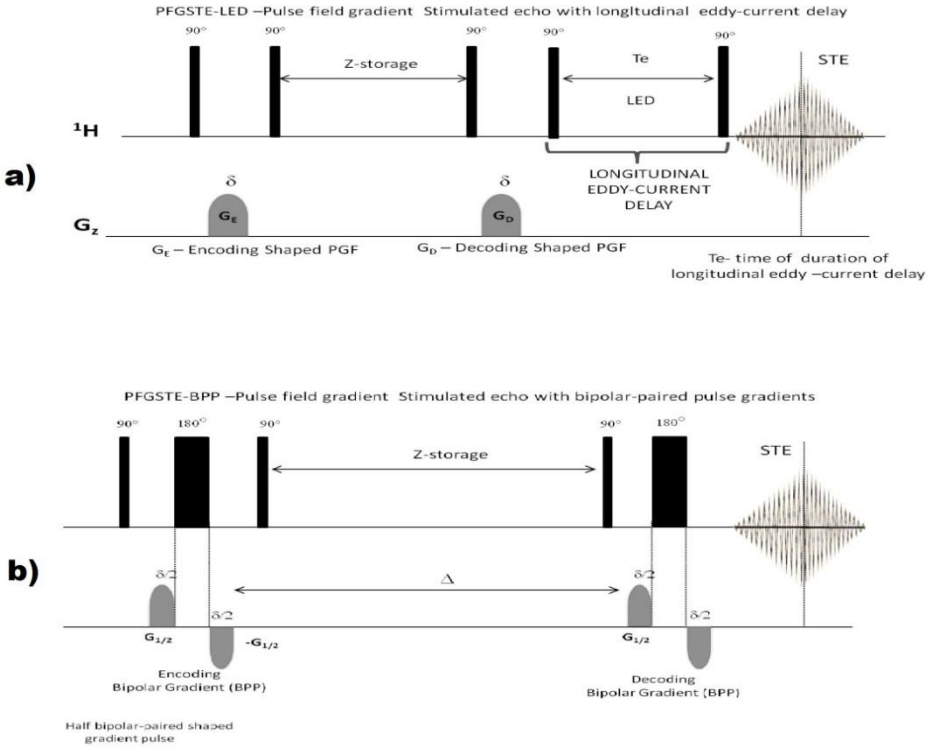

Figure 10. Improvements introduced to minimize eddy currents: a) longitudinal eddy-current delay (LED) and b) bipolar-pair pulses (BPP). All gradients are represented as a shine-shaped PFG.

2.3.4.2.3. 1D ^{1}H NMR diffusion of serum/plasma samples. For quick acquisition and easy handling of the lipoprotein profile, 1D diffusion-edited ^{1}H spectra of lipoproteins can be efficiently measured using a diffusion pulse sequence with bipolar gradients and the longitudinal eddy-current delay (LED) scheme **[100]** with two spoil gradients to destroy unwanted coherences (**Figure 11**). This pulse sequence (see **Figure 4** for the legend) in equipment with a standard Z-axis gradient amplifier unit (with a maximum strength of 50 gauss/cm) it is typically performed with a sine-shaped bipolar pulse of 2–3 ms in total (1–1.5 ms each gradient G_1) at 95% of gradient strength (which is around 33 gauss/cm for a sine shaped gradient). The diffusion delay (Δ), during which the molecules diffuse and their signal attenuates, is set from 100 to 120 ms for optimum NMR signal free of low molecular weight metabolites such as lactate, glucose and alanine. The 90º pulse–G_2–90º pulse block,

during diffusion time and LED delays, stores the magnetization on the Z axis to prevent losses through transverse (T_2) relaxation, which is usually faster than longitudinal relaxation (T_1). No water suppression is needed in serum or plasma because the water signal is strongly attenuated by diffusion. This sequence has a longitudinal eddy-current delay of 5 ms (t_{led}) to allow these currents to decay before acquisition (ACQ). Thus, 1D ^{1}H NMR diffusion-edited acquisition has also been discussed for metabolic profiling **[63]** and lipoprotein quantification **[86]** but it has not been put to more general use in lipoprotein studies.

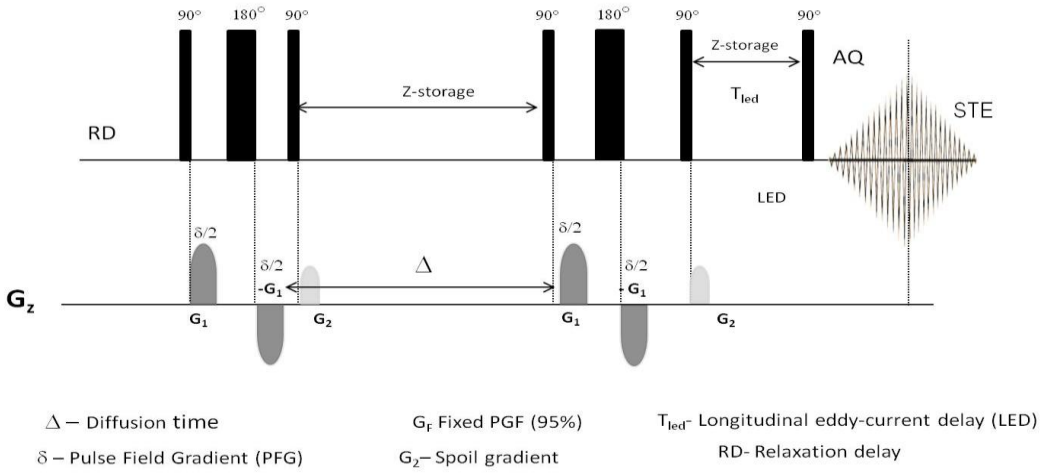

Figure 11. Diffusion pulse sequence with bipolar gradients and the longitudinal eddy-current delay for 1D diffusion measurements on serum/plasma.

2.3.4.3. 2D ^{1}H NMR diffusion measurements

As has been mentioned above, the acquisition of a set of diffusion-edited NMR spectra with an increasing strength in gradient enables the translational diffusion coefficient (D) of a certain molecule to be calculated. This parameter is a direct measure of self-diffusion and is related to its size in a solution. This approach has been reported to enhance information recovery from plasma NMR spectra **[101]**.

2.3.4.3.1. Convection flows. Another problem in gradient experiments is the presence of

convection flows. This source of signal attenuation is easily confused with attenuation from true diffusion and thus distorts the diffusion coefficient (*D*) calculation. The convection is due to temperature gradients in the sample tube and can be partially reduced by improving temperature control. The convection attenuation makes the measured diffusion coefficient increase with diffusion time (*Δ*), and interferes with the exponential decays of intensities in the PFG of the experiment. This unwanted flow increases the distance traveled by the molecule and the attenuation shows a non-linear behavior.

Various experimental strategies have been recommended to avoid convection effects: increasing the flow of the gas heater to reduce temperature gradients **[87]**, reducing sample height or using a smaller sample tube (see, for example, Shigemi **[102]**, inner capillary tubes **[103]**) or spinning the sample to break convection flows **[104]**.

In addition, the regular and ordered nature of convection flows means that a modified pulse sequence can be used to compensate for their effects. This compensation is based on double stimulated echo (DSTE) **[105]** the pulse sequence of which splits the diffusion time (*Δ*) into two gradient encoding–decoding periods that refocus all constant-velocity terms (flow and convection) (**Figure 12**).

Although the shortest and simplest PFG sequences should provide better sensitivity and signal-to-noise ratio, in the case of the lipoprotein diffusion measurement, the heterogeneity of the serum sample can cause non-uniform temperature and magnetic gradients. Thus, the use of a more complex DSTE pulse is recommended at the expense of its lower sensitivity. Also, a longitudinal eddycurrent delay (LED) and alternated bipolar pair pulses (BPP) should be used to avoid the deleterious effects of eddy currents (**Figure 13**).

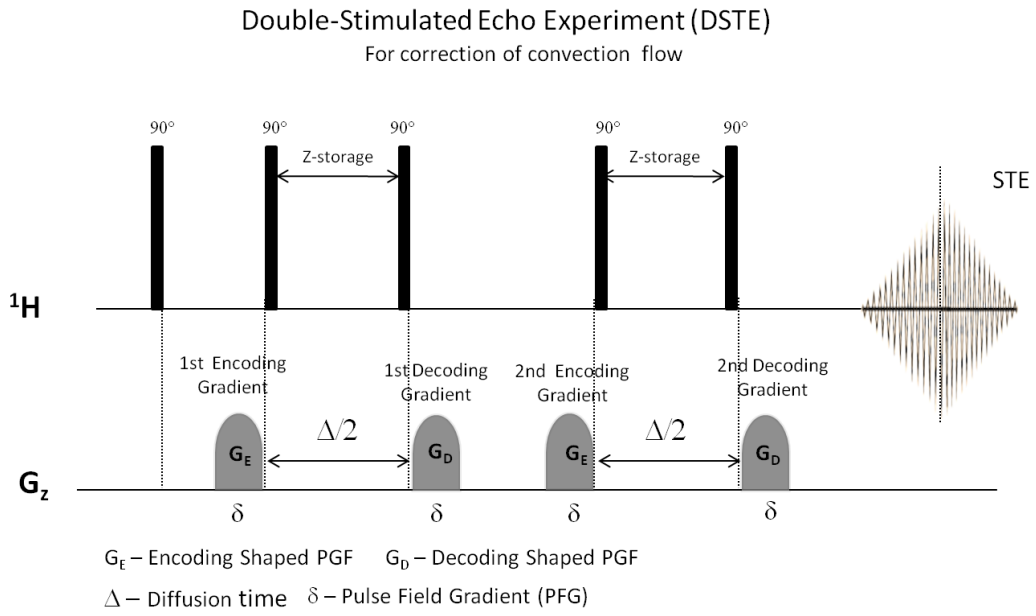

Figure 12. Double Stimulated Echo pulse sequence to compensate for convection in the sample.

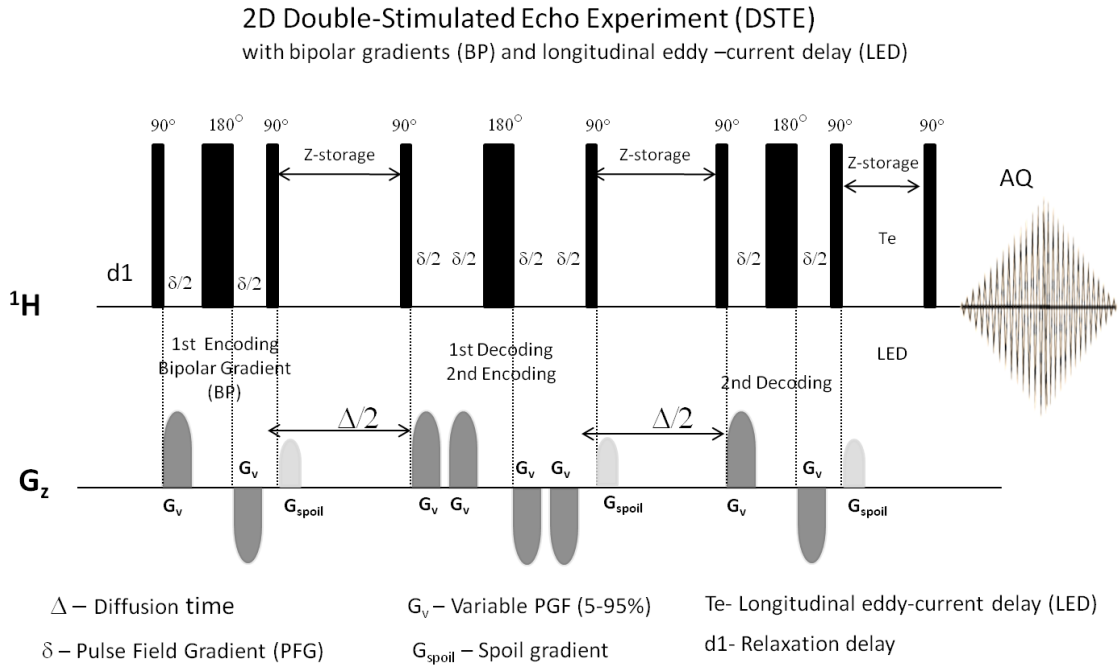

Figure 13. 2D Double-Stimulated echo experiment with bipolar gradients and the longitudinal eddy-current delay for diffusion measurements on serum/plasma.

2.3.4.3.2. 2D ^{1}H NMR diffusion measurements of serum/plasma samples. As a rule of thumb, 2D diffusion-edited ^{1}H NMR measurements can be performed with a coaxial double-tube system that reduces convection. The inner capillary contains a deuterated water solution of 3-(trimethylsilyl) 2,2,3,3-tetradeutero-propionic acid (TSP) that gives lock and reference signal (TSP, δ=0 ppm) without disturbing or interacting with the outer serum. The diffusion spectrum is recorded at 310 K (the physiological temperature) to obtain the full signal from melted lipids in lipoproteins and the heating air flow rate is set to the maximum before vibration occurs in the sample (typically 670–800 l/h). However some effects from convection currents still remain and can be observed if a single stimulated echo pulse is used. **Figure 14** shows that there is a systematic error due to convection currents when we compare the *D* estimated using DSTE or STE from the same samples. Therefore, a double-stimulated echo pulse sequence is recommended to efficiently compensate for this effect.

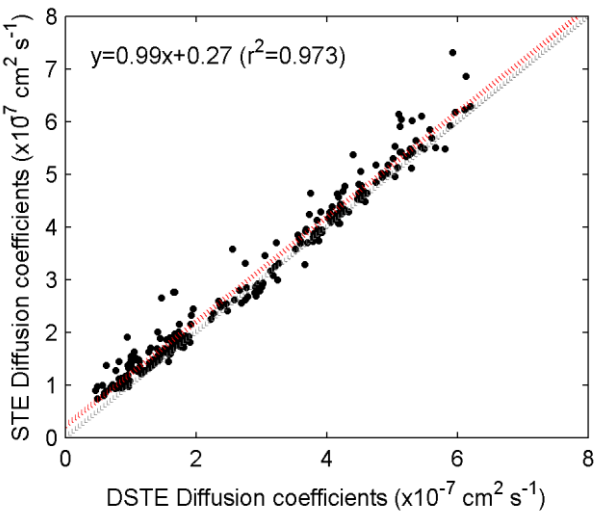

Figure 14. Diffusion coefficient calculated over the same serum sample with single stimulated echo (STE) and double stimulated echo (DSTE) pulse sequences. The grey line represents the ideal situation while the red line expresses the actual regression, which shows that STE pulses are affected by some convection flows. Therefore, it is recommended that DSTE be used for flow compensation.

In the spectrometer setup, a diffusion time (*Δ*) of 100–200 ms and a gradient pulse duration (*δ*) of 8–12 ms have to be applied to attenuate the lipoprotein signals to a reasonable extent with a good signal-to-noise ratio in 32–64 scans for each gradient. With this long gradient pulse it is important to minimize eddy currents. Thus, sine-shaped gradients are used, the length of the gradient pulse is divided into a bipolar pair pulse, each one of which is 4–6 ms, and a LED scheme of 5 ms is injected before acquisition. Additionally, three spoil gradients are used during the z-storage periods of the two stimulated echoes and the LED to shorten the phase cycle and remove unwanted magnetization.

Once the gradient pulse (*δ*) and diffusion time (*Δ*) have been optimized and fixed, the gradient pulse strength can be gradually increased into the linear range of the gradient amplifier to generate spectra in the diffusion dimension (typically, in equipment with a standard Z-axis gradient amplifier unit of about 55 G/cm, linearity ranges from 5% to 95%). Depending on the resolution needed in the diffusion dimension, 20–50 diffusion spectra can be measured in an exponential, squared or linear distribution of gradient values.

The spectra in **Figure 15** were obtained from serum with the pulse sequence described. This type of dataset includes a huge amount of highly overlapped chemical information that configures a hilly and continuous mountain-shaped signal without baseline in the direct dimension F2 and, in the indirect dimension F1, the attenuation profiles of which are used to measure diffusion coefficients and calculate molecular sizes.

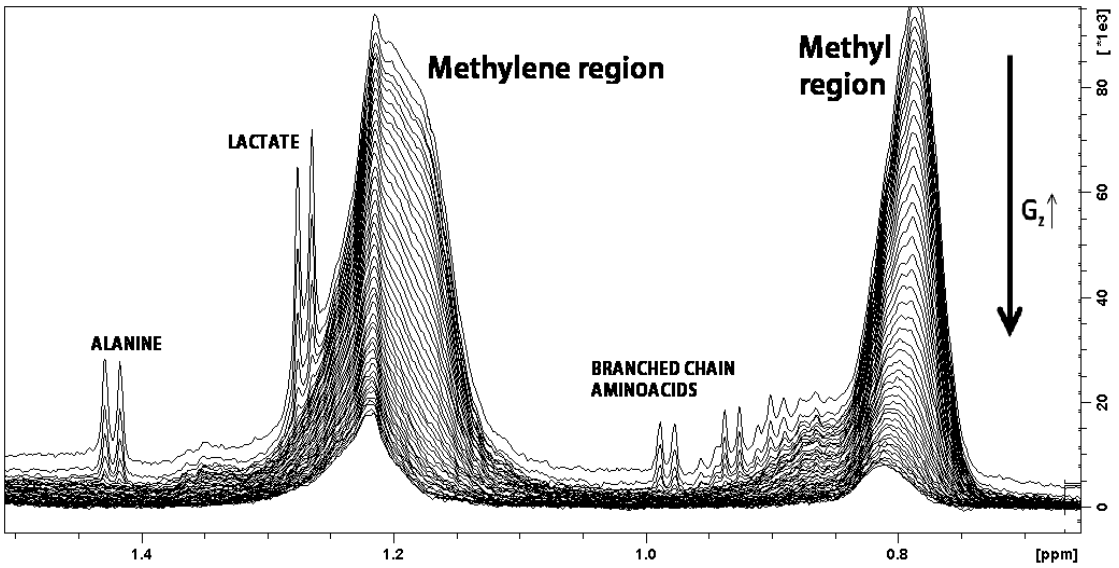

Figure 15. Detail of NMR signal attenuation of plasma lipoproteins in a 2D diffusion experiment.

DOSY experiments require spectra to be of high quality if they are to be processed; even small deviations from expected behaviour in NMR signals can cause significant distortions in the diffusion domain. In addition, depending on the serum or plasma sample, acquisition quality, signal-to-noise ratio and experimental conditions, different processing methods can be used **[106]**. Specific methods for analyzing plasma and serum lipoproteins by DOSY acquisitions have even been developed: for example, mathematical deconvolution and direct calculation of diffusion coefficient and average size (*D*, *r*) **[26,103]** and multivariate methods **[86,107]**. These methods for extracting useful data from the NMR measurement of serum, plasma or lipoprotein fractions and their usefulness for studying diabetes and metabolic diseases are discussed in detail in the sections below.

2.4. Lipoprotein analysis using NMR data

2.4.1. Curve fitting methods for ^{1}H NMR serum/plasma spectra

Since the beginning of the 1990s **[108]**, two groups have taken huge strides in the analysis of NMR lipoprotein profiles. The first group, led by Otvos, created and perfected the LipoProfile® test, which was made commercially available in 1997 by Liposcience Inc. And the second group, led by Ala-Korpela, has been making in-depth studies of the possibilities of ^{1}H NMR in the analysis of lipoproteins and the use of NMR spectra in the assessment of risk factors in population studies. In this section we shall also discuss the findings of Le Moyec, whose group has assessed the effects of some lipid lowering drugs by NMR spectroscopy.

Mathematical curve fitting methods go beyond the scope of this study, but a description can be found elsewhere **[109]**. For purposes of illustration, **Figure 16** shows a methyl peak spectra fitted with nine Lorentzian functions.

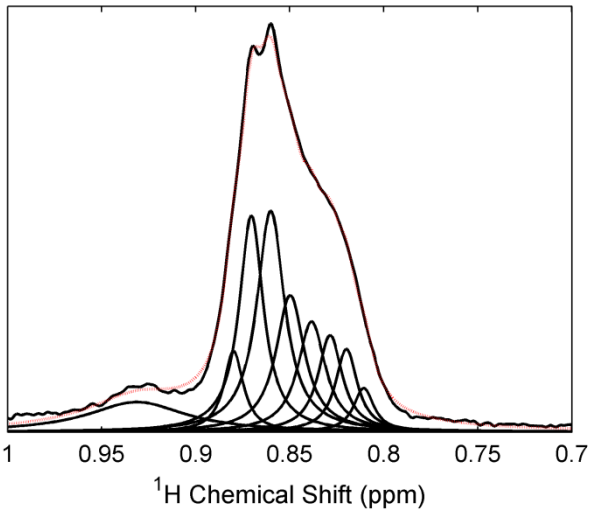

Figure 16. Illustration of a methyl envelope fitted with nine Lorentzian functions.

2.4.1.1. The method of Otvos

The first studies reported by Otvos appeared in the early 1990s **[110,111]**. They described a curve fitting method for quantifying the particle concentration and mean particle size of different lipoprotein subclasses. First, the main lipoprotein fractions (chylomicrons, VLDL, IDL, LDL and HDL) obtained by sequential ultracentrifugation **[112,113]** were used to obtain highly purified lipoprotein subfractions with a range of particle diameters that were as homogeneous as possible. Agarose gel filtration chromatography was used in combination with ultracentrifugation. The lipid content (cholesterol and triglycerides) of lipoprotein subfractions was measured by standard biochemistry methods and the particle size for the purified VLDL and LDL subclasses were assessed by Transmission Electron Microscopy (TEM). Additionally, non-denaturing gradient gel electrophoresis **[114]** was used to measure the particle sizes of the LDL **[115,116]** and HDL **[117–119]** subfractions. Then, ^{1}H NMR spectra were acquired of all plasma and isolated lipoprotein samples on a 400 MHz NMR spectrometer equipped with a flow probe and a sample volume of 120 µl. All NMR measurements for lipoprotein analyses were performed at 47 ºC, a temperature at which the molecules in the core of the particles have an NMR-isotropic behavior.

The basic principle of the method is the specific magnetic property of lipoproteins: i.e. that lipids in smaller lipoproteins broadcast signals at lower frequencies than lipids in larger ones resulting from the different compositions of the lipoproteins **[80,120]**. Particle concentrations are obtained by fitting the methyl envelope arising at around 0.8 ppm of the ^{1}H NMR spectra of plasma samples. The adjusted curve is calculated using a linear combination of ^{1}H NMR spectra from individual lipoprotein subfractions taken from a reference library **[6,120]** in a linear least squares process **[39]**. Once the amplitude of each lipoprotein subfraction reference has been calculated, the particle concentration is determined (in nmol/L for VLDL and LDL, and µmol/L for HDL), using the appropriate

conversion factor, calculated from the lipid concentration of the core lipoprotein fraction and the lipoprotein's radii **[121]**. This spectral library of lipoprotein subfractions, measured by ^{1}H NMR, is representative of the different subfractions that can be found in human plasma samples of patients with either normolipidemia or dyslipidemia. It is worth mentioning that the area of the methyl envelope is proportional to the number of protons (3) for every methyl termination of the core lipids (i.e. 3 for TG and CE). This means that the amplitude is insensitive to compositional variability in subclasses (cholesterol ester–triglyceride exchange mediated by cholesterol ester transport protein, CETP) **[122]**.

The mean particle sizes estimated by LipoProfile® have been compared with those measured by TEM (VLDL and LDL fractions) **[123]** and GGE (LDL and HDL fractions) **[115–119]**. Agreement with GGE was excellent, although some offset differences in mass estimation were found between LDL sizes measured by NMR and TEM.

Nowadays, the LipoProfile® test is the only commercially available method that can quickly and accurately give information about the size and particle number of several lipoprotein fractions in plasma (or serum) samples **[5]**. It is also one of the few methods used in the study of lipoprotein fraction size, with the advantage that a prior separation process is not needed **[49]**. The number of fractions obtained in the LipoProfile test has finally been fixed at 10 (see **Table 2**) **[6,120]**. The NMR LipoProfile® test **[124]** has been available in United States hospitals since 1997 for clinical analysis. Since then, millions of analyses have been performed in the LipoScience laboratory in North Carolina, and more than 250 clinical studies have been completed.

Table 2. Research papers on lipoprotein characterization based on ^{1}H-NMR spectroscopy.

Author [Ref.]	Sample set	NMR Settings	Feature extraction method	Calculated parameters
Otvos et al. **[6]**	Many clinical trials (Comercial name: *LipoProfile*® test)	90º One-pulse presaturation sequence 250 MHz-400MHz Temp.= 47 ºC	Proprietary least squares deconvolution process on methyl peak	Particle Number and size of: $VLDL_{Large}$, $VLDL_{Medium}$, $VLDL_{Small}$, IDL, LDL_{Large}, LDL_{Medium}, LDL_{Small}, HLD_{Large}, HDL_{Medium}, HDL_{Small}
Ala-Korpela et al. **[2,164]**	Many serum sample sets	1D noesy presat 500 MHz Temp.= 37 ºC	Combination of different regression and statistical methods	Concentration of total Lipids, C, PL and TG, Apo A-I and ApoB, particle concentration and size in up to 14 subfractions (CM, 5 VLDL, 1 IDL, 3 LDL and 4 HDL)
Le Moyec et al. **[165]**	60 serum samples	90º One-pulse presaturation sequence 500 MHz T = 40 ºC	Lineshape fitting of methyl peak	PCA+HCA with the area of 11 deconvoluted Lorentzian functions
Bathen et al. **[133]**	52 plasma samples	90º One pulse sequence 600 MHz Temp.= 44 º C	PCA and PLS (for calibration models) from the methyl and methylene peaks	Plasma-TG, Plasma-CH, VLDL-TG, VLDL-C, IDL-TG, IDL-C, LDL-TG, LDL-C, HDL TG, HDL-C, Plasma- ApoA1, HDL-ApoA1, Plasma-ApoB, LDL ApoB
Petersen et al. **[4]**	103 plasma samples	1D longitudinal eddy-current delay (LED) gradient 600 MHz Temp.=30 ºC	PLS regression models	Total-C, Plasma TG, VLDL-C, VLDL-TG, IDL-C, IDL-TG, LDL-C, IDL-TG, LDL1-C, LDL1-TG, LDL2-C, LDL2-TG, LDL3-C, LDL3-TG, HDL-C- HDL-TG, HDL1-C, HDL1-TG, HDL2-C, HDL2-TG, HDL3-C, HDL3-TG
Kristensen et al. **[134]**	68 rat serum samples	Composite 90º pulse sequence with presaturation 400 MHz Temp.=38 ºC	PLS regression models	Total C, VLDL-C, IDL-C, LDL-C, HDL-C
Serrai et al. **[135]**	9 blood samples	90º pulse and presaturation 500MHz Temp.=25 ºC	Wavelet transform from FID's	Lipoprotein visible lipid quantifications and an estimation of non-NMR visible protein and free cholesterol in VLDL, IDL+LDL and HDL
Savorani et al. **[136]**	18 serum samples	Use of*zgcppr* Bruker pulse sequence NMR. Temp. = 37°C on a 500 MHz spectrometer	PLS regression in methylene peak	TG content of CM

2.4.1.2. The method of Ala-Korpela

During the first half of the 1990s, Ala-Korpela set up a lineshape fitting model for deconvoluting the overlapping information in the methyl and methylene peaks of ^{1}H NMR spectra of plasma samples **[108,125,126]**, measured at the physiological temperature of 37 ºC. In these studies, VLDL, LDL and HDL fractions were modeled by adding three Lorentzian components for VLDL and LDL fractions and one for HDL. The algorithm used for lineshape fitting analysis was FITPLAC **[108,125,126]**, developed by Ala-Korpela himself, and the parameters that are mathematically optimized for every Lorentzian are half-line width, resonance frequency and intensity. The model also includes background resonances related to residual water, and albumin-bound immobile fatty acids or proteins (see Ref. **[125]** for more details). The areas of the functions are scaled using the area of an external reference signal **[127]** in a concentric coaxial tube. The highly overlapping nature of the fraction spectra means that, depending on the initial parameters chosen for lineshape fitting, numerous solutions are mathematically possible. To overcome this problem, FITPLAC was run many times on every spectrum, with the initial parameters being randomly chosen between reasonable (wide) limits. The solution selected was the one that gave the lowest RMS error and was physically meaningful.

One of his reports **[125]** described the use of plasma from a set of 15 volunteers with highly different lipid profiles and 51 plasma samples from 43 individuals (double-blind test). The fractions of the 15 volunteers and 5 of the 43 individuals in the double-blind test were sequentially ultracentrifuged to obtain their VLDL, IDL, LDL and HDL fractions. The lineshape fitting analysis of the methyl peak using the FITPLAC algorithm confirmed that the parameters of the Lorentzians for every fraction were very similar for all the individuals, so the half-linewidth and position of every Lorentzian was set at a constant value. This indicated that the main variable in the ^{1}H NMR spectra of the lipoprotein fractions of

different people is the total amount (i.e. the fraction concentration). All these restrictions also improved the mathematical uniqueness of the solutions. The linear correlation between the fraction areas found by NMR and the biochemistry of the VLDL, LDL and HDL fractions made it possible to estimate the concentration of triglycerides, phospholipids, total cholesterol, free cholesterol, esterified cholesterol, total proteins, and total masses. In particular, correlation coefficients were found to be high (e.g. 0.98, 0.88, and 0.93) in the VLDL triglycerides, LDL cholesterol, and HDL cholesterol, respectively, of plasma samples. On the other hand, it was not possible to include the IDL fraction in the lineshape fitting model, since it required new free adjusting parameters to be introduced in the model. Moreover its contribution to the spectra was determined to be much lower than the other fractions.

Several years later, Ala-Korpela demonstrated that the inherent accuracy of lipoprotein subclass quantification by NMR is subclass dependent **[128]**. In particular, the classes with maximum response at the higher or lower limits of the methyl peak (VLDL and HDL3, respectively) have an error that is ten times lower than LDL1, the least accurate. Errors were 0.5% and 5%, respectively. This is consistent with the increasing overlap of the spectra in the center of the methyl peak.

2.4.1.3. The results of Le Moyec

Le Moyec et al. **[129]** analyzed the methyl region (between 0.64 and 1.08 ppm) of ^{1}H NMR serum, using 11 Lorentzian functions, 6 of which represented lipids. The approach they used allowed them to assess the cholesterol reduction in hypercholesterolemic patients (n = 60) who were taking simvastatin (n=30) or atorvastatin (n=30). The integral areas of the 6 Lorentzian functions were used as input variables in a Principal Component Analysis (PCA) coupled with a Hierarchical Cluster Analysis (HCA) algorithm. The results

revealed that the features used from the Lorentzians could be used to cluster the patients before and after each treatment, although some overlapping regions were evident. They concluded that there is a decrease in LDL cholesterol and an increase in HDL cholesterol, and they assigned some function(s) to each fraction, although they could not find any rigorous relationship between the six functions and the lipoprotein fractions. In their report they do not give details about the lineshape fitting method nor the mathematical uniqueness of the solutions.

2.4.2. Statistical correlation methods for ^{1}H NMR spectra plasma/serum measurements

In **Section 2.4.2.1** we shall review all the lipoprotein characterization methods that use the ^{1}H NMR intact serum spectra envelop of the lipids peaks (i.e. with no previous lineshape peak deconvolution) for statistical correlation with the biochemical data obtained by conventional biochemistry in the lipoprotein subfractions isolated from the serum of the same individuals. In **Section 2.4.2.2** we shall discuss the clustering of the samples according to their health status.

2.4.2.1. Predictive methods

In 1995 **[130,131]**, Ala-Korpela's group published the results of a study of 57 subjects with a wide range of plasma lipoprotein lipid values, including abnormalities such as hyperlipidemia, hypercholesterolemia and hypertriglyceridemia. VLDL triglycerides, as well as LDL, IDL and HDL cholesterol concentrations were available from these subjects and Artificial Neural Networks (ANN) were used to quantify lipid concentrations from the NMR spectra.

A total of 50% of the subjects were used for training, and the rest for validation. The methyl and methylene portions of the spectra (as well as the TSP region considered for quantification) were used as the input signals for a neuronal network with 50 hidden neurons and 4 neurons in the output layer. These four neurons corresponded to the four biochemistry values to be quantified: VLDLTG, IDL-C, LDL-C and HDL-C. The correlations with the validation group for each lipid value were 0.99, 0.74, 0.87 and 0.84, respectively. In the same study a network designed for quantifying phospholipids and their performance was also reported.

Vehtari et al. have also demonstrated the usefulness of Bayesian methods in the quantification of serum lipids **[85]**. They used the Markov Chain Monte Carlo (MCMC) method in a Bayesian inference algorithm to build quantitative models for lipid subclasses based on their ^{1}H NMR spectra. The model, was built and trained with a set of 75 ^{1}H NMR spectra of serum samples representing broad lipoprotein profiles and biochemistry values of the main fractions (VLDL-TG, IDL-TG, IDL-C, LDL-C and HDL-C), obtained by ultracentrifugation. The correlations (R-values) were very good for VLDL-TG (0.985), acceptable for IDL-C, LDL-C and HDL-C (0.787, 0.943 and 0.933 respectively), but only poor for IDL-TG.

In 2009, Ala-Korpela's group set up a high throughput method for serum analysis **[2,22,23]**, which included the analysis of lipoproteins. The method can quantify the total concentration of TG and C in serum, as well as VLDL-TG, IDL-C, LDL-C and HDL-C. They also reported that they were able to quantify total lipid content, and particle concentrations and size in 14 lipoprotein subclasses, distributed in chylomicrons, 5 VLDL, 1 IDL, 3 LDL and 4 HDL. The lipoprotein size of these subclasses was calibrated with High Performance Liquid Chromatography **[54]**, although no details about the calibration process were given (**Table 2**). The concentrations of Apo A-I and ApoB are also available and have been calculated

from the Friedewald parameters **[132]**.

This method has been applied successfully in genome-wide association studies – showing a high correlation between the variant rs7575840 and the ApoB-containing lipoproteins **[24]** – and in epidemiological experiments, showing associations between metabolic phenotypes and subclinical atherosclerosis **[23]**.

Bathen et al. **[133]** used PCA, partial least squares regression (PLS) predictors and ANN to quantify the TG and C of the main fractions, and the Apo A and Apo B apolipoproteins. In a study of 52 individuals (controls and subjects) with cancer and other diseases, the lipid and apolipoprotein values predicted when PLS models were constructed with the methyl and methylene peaks of a ^{1}H NMR spectra correlated very well with the data obtained by standard clinical bioanalysis of the main fractions obtained by ultra-centrifugation; the correlation coefficient for total plasma triglyceride was 0.99, for total plasma cholesterol 0.98, for LDL cholesterol 0.97, and for HDL cholesterol 0.88. Of the 52 subjects, 44 were used for model calibration and the rest for validation. ANN-based strategies, involving either general regression or polynomial neural networks in combination with genetic adaptive components for parameter optimization, have been very effective when used for lipid and apolipoprotein quantification.

Petersen et al. **[4]** used a similar approach to Bathen et al. **[133]** and applied a PLS regression model to diffusion-edited NMR spectra in a sample set of 103 individuals. The model was built by applying one-dimensional longitudinal eddy current bipolar pulses and biochemistry data obtained from 10 lipoprotein fractions (VLDL, IDL, total LDL, LDL-1, LDL-2, LDL-3, total HDL, HDL-1, HDL-2, HDL3). They used the spectrum region between 5.7 and 0.2 ppm (excluding some small areas to prevent water and the regions containing EDTA peaks from interfering). Full cross-validated models were built for every fraction of TG, C, total serum C and TG. They demonstrated that PLS models can be used to quantify lipids in

lipoproteins, although the resolution when multiple subclasses need to be quantified is rather poor, especially if the concentration is low.

In a recent study **[134]** with a sample set of 68 rat serums, PLS models were constructed for VLDL, IDL, LDL and HDL-C by using the information from the ^{1}H NMR spectral region between 1.36 and 0.57 ppm and the cholesterol value measured after ultracentrifugation. It is worth noting that this is the first study to have been made of rat serum characterization. Unlike for human samples, there are no commercial kits for animal serum lipid analysis, making NMR serum characterization even more interesting. The correlation coefficients ranged from 0.95 (HDL C) to 0.48 (IDL-C).

Unlike preceding studies, Serrai et al. **[135]** used the FID signal instead of its Fourier Transform and applied a Wavelet Transform in the time domain to quantify the lipids and proteins in the main fractions (i.e. total and free C, TG, PL and proteins). In an experiment, with only nine serum samples, they correlated the biochemistry values of the main fractions with the Wavelet Transform coefficients extracted from the FID. The correlation coefficients obtained were 0.79, 0.84 and 0.92 (for LDL + IDL, VLDL and HDL, respectively) for visible lipids and 0.81, 0.87 and 0.88 (for VLDL, IDL + LDL and HDL, respectively) for the non-visible lipids (proteins and free cholesterol) (**Table 2**).

Finally, using data from the methylene peak spectra in human plasma coupled with multivariate calibration analysis, Savorani et al. **[136]** were able to predict the TG content of chylomicrons. The method is rapid and robust, creating new opportunities for research in lifestyle diseases and obesity.

2.4.2.2. Clustering methods

For the first time in 1998 Ala-Korpela et al. **[137]** used Self Organizing Map (SOM)

networks **[138,139]** to represent the data derived from the methyl peak of plasma samples in 2D plots. The samples were placed on the surface according to their similarity, with an unsupervised approach and or definition of classes. The model was first trained with all the 65 samples and then with a training set of 50 samples and a validation set of 15. In both cases the model successfully represented the different lipoprotein profiles: normal and Types IIa, IIb and IV hyperlipidemias. The map was also calibrated for different risk factors (i.e. TG, TC, HDL-C and LDL-C) and in all cases the samples were coherently positioned on the map. The SOM approach could be used to visualize the overall CHD risk, not related to the known individual risk factors. These results have been confirmed in a study by the same research group on 69 individuals, with considerable differences in lipid profiles **[140]**. A SOM representation of the choline and methyl ^{1}H NMR regions showed a sample clusterization with meaningful clinical information related to CHD risk. In the same article, they showed the SOM analysis of simulated spectra, constructed by adding 11 subfractions obtained by ultracentrifugation from one individual. They defined normal spectra (N), metabolic syndrome spectra **[141]** and metabolic pathway spectra (MP), and built families of simulated spectra by adding different proportions of each subclass. The maps revealed a coherent arrangement of the simulated samples, according to the degree of metabolic syndrome. An interesting conclusion is that the choline region (around 3.2 ppm) is the most informative part of the aliphatic region (0.5–3.5 ppm). These studies by Ala-Korpela, together with **[129]**, showed that the ^{1}H NMR information that gives the lipoprotein peaks per se (i.e. without using subfraction lipid information) is clinically relevant.

Using the same samples set as in **[133]**, Bathen et al. **[142]** applied Kohonen Neural Networks (KNN) and generative topographic mapping (GTM), two unsupervised algorithms, which displayed a very heterogeneous group of samples according to their lipid values.

2.4.3. Lipoprotein subclass surface fitting in PGSE spectroscopic ^{1}H NMR signals

In 1993, Boerner **[143]** used pulse field gradient NMR to implement a diffusion ordered spectroscopy. He obtained a 2D spectrum consisting of a ^{1}H NMR spectrum in the first dimension and the diffusion coefficient for the different molecules in the second dimension. For this purpose, he programmed a new algorithm called CUMULANT with which he obtained accurate mean diffusion coefficients; however, he could not resolve the lipoprotein peaks.

In 1996, Liu et al. **[74]** developed a new approach for characterizing biomolecules that took into account both diffusion and relaxation parameters. They demonstrated that the use of field gradient pulses could produce ^{1}H–^{1}H diffusion-edited TOCSY spectra of human blood plasma in which the response of low- and medium-weight components was filtered and only the response of macromolecules, characterized by a slow diffusion coefficient, remained. They proved that diffusion-edited spectra together with the ^{1}H NMR relaxation times could increase the discrimination capabilities among samples in a metabolomic experiment with biofluids. The same group used two-dimensional diffusion-edited total-correlation NMR spectroscopy (DETOCSY **[144]**) to estimate the diffusion coefficient of all the molecules in complex biofluids (i.e. serum), by measuring the slope of the metabolite area attenuation induced by the diffusion field strength. Nevertheless, they were unable to resolve the overlapped lipid peaks and separate the response of the different lipoprotein fractions. They argued that the interpretation of the derived diffusion coefficients could reveal changes in the aggregation or bonding circumstances of the molecules. They suggested that it is intrinsically not possible to resolve the lipid fraction peaks using higher field NMR spectrometers, because lipoprotein size is a continuum function in blood. In an experiment with serum rat samples **[145]** they demonstrated that control animals were better classified from those treated with a hepatotoxin model using

standard one-dimensional 1H NMR together with diffusion-edited spectra. The reason is that a better appreciation of lipid profiles is possible using diffusion-edited spectra. The same group developed the so-called diffusion-ordered NMR spectroscopy with statistical total correlation spectroscopy (STOCSY) **[146]**. This development displays the covariance intensity of the various nuclei in the same molecule across a series of spectra, including the diffusion coefficient calculated across the different gradient intensities. They named this technique statistical diffusion-ordered spectroscopy (S-DOSY).

Numares, a German company **[147]**, has described a procedure for determining the concentration and size of lipoprotein subclass particles, and a patent application submitted for the invention **[148]**. The serum/plasma samples are measured at different temperatures (typically 278 K, 298 K, 308 K and 318 K). As the temperature rises, the relaxation times T_1 and T_2 increase and the lineshape narrows. These measuring conditions, combined with three diffusionweighted ^{1}H NMR measurements carried out with a modified STE–LED (stimulated echo and longitudinal eddy-current delay) pulse sequence, produce a range of different spectra, which depend on the attenuation of the lipoprotein fractions under each of the measuring conditions. Previously, the different lipoprotein classes were characterized individually and their characteristic parameters (chemical shift, shape of NMR signal intensities, signal width and line form) extracted for each temperature and gradient strength. Then, the simulated NMR spectrum is calculated and adjusted to the experimental spectrum by means of multidimensional optimization processes. The lipoprotein size limits of the different fractions are calculated once the particle density is known through their lipid contents. This method makes it possible to determine 15 different fractions (**Table 3**) – including CM, VLDL, IDL, LDL, HDL fractions – and Lp(a) proteins. The determination of the latter is clinically very relevant because of the high health risk associated with this protein **[45]**. The method has been used to demonstrate that macrophage cholesterol efflux is inversely correlated with lipoprotein particle size and

risk of CVD **[149]**. One study **[114]** investigated the heritability of the entire LDL and HDL subfraction profile using NMR spectrometry and found significant evidence of linkage for HDL size on chromosome 12 and for HDL particle concentration on chromosome 18.

Dyrby et al. **[107]** applied multi-way calibration by N-PLS and multi-way curve resolution using PARAFAC to 2D diffusion-edited ^{1}H NMR spectra of human blood samples. The application of PARAFAC with four components extracted from the methylene peak revealed that these components correspond well with the four main lipoprotein groups (VLDL, IDL, LDL and HDL) because the diffusion coefficients (and hence their derived radii) that represent the extracted coefficients correspond quite well with the size characteristics of each fraction. However, the correlation between them and the concentration of the fractions is poor, probably because of the limited number of samples (n = 17). N-PLS calibration led to better lipoprotein lipid correlations for the four main fractions, and the 11 subfractions considered (the correlation coefficient r had values between 0.75 and 0.98) (**Table 3**).

Liu et al. **[26]** mathematically deconvoluted the methyl and methylene peaks in the diffusion-edited ^{1}H NMR spectra of one plasma sample into six Lorentzian functions, each one of which was characterized by amplitude, position, width and a diffusion coefficient. They demonstrated that the hydrodynamic radii estimated from the diffusion coefficients for every function has a good correlation with the radii of the conventional subfractions. The measurements were made at 25 ºC, a temperature at which some lipid fractions have reduced visibility in the NMR spectra. The experiment had no clinical implications, since only one plasma spectrum was processed.

Recently, Mallol et al. **[103]** used a mathematical deconvolution of the 2D diffusion-edited spectra of plasma samples into 8 Lorentzian functions to demonstrate that the extracted areas and diffusion coefficients in a set of 26 plasma samples separate the

different lipoprotein profiles (normal, high triglycerides, low HDL/LDL ratio, and both risk factors) in a PCA score plot much better than the original spectra. They also showed that the relationship between the shift position of the Lorentzian functions and the lipoprotein radii calculated from the experimental diffusion coefficient agrees with results found by other authors **[80,120]**, particularly in normolipidemic samples.

Table 3. Research methods on lipoprotein characterization based on diffusion-edited NMR spectroscopy.

Author [Ref.]	Sample set	NMR settings	Feature extraction method	Calculated parameters
Smith et al. **[101]**	Serum samples	Bipolar pulse-pair and LED (BPP-LED) pulse sequence. 400, 600 and 800 MHz.	Extraction of diffusion coefficient at each ppm spectra	Statistical Diffusion-Ordered Spectroscopy (S-DOSY) and Diffusion-Ordered Projection (DOPY) Spectroscopy
Liu et al. **[26,166]**	1 plasma sample	BPP-LED pulse sequence with water presaturation. 600 and 800 MHz Temp.=25 ºC	Lineshape fitting with six Lorentzian functions at methyl and methylene peaks through the attenuated spectra	Area, diffusion coefficient and hydrodynamic radii of every Lorentzian function.
Dyrby et al. **[167]**	17 plasma samples	Double stimulated echo (DSTE) experiment with BPP-LED 500MHz Temp. = 45 ºC	Multi-way calibration by N-PLS and multi-way curve resolution by PARAFAC at methyl and methylene peaks	- N-PLS Model: VLDL-TG, VLDL1-TG, VLDL2-TG, IDL-TG, and LDL-C, LDL1-C, LDL2-5-C, LDL6-C, HDL-C, HDL1-C, HDL2-C, HDL3-C - PARAFAC Model: VLDL-TG, IDL-TG, LDL-CH and HDL-C and the diffusion coefficient (and radii) of the main fractions
Kremer et al. **[25]**		Stimulated-Echo with LED. Temp.=5 ºC, 25 ºC, 35 ºC, 45 ºC	Lineshape fitting with Lorentzian and Gaussians functions of methyl and methylene spectra acquired at different temperatures and diffusion gradients	Concentration and size of : CM-C, CM-B, CM-A, CM-Remnants, VLDL-C, VLDL-B, VLDL-A, IDL, LDL-C , LDL-B, LDL-A, LDL (small Dense) Lp(a), HDL2, HDL3
Mallol et al. **[103]**	26 plasma samples	DSTE-BPP-LED 600 MHz Temp.=37 ºC	Lineshape fitting with eight Lorentzian functions at methyl peak through the attenuated spectra family	Area, position, width diffusion coefficient of eight Lorentzian functions. Calculation of associated hydrodynamic radii by the Einsten-Stokes equation

2.5. NMR lipoprotein analysis in the study of diabetes and related diseases

The aim of this section is to summarize some of the main applications of NMR spectroscopy in the study of lipoproteins in diabetes and related metabolic diseases. Some articles in the literature discuss some of these studies **[6,19,150]**. Our aim here is to discuss the results of various studies that have tried to characterize diabetes using either the LipoProfile® Test or the platform developed by Ala-Korpela for serum analysis. This is expected to serve as a guide for future studies so that the outcomes can be compared and contrasted.

The main application of ^{1}H NMR spectroscopy in the study of the metabolism of diabetes is the quantification of lipoprotein subclasses in T1DM and T2DM patients using the LipoProfile® test. This method has made it possible to relate different lipoprotein parameters and diabetes, and also other risk factors, in a univariate fashion. A more recent application led by Ala-Korpela has focused on the classification and characterization of diabetic states in order to highlight patho-physiological mechanisms and come to a holistic understanding of the disease.

In the first subsection, we shall discuss the results from the studies based on the LipoProfile® test and, in the second subsection, we shall list some of the applications of clustering methods for discovering the main characteristics of Diabetes Melitus.

2.5.1. Quantification of lipoprotein subclasses based on the LipoProfile® test

LipoScience Inc. has carried out numerous studies on the quantification of lipoprotein subclasses with diabetic patients. These studies deal with a variety of issues

such as case-control comparisons, treatment effects, or associations between lipoprotein subclasses, diabetes and individual risk factors (**Table 4**). In casecontrol studies, the common conclusion was that VLDL size increased and LDL size decreased in diabetic patients. Most of the studies agree that HDL size appears to decrease in diabetic patients, except in the study by Colhoun et al. **[151]**, who found an increase in size in T1DM. On the other hand, there is general agreement that there is an increase in the values of total, large and small VLDLs, and total, medium and small LDLs. There is also general agreement that the values of large LDL and total HDL are lower in diabetic patients.

Although these were case-control studies, it should be pointed out that they differed not only in the type of case and controls but also in the type of diabetes. For instance, Colhoun et al. compared the NMR-derived lipoprotein concentrations between control and T1DM subjects and between men and women **[151]**. They also studied the relationship between particle sizes and coronary calcification. However, no relationship was found between this measure of atherosclerosis and particle size in diabetic subjects.

Another study on the effects of T2DM on the size and concentration of lipoprotein subclass particles determined by NMR **[152]** showed that the decrease in LDL size is the result of an increase in small LDL and a reduction in large LDL concentration, which together lead to a minimal difference in LDL cholesterol. The changes in the NMR lipoprotein subclass profile predictably increase the risk of cardiovascular disease and these were not fully apparent in the conventional lipid panel (see **Section 2.2.3.1**).

Table 4. Research methods on lipoprotein characterization in diabetic patients using ^{1}H NMR spectroscopy

							Particle Concentration											
				Size (nm)			VLDL (nmol/L)				LDL (nmol/L)				HDL (umol/L)			
Author (Year) [Ref]	**Type of Diabetes**	**Experiment groups**	**Genre**	**VLDL**	**LDL**	**HDL**	**Total**	**Large**	**Medium**	**Small**	**Total**	**Large**	**Medium**	**Small**	**Total**	**Large**	**Medium**	**Small**
Colhoun H. (2002) [158]	T1DM	T1DM Vs Control	Men			↑	↓		↓							↑		↓
			Women	↑	↓	↑			↓			↓		↑		↑		↓
Garvey W. (2003) [159]	T2DM	T2DM Vs IS (Control)	Both	↑	↓	↓	↑	↑			↑	↓	↑	↑				↑
Soedamah-Muthu S.* (2003) [9]	T1DM	T1DM CAD Vs T1DM no-CAD	Both		↓	↓	↑	↑	↑	↑	↑		↑	↑	↓	↓	↑	
Festa A. (2005) [160]	T2DM	Prediabetic Vs Nondiabetic	Both	↑**	↓	↓	↑	↑			↑			↑		↓		↑**
Hodge A. (2009) [161]	T2DM	Diabetic vs Nondiabetic	Both	↑	↓	↓***	↑***	↑	↑	↑	↑	↓	↑	↑		↓		↑
Mora S. (2010) [162]	T2DM	Diabetic vs Nondiabetic	Women	↑	↓	↓	↑	↑		↑	↑	↓	↑	↑	↓	↓		↑
Soedamah-Muthu S. (2003) [173]	T2DM	Atorvastatin Vs Placebo	Both			↑			↓	↓		↓		↓		↑		

An improvement in CAD prediction in T1DM patients has also been reported **[9]**. The particle concentrations of VLDL subclasses, small LDL, medium LDL, and medium HDL were found to be higher in CAD cases than in controls, while the concentration of large HDL particles was lower. Mean LDL and HDL particle sizes were lower in T1DM individuals. VLDL particle concentration was an independent predictor of CAD. While total LDL cholesterol was not related to CAD, LDL particle concentrations measured by NMR were strongly associated with CAD. This result is because small and medium LDL subclasses are the LDL fractions associated with CAD. Since small LDL particles contain considerably less cholesterol than larger LDL, they make a smaller contribution to total LDL cholesterol mass than to the total LDL particle number. However, LDL particle concentrations were not able to predict CAD independently, probably because these measures were highly correlated to other lipids and lipoproteins. The final conclusion was that the use of individual lipoprotein subclass levels might be more useful than overall mean particle sizes, as the latter did not improve the prediction of CAD.

Another study dealt with the characterization of a proatherogenic state in healthy, non-diabetic subjects who subsequently develop diabetes **[153]**, and in whom increased small HDL particle concentration and VLDL particle size at baseline was related to incident diabetes at a mean follow-up of 5.2 years. Hodge et al. predicted T2DM patients **[154]** because values of NMR lipoprotein subclasses differed between subjects who developed diabetes and those who did not. Finally, Mora et al. characterized the proliferation of T2DM in women **[155]**. In their report, NMR lipoprotein parameters remained significantly different between controls and T2DM patients after adjustment for established risk factors.

Other studies have been made on the effects of atorvastatin on NMR-derived lipoprotein fraction concentrations **[156]**, the relationships between apolipoprotein C-III concentrations and lipoprotein subclasses **[157]**, the interrelations between poor glycemia

and lipoprotein metabolism **[158]**, the association between retinopathy and lipoprotein subclasses in T1DM **[159,160]** and T2DM **[160]** diabetic patients, and the interrelations between diabetic nephropathy and lipoprotein metabolism **[159]**. It should be pointed out that the new associations found between retinopathy and lipoprotein subclasses were previously unknown and could not be detected using conventional lipid profiles.

2.5.2. Characterization of metabolic features of diabetic patients using NMR and SOM

Makinen et al. used ^{1}H NMR spectroscopy for the clinical diagnosis of diabetic nephropathy in T1DM patients **[161]**. They used two molecular windows: LIPO and LMWM. After a classification step, NMR was well suited to distinguish among the metabolic characteristics of T1DM. Finally, they showed the quantitative nature of ^{1}H NMR for the determination of lipoprotein lipids, apolipoprotein components and low-molecular-weight metabolites.

Subsequently, the same workers characterized the metabolic features of Diabetic Kidney Disease (DKD) and the interrelations between DKD, the metabolic syndrome and other complications in T1DM patients **[10]**. They used classical biochemistry measures and found high triglyceride levels and low HDL levels. Recently, Valcárcel et al. used differential networks to characterize differences of lipoprotein metabolism-related diabetic dyslipidemia in prediabetic patients **[162]**. These differences were not shown by classical biochemical parameters. Finally, the addition of a third molecular window, the LIPID window, to the metabonomics platform led by Ala-Korpela enabled sphingomyelin and other lipidand lipoprotein-related parameters to be associated with kidney disease in patients with T1DM **[163]**.

2.6. Final conclusions

Each NMR spectrum from a serum/plasma sample contains a wealth of information about lipoproteins. NMR spectroscopy is the only technique that allows a full lipoprotein subfraction analysis of serum/plasma samples. On the basis of the various analysis methods reported in **Section 2.4**, we conclude that the data they provide enable the lipid concentration (mainly for cholesterol and triglycerides), and particle numbers and sizes for several subfractions (typically between 9 and 14) to be correctly measured.

However, there is a lack of standardization among the methods presented. One fundamental reason for this is that the discretization of the continuous lipoprotein sizes in blood is method dependent, which makes decomposing them into subfractions a rather arbitrary task. The traditional biochemical methods for fraction isolation (reviewed in **Section 2.2.3.2**) suggest that the number of fractions is method dependent, which makes direct comparison between them impossible. The calibration and validation procedures used to set up new NMR methods rely on the correlations made between the NMR spectra and the lipids (generally cholesterol and triglycerides) measured in a particular set of subfractions, all isolated by ultracentrifugation. In many experiments, there was a limited availability of a high number of serum/plasma samples representing a wide spectrum of dyslipidemias, which makes it very difficult to generalize the method to all sample sets.

The methods reviewed in **Section 2.4.2** use statistical correlations for the quantification of cholesterol and triglycerides in several subfractions. In general, correlations are very good for the fractions with signals at the ends of the peak (mainly VLDL and HDL) and good for the fractions with signals in the middle of the peaks (i.e. IDL and LDL). The calculation of particle number is based on the decomposition of the methyl peak. Provided that the number of methyl terminations is the same in C and TG (three in both cases), the methyl signal amplitude remains constant, even if the cholesterol ester and

triglyceride composition of the lipoprotein cores varies. As has been mentioned in **Section 2.2**, LDL-P is a new cardiovascular risk factor that could be accepted by the FDA administration as a clinical test. This parameter is calculated by the lineshape fitting of the methyl peak to obtain the area of the individual subfractions. Peak overlapping is intense, which makes it mathematically very difficult to find the individual subfractions, and the system is prone to finding multiple solutions, especially when the number of subfractions to be obtained is high. The details of the decomposition algorithms used by the groups have not been revealed **[6]**, which makes it very difficult to evaluate the reported methodology and for other researchers to reproduce the approach.

The size of the lipoprotein subfractions is only given by the methods proposed by the groups of Otvos and Ala-Korpela. In the first case, the size of the subfractions are those of the proprietary library subfractions used in the decomposition process and in the second case, the given sizes have been calibrated using those obtained by a size-exclusion chromatography technique. In neither case were they measured directly. Diffusion NMR spectroscopy can provide a direct measure of lipoprotein radii by using the diffusion coefficient and applying the Einstein–Stokes equation. Nevertheless, the determination of the serum/plasma viscosity parameter in the formula is not straightforward. In this kind of experiment, the existence of a third dimension (diffusion coefficients) makes the fitting process more reliable, because the lipid peaks are substituted by a surface. However, at the present time, this approach still requires further development if it is to give reliable and meaningful results for clinical applications.

Although most of the methods reported aim to predict and quantify the lipid concentration, and the number or size of particles in a set of lipoprotein subfractions, some studies use the features extracted from the lipid peaks to find associations between them and different diseases and the identification of risk pathways. This approach goes beyond

the CVD risk factors proposed by the lipid panels and could, in the future, characterize the risk of CVD better and identify new phenotypes.

In **Section 2.5** we reviewed many studies that use NMR spectroscopy to characterize diabetic dyslipidemias. The number and size of the particles in the lipoprotein fractions provided by this technique have played an essential role in obtaining insight into this complex metabolic disease.

Finally, we would like to make two points: the first is that very few methods can make a comprehensive analysis of lipoproteins by NMR, and it is very difficult to compare them because they give different parameters. That is why there are no studies comparing these methods among themselves or with classical methods. The techniques must be standardized and validated if they are to be used in the future in clinical diagnosis. If they are, then NMR spectrometers could be introduced into hospitals.

The second point concerns the lack of lipid and lipoprotein databases for use in NMR metabolomic studies with serum/plasma. As well as the groups whose work has been reviewed in this article and which specialize in lipoprotein analysis, the scientific community also performs qualitative lipopoprotein characterizations in the area of NMR-metabolomics. In other words, there is a pressing need to create measurement databases that can be accessed freely, as is the case for the low-weight metabolites found in serum/plasma, thus enabling the serum/plasma samples in research experiments to be completely profiled.

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3. DEVELOPMENT AND EVALUATION OF A NOVEL NMR-BASED ADVANCED LIPOPROTEIN TEST

3.1. "Particle size measurement of lipoprotein fractions using diffusion-ordered NMR spectroscopy"

3.1.1. Abstract

The sizes of certain types of lipoprotein particles have been associated with an increased risk of cardiovascular disease. However, there is currently no gold standard technique for the determination of this parameter. Here, we propose an analytical procedure to measure lipoprotein particles sizes using diffusion-ordered nuclear magnetic resonance spectroscopy (DOSY). The method was tested on six lipoprotein fractions, VLDL, IDL, LDL_1, LDL_2, HDL_2, and HDL_3, which were obtained by sequential ultracentrifugation from four patients. We performed a pulsed-field gradient experiment on each fraction to obtain a mean diffusion coefficient, and then determined the apparent hydrodynamic radius using the Stokes–Einstein equation. To validate the hydrodynamic radii obtained, the particle size distribution of these lipoprotein fractions was also measured using transmission electron microscopy (TEM). The standard errors of duplicate measurements of diffusion coefficient ranged from 0.5% to 1.3%, confirming the repeatability of the technique. The coefficient of determination between the hydrodynamic radii and the TEM-derived mean particle size was r^2=0.96, and the agreement between the two techniques was 85%. Thus, DOSY experiments have proved to be accurate and reliable for estimating lipoprotein particle sizes.

3.1.2. Introduction

Interest in the assessment of the size profiles of lipoprotein particles has been increasing due to the role of this parameter in cardiovascular disease (CVD) risk prediction [1]. For example, small, dense low-density lipoprotein (sdLDL) particles are removed slowly from the blood stream, which subjects them to oxidation processes and leads to the formation of atheroma **[2,3]**. Moreover, a predominance of sdLDL is associated with an

atherogenic lipoprotein phenotype that is characterized by high concentrations of plasma triglyceride, low concentrations of high-density lipoprotein (HDL) cholesterol and apoA-I, and high insulin resistance **[4–7]**. In contrast, HDL particles are considered to be anti-atherogenic lipoproteins because they help reverse cholesterol transport **[8,9]**. In addition, when HDL particles are divided into large and small HDL subclasses, a decrease in the number of larger particles has a stronger influence on the development of CVD. The summary described above is based on several epidemiological studies; however, other studies contribute to the controversy regarding (1) whether sdLDL particle numbers may be considered an independent risk factor for CVD, (2) which HDL subclasses are more protective against CVD, and (3) whether new lipoprotein analytical methods improve risk assessment compared with standard lipid panels **[10–13]**.

Several methods have been developed to determine the particle sizes of different lipoprotein fractions. The Vertical Auto Profile-II or VAP-II test (Atherotec Inc.) fractionates lipoproteins on the basis of differences in density **[14]**. In contrast, the nuclear magnetic resonance (NMR) LipoProfile-II test (LipoScience Inc.) distinguishes among the different lipoprotein particles using the chemical shift value of the methyl NMR signal **[15]**. Lipoprotein particle sizes are then estimated based on a proprietary library containing the NMR signals of lipoproteins of known size. Other methods are based on gradient gel electrophoresis (GGE), including segmented GGE (Berkeley HeartLab), which separates lipoproteins based on their size, and the Quantimetrix Lipoprint LDL System, which estimates LDL particle sizes by comparing their electrophoretic mobility to the electrophoretic mobilities of particles of known size **[16,17]**. All of the methods described above are based on different physicochemical properties, which make them difficult to compare. In addition, they estimate lipoprotein sizes using different assumptions and approximations. Consequently, recent reviews have emphasized their divergence **[18–22]**. A recent study by Ensign et al. that compared the LDL particle sizes obtained using these four

methods reported a total agreement of only up to 8% according to LDL subclass phenotyping (preponderance of large, intermediate, or small LDL particles) **[19]**. Clearly, there is a need for standardization **[23]**. Another disadvantage of the methods that are currently available is that information is delivered on a reduced set of lipoprotein subclasses. Because LDL is usually considered the most clinically relevant lipoprotein class, most of the studies published to date have been focused on LDL phenotyping, and other lipoprotein classes, such as very low-density lipoprotein (VLDL) or intermediate density lipoprotein (IDL), are seldom reported. Therefore, it would be beneficial to consider a complete profile of lipoprotein particle sizes.

In order to measure lipoprotein particles sizes, diffusion-ordered NMR spectroscopy (DOSY) might be thought as a good alternative due to its robustness and simple sample manipulation **[24–26]**. DOSY has been extensively used to measure the size distribution of different materials, including lipid vesicles and gold nanoparticles **[27, 28]**. In this study, the particle sizes of six lipoprotein fractions, VLDL, IDL, LDL_1, LDL_2, HDL_2, and HDL_3, were assessed using DOSY. To the best of our knowledge, this is the first reported work that attempts to assess the particle sizes of ultracentrifuged lipoprotein fractions using this technique. First, the attenuation of the methyl signal was used to obtain a diffusion coefficient for each fraction. We evaluated the diffusion coefficients that were obtained in terms of the signal-to-noise ratio (SNR), and we then constructed a DOSY schematic map to deliver a qualitative visualization plot for lipoprotein analysis. Second, the hydrodynamic radii of the lipoprotein fractions (R_H) were derived using the Stokes–Einstein equation **[24]**:

$$\boldsymbol{R_H = \frac{kT}{6\pi\eta D}} \quad \textbf{(1)}$$

where k is the Boltzmann constant, T is the absolute temperature, and η is the solvent or solution viscosity. **Equation 1** can be decomposed into its component pieces, i.e., the Einstein relationship between a thermal, stochastic property (diffusivity) and a

deterministic, mechanical property (mobility) **[29]**:

$$\boldsymbol{M = \frac{D}{kT}} \quad \textbf{(2)}$$

and the Stokes relationship that relates the deterministic probe response (mobility) to the rheological properties of the material (viscosity):

$$\boldsymbol{R_H = (6\pi\eta M)^{-1}} \quad \textbf{(3)}$$

While the Einstein relationship is strictly valid in this study, the Stokes relationship may fail since it assumes infinite dilution conditions and a continuum solvent. To enable some corrections to be made for obstruction effects, we therefore also measured the experimental viscosity. The feasibility of using **Equation 1** will be explored, comparing the mobility and hydrodynamic radii to the mean lipoprotein sizes measured using transmission electron microscopy (TEM), which has been extensively used in the characterization of lipoprotein fractions **[30]**.

3.1.3. Materials and methods

3.1.3.1. Patient selection

Four patients attending the Lipid Clinic of Sant Joan University Hospital in Reus with different hyperlipoproteinemic phenotypes to cover a broad range of lipid and lipoprotein concentrations were recruited. Patients 1 and 3 were diabetic and suffered from lipoprotein lipase deficiency (Type I) and severe hypertriglyceridemia (Type V), respectively. Patients 2 and 4 had dis-β-lipoproteinemia (Type III) and polygenic hypercholesterolemia (Type IIa), respectively. Blood samples were obtained after a 12-h overnight fasting period; samples were withdrawn into EDTA-containing tubes and centrifuged immediately for 15 min at 4 ºC and 1,500×g to obtain plasma. The study protocol was approved by the Ethical Committee

of Sant Joan University Hospital. All patients gave their written consent to participate in the research program.

3.1.3.2. Lipoprotein fractionation

Six lipoprotein fractions were obtained from the collected plasma by sequential preparative ultracentrifugation in a Kontron ultracentrifuge T-1075 rotor TFT 45.6 at 4 ºC, as previously described **[31]**. The lipoproteins isolated included VLDL (0.95–1.006 g/ml), IDL (1.006–1.019 g/ml), LDL_1 (1.019–1.044 g/ml), LDL_2 (1.044–1.063 g/ml), HDL_2 (1.063–1.0125 g/ml), and HDL_3 (1.125–1.210 g/ml). Sucrose was added to the samples to maintain the physicochemical properties of all the fractions prior to freezing, as previously described **[32]**. All lipoprotein fractions were concentrated two-fold prior to NMR analysis, with the exception of the LDL_2 fraction of patient 2.

The total cholesterol and levels of triglycerides, direct LDL cholesterol, HDL cholesterol, and ApoB-100 in the plasma samples, as well as the lipid and apolipoprotein levels in the lipoprotein fractions, were measured using enzymatic and immunoturbidimetric assays. In **Section 3.1.8**, the analytical lipid and lipoprotein values are detailed (**Table S1**).

3.1.3.3. Diffusion-ordered NMR spectroscopy (DOSY)

To prepare samples for DOSY, the lipoprotein fractions (430 µl) were transferred into NMR tubes (o.d. 5 mm). An internal reference tube (o.d. 2 mm, supported by a Teflon adapter) containing 9.9 mmol/l sodium 3-trimethylsilyl [2,2,3,3-d_4]propionate (TSP) and 0.47 mmol/l $MnSO_4$ in 99.9% D_2O was placed coaxially into the NMR sample tube. The tubes were maintained at 4 ºC in the sample changer until the time of analysis.

^{1}H NMR spectra were recorded on a Bruker Avance III 600 spectrometer operating

at 600.20 MHz. Diffusion measurements were performed at 310 K to obtain a full signal from the melted lipids in the lipoproteins and to avoid serum degradation during measurement. The double-stimulated echo (DSTE) pulse program was used, with bipolar gradient pulses and a longitudinal eddy current delay (LED) **[33]**. This pulse program consists of a DSTE sequence followed by z-storage of the magnetization, and allows longer diffusion delays that are limited only by the longitudinal relaxation of the molecules. This requirement is compulsory if the pulse sequence is to be applied to lipoproteins, in which $T_1 >> T_2$. A DSTE sequence efficiently compensates for flow convection currents that can develop in plasma samples at elevated temperatures. Additionally, three spoil gradient pulses were employed during the z-storage periods and during the recycle delay, to shorten the phase cycle and eliminate accidental refocusing of unwanted magnetization. To minimize the possibility of convection currents, the heating air flow rate was set to 670 l/h. A spectral window of 18,000 Hz was used, with an acquisition time of 1.82 s.

During the experiment, the relaxation delay was 2 s, the FIDs were collected into 64 k complex data points, and 32 scans were acquired for each sample. The gradient pulse strength was increased from 5% to 95% of the maximum strength of 53.5 G cm^{-1} in 50 steps, in which the squared gradient pulse strength was exponentially distributed. A diffusion time (Δ) of 120 ms and bipolar half-sine-shaped gradient pulses (δ) of 6 ms were applied to obtain a reasonable amount of lipoprotein signal attenuation:

$$\boldsymbol{I = I_0 e^{-kDG^2}} \quad \textbf{(4)}$$

where $\boldsymbol{k = (2a\gamma\delta)^2(\Delta - 5\delta/4 - \tau)}$, $\boldsymbol{a = (2/\pi)}$ is a gradient shape factor for the half-sine shape, and τ is the short delay between the pulses in a gradient pulse pair. The total experiment time was 1 h 45 min per sample. All spectra were Fourier transformed after applying an exponential function equivalent to 2 Hz Lorentzian line broadening, phase corrected, baseline corrected, and referenced to the TSP reference signal at 0 ppm. The SNR

was defined as the ratio of the methyl signal maximum in the least attenuated (lowest gradient) spectrum to the standard deviation of the noise height in the baseline.

Diffusion coefficients were obtained using a surface fitting approach, as described previously **[34]**. In this case, only one Lorentzian function was used to fit the methyl surface (**Figure 1**). The estimated diffusion coefficients and peak positions were used, together with their standard errors (SE), to build a schematic DOSY peak map showing the distribution of the different lipoprotein fractions in terms of their chemical shifts and diffusion coefficients.

3.1.3.4. Viscosity measurements

Lipoprotein solution viscosities were measured at 37 ºC with a Cannon–Manning semi-micro capillary viscometer. To obtain the viscosity in millipascal seconds, the density of each fraction was calculated by weighing a volume of 250 µL. The kinematic viscosity was then measured by multiplying the efflux time of the sample between two reference lines by the viscometer constant provided by the manufacturer. Finally, the kinematic viscosity and the density were multiplied to obtain the viscosity.

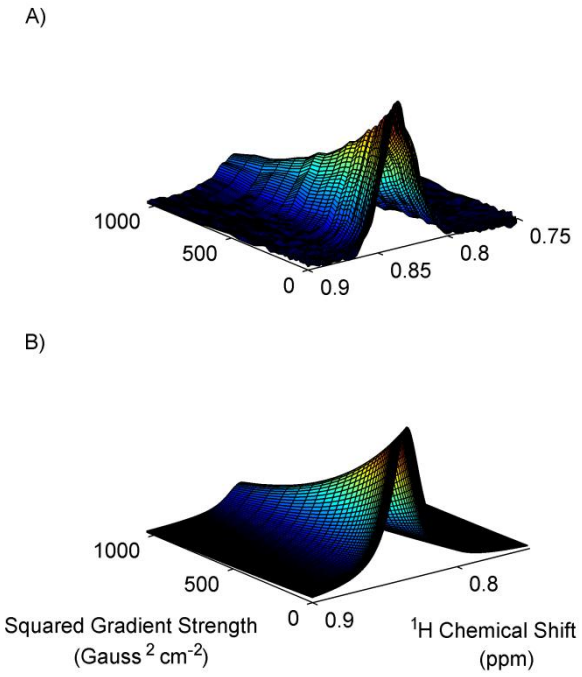

Figure 1. Surface fitting of the attenuated methyl signal of a VLDL fraction (A) using an individual Lorentzian function (B).

3.1.3.5. Transmission electron microscopy (TEM)

TEM samples were prepared by placing a drop of each lipoprotein fraction onto a formvar carbon film for 2 min and then blotting the excess liquid. Negative staining was performed with 2% phosphotungstic acid adjusted to pH 6.6; this solution was applied for 1 min and then blotted dry. The gridded samples were examined on a JEOL JEM-1011 TEM at an accelerating voltage of 80 kV. The particle sizes of the lipoprotein fractions were calculated using the IMAQ Vision software (National Instruments Inc.). Each lipoprotein fraction was analyzed using a different number of micrographs. Some images were filtered with a Gaussian filter prior to analysis to avoid interference from noise. All images were then truncated to black and white, and particles at the borders were discarded. All particles with a circularity factor close to 1 were selected. The number of particles considered ranged from 100 to 800. The HDL fractions were particularly difficult to measure via TEM; only the HDL_2 fraction from patient 2 and the HDL_3 fractions of patients 2 and 3 were entered in the

regression analysis because these samples were the only HDL fractions that exhibited reasonable particle sizes.

3.1.3.6. Statistical analysis

The diffusion coefficients among the lipoprotein subclasses were compared using the nonparametric Kruskal–Wallis test. The resulting P value was used to test the null hypothesis of all the subfractions belonging to the same subclass. $P<0.05$ was considered to be statistically significant in order to reject the null hypothesis. A simple linear regression analysis was used to examine the relationship between the lipoprotein mobilities and hydrodynamic radii obtained via NMR, and the lipoprotein particle sizes obtained via TEM. The parameters used to evaluate the linear regressions were the coefficient of determination ($0 \leq r^2 \leq 1$) and the regression lines. In order to evaluate the agreement between the two techniques, the root mean squared percentage error (RMSPE) of the differences between NMR and TEM was calculated according to the following formula:

$$\boldsymbol{RMSPE}(\%) = \sqrt{\frac{\sum_1^n \left(\frac{(NMR-TEM)\times 100}{TEM}\right)^2}{n}} \quad \textbf{(5)}$$

where NMR refers to the NMR-derived sizes, TEM to the TEM-derived sizes, and n the number of lipoprotein fractions. All of the analyses were performed with MATLAB Version 7.10.0.499 R2010a (MathWorks).

3.1.4. Results

3.1.4.1. Calculation of the diffusion coefficients

DOSY experiments are vulnerable to some experimental limitations, such as non-uniform field gradients, temperature gradients, and low solute concentrations **[25]**. The last

limitation is of particular concern in the analysis of lipoprotein fractions because these are typically diluted during several sample manipulation steps; consequently, pulsed-field gradient (PFG) experiments on lipoprotein fractions may yield spectra with low SNR. The VLDL and HDL_3 fractions of patient 1 were therefore evaluated, as they represent two extreme cases for SNR (250:1 and 15:1, respectively). **Figures 2A-B** illustrate the attenuation of the methyl signal of these samples in a PFG experiment. While the VLDL fraction did not exhibit complete attenuation of the signal over the range of gradient amplitudes used, the HDL_3 fraction was completely attenuated at high gradient strengths because of the faster diffusion of their particles. The HDL_3 fraction also exhibited lower SNR even at low gradient strengths. The SNR differences between the VLDL and HDL_3 fractions arose because of the different concentrations; the VLDL fraction of patient 1 contained 1.31 and 3.66 mmol/L of cholesterol and triglycerides, respectively, whereas the HDL_3 fraction from the same patient contained 0.16 and 0.09 mmol/L, respectively (**see Table S1 in Section 3.1.8**). Despite the low concentrations found in the HDL_3 fraction, the nonlinear least squares fitting of the experimental data to **Equation 4** yielded an acceptable fitting error of ~3% (**Figures 2C–F**). The use of integral area attenuation to fit with **Equation 4**, instead of intensity attenuation as is more common in DOSY experiments, reduces the amount of uncertainty in signal attenuation due to noise.

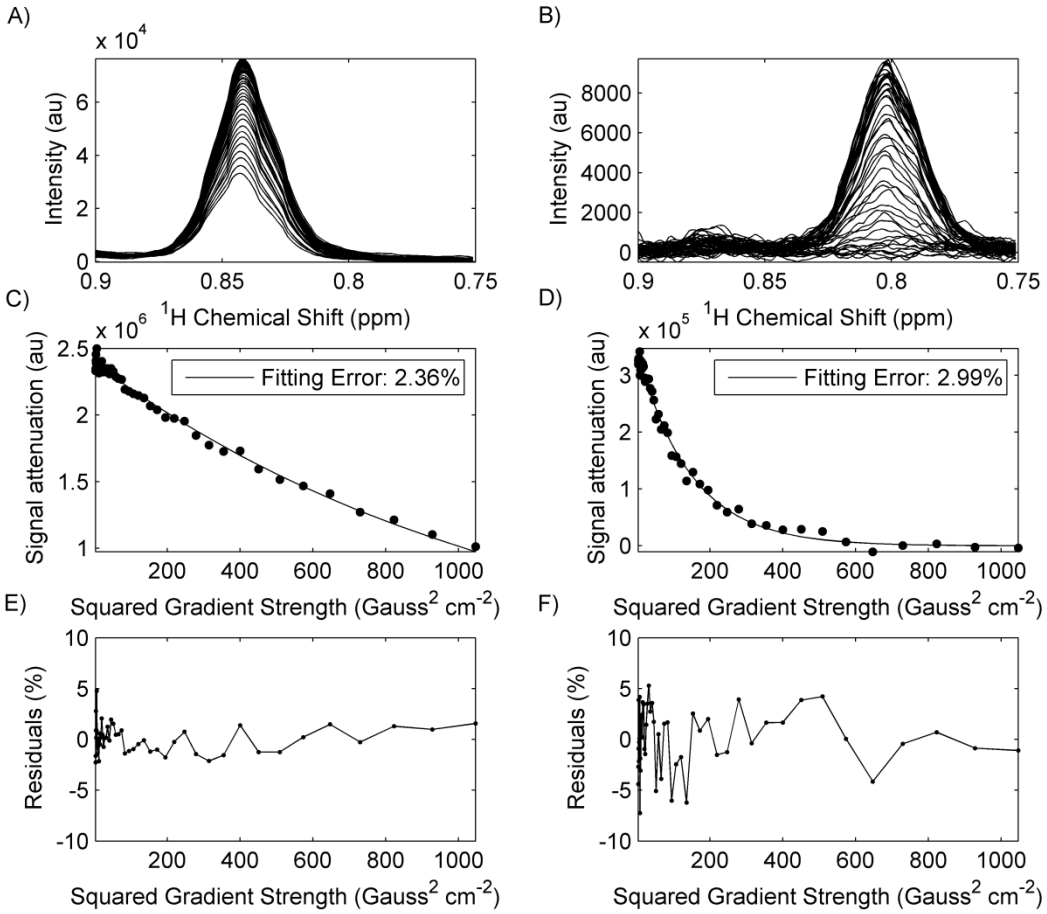

Figure 2. SNR analysis of DOSY spectra. A-B) Signal attenuation of the VLDL and HDL_3 fractions from patient 1. C-D) Fitting of the integral area to Equation 4. E-F) Residuals of the fittings.

Figure 3 shows a schematic DOSY peak map of the diffusion coefficients that were obtained for all the fractions, details of which are also summarized in **Table 1**. Spectra of the different lipoprotein fractions from patient 3 are shown along the top as a reference. On the right side, projections of the diffusion coefficients are depicted. For each patient, the methyl signal of the neutral lipids in the lipoprotein shows faster diffusion as the lipoprotein densities increase. In general, larger lipoprotein subclasses yielded lower SEs. The maximum SE value of an estimated diffusion coefficient was ~4%, and was obtained for the LDL2 fraction of patient 2; the estimated SE for the remaining samples was less than 1.5%. A high SE was observed for this particular LDL_2 fraction because it was highly diluted compared

with the other fractions; therefore, its SNR (4:1) was around the detection level (5:1). We did not use the diffusion coefficient obtained for this lipoprotein fraction in further analysis.

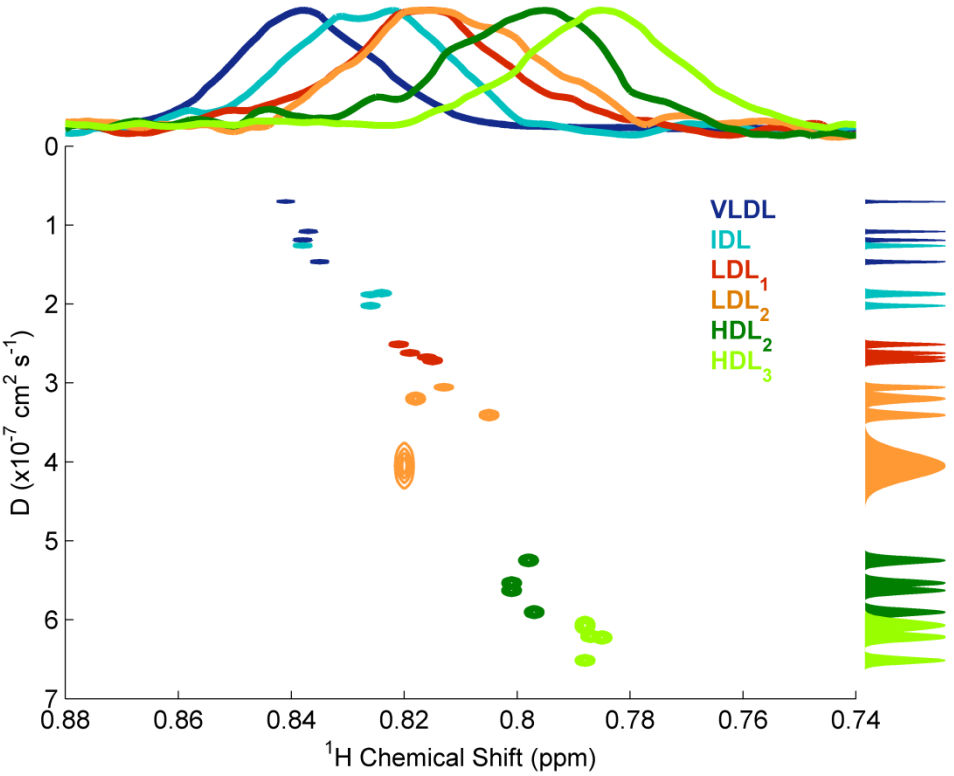

Figure 3. Schematic DOSY peak map showing the diffusion coefficients obtained by fitting **Equation 4** to each dataset. The width along the chemical shift axis of the Gaussian curves represents the SE in estimating the position, while the width along the diffusion dimension represents the SE in estimating the diffusion coefficient. The superimposed spectra at the top of the figure are those acquired for the lipoprotein fractions from patient 3 and have been normalized for visualization purposes; the Gaussian functions at the right side correspond to the projection of the diffusion spectra obtained for all samples.

Table 1. Results from all of the measurements. *D* is the diffusion coefficient (% SE), *M* is the mobility, η is the viscosity expressed as mean±standard deviation, R_H is the hydrodynamic radii, and the TEM-derived particle sizes are expressed as mean±standard deviation (number of particles measured); Nd = Not determined.

Fraction	Patient	SNR	*D* / (cm^2 s^{-1})	*M* / (cm^2 s^{-1} J^{-1})	η / (mPa s)	R_H / (Å)	TEM Size / (Å)
VLDL	1	252	0.70 (0.8)	151	0.81	402	265±113 (400)
	2	208	1.07 (0.8)	233	0.81	261	228±110 (803)
	3	506	1.18 (0.7)	256	1.12	171	136±49 (746)
	4	43	1.46 (0.7)	315	0.80	195	184±44 (625)
IDL	1	14	1.25 (1.0)	271	0.77	236	179±72 (296)
	2	18	2.01 (0.8)	435	0.71	159	169±36 (450)
	3	31	1.88 (0.8)	405	0.87	139	135±26 (106)
	4	17	1.86 (1.0)	401	0.76	160	155±57 (532)
LDL_1	1	14	2.69 (0.9)	585	0.79	107	96±63 (97)
	2	29	2.50 (0.7)	541	0.71	128	114±28 (277)
	3	23	2.66 (0.7)	576	0.77	111	118±36 (95)
	4	45	2.61 (0.6)	564	0.76	114	130±24 (98)
LDL_2	1	11	3.38 (1.0)	734	0.76	88	83±28 (101)
	2	4	4.16 (3.8)	872	0.73	75	86±21 (98)
	3	18	3.05 (0.7)	658	0.85	88	80±19 (162)
	4	9	3.20 (1.3)	689	0.84	84	87±23 (166)
HDL_2	1	25	5.63 (0.7)	1212	0.77	52	Nd
	2	23	5.89 (0.7)	1272	0.72	54	45±13 (18)
	3	19	5.23 (0.8)	1130	0.73	59	Nd
	4	19	5.53 (0.7)	1192	0.71	58	Nd
HDL_3	1	15	6.09 (1.0)	1308	0.72	52	Nd
	2	49	6.21 (0.6)	1338	0.71	51	41±11 (122)
	3	52	6.22 (0.7)	1341	0.74	49	39±17 (339)
	4	41	6.51 (0.5)	1403	0.71	49	Nd

Lipoprotein subclasses could be clearly distinguished using their average diffusion coefficients (*P*=0.00073). The minimum difference in diffusion coefficient between two subclasses was 2.8%; the two subclasses involved were the two HDL subclasses. The SE for the fastest HDL_2 fraction and the slowest HDL_3 fraction were 0.7% and 1%, respectively, and these subclasses could still be statistically distinguished (P=0.021). However, there was a

lipoprotein fraction that exhibited an average diffusion coefficient that was out of its subclass range. This was the IDL fraction of patient 1, a sufferer from lipoprotein lipase deficiency, and exhibited an average diffusion coefficient within the range of the VLDL fractions. Despite the normal lipid values exhibited by this IDL fraction (**see Table S1 in Section 3.1.8**), its low diffusivity suggests that larger and more lipid-rich particles than expected are present. These larger particles may correspond to VLDL particles because complete separation of these lipoprotein fractions by ultracentrifugation cannot be achieved in subjects with this pathology due to the lactescent state of the plasma sample.

3.1.4.2. Determination and validation of lipoprotein hydrodynamic radii

We obtained a mobility and hydrodynamic radius for each lipoprotein fraction using **Equations 2 and 3**, respectively (**Table 1**). To evaluate the validity of the Stokes relationship, we performed two linear regression analyses to assess the relationships between the two variables and the TEM-derived particle sizes. **Figure 4A** illustrates the regression model constructed using the calculated lipoprotein mobilities as a prediction variable. The two variables correlated satisfactorily (r^2=0.78). Using the measured solution viscosity (as a partial correction for the effects of other solution components and of obstruction) and performing the linear regression analysis for hydrodynamic radius instead of mobility resulted in a stronger linear relationship (r^2=0.90), as shown in **Figure 4B**. In this figure, dotted rectangles identify the areas in which the different lipoprotein fractions used in this study should be placed according to the literature (VLDL, 150–400 Å; IDL, 125–175 Å; LDL, 90–140 Å; HDL, 25–60 Å) **[35]**. These rectangles represent the main lipoprotein classes since the size ranges that define the subsequent subclasses are study dependent. As shown in **Figure 4B**, most of the lipoprotein samples lie within their theoretical range, but all of the LDL_2 fractions had a hydrodynamic radius slightly smaller than the minimum expected

value.

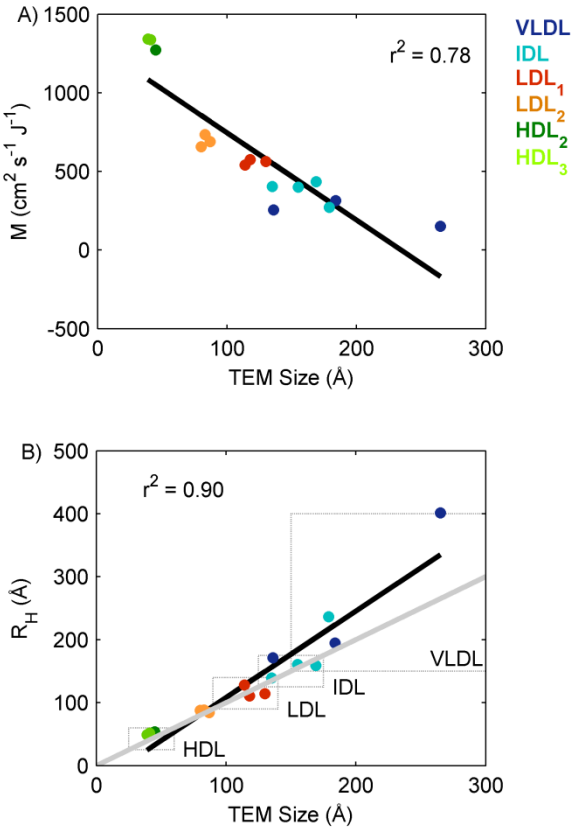

Figure 4. Regression between NMR-derived data and TEM-derived particle sizes. A) Relationship between mobility (*M*) and TEM-derived particle sizes (*y=-5.5x+1300*), B) Relationship between hydrodynamic radii (R_H) and TEM-derived particle sizes (*y=1.37x-29*). The gray line correspond to the identity line (*y=1.00x+0*).

Although we found a high degree of correlation between the two techniques, their agreement was moderate in terms of absolute values (RMSPE of 20%). This is not surprising: The relationship between measured diffusion coefficient and particle size is complicated by obstruction effects, polydispersity, shape and flexibility effects, and other limitations of the simple Stokes–Einstein model. Of these problems, polydispersity is expected to be one of the most serious. The signal measured in an NMR experiment like DOSY is proportional to the number of spins present, so larger particles will contribute much more strongly to the

NMR data than smaller, in proportion to the cube of the radius **[36]**. Thus, the diffusion coefficient obtained by NMR is expected to correspond to an "average" size larger than the mean of the radii obtained by TEM.

To assess the importance of polydispersity, we simulated an attenuating NMR dataset, corresponding to the parameters used for the experimental measurements, for each sample, using the experimental size distributions found with TEM (**see Appendix in Section 3.1.8**). These datasets were then fitted to the Stokes–Einstein equation as above and used to derive apparent hydrodynamic radii. For each sample, a correction factor for the effects of polydispersity on the NMR data was then calculated from the ratio of the mean TEM size listed in **Table 1** to the size obtained by fitting the synthetic data. Finally, this correction factor was applied to the NMR data, allowing the TEM size to be compared to the NMR sizes corrected for the bias introduced by polydispersity. **Figure 5** shows the linear regression between the corrected hydrodynamic radii obtained from NMR and the mean TEM sizes. As can be seen, this correction not only improved the correlation between the two techniques (r^2=0.96) but also improved the agreement (RMSPE of 15%), suggesting that the dominant systematic factor leading to differences in apparent size was polydispersity. It must be stressed that this correction was aimed at evaluating the importance of polydispersity in the agreement between the two techniques, and that it is not expected to be used routinely. It should perhaps be noted that there is no reason a priori to prefer the bias towards small particles inherent in using average TEM radius to the bias towards larger particles inherent in DOSY, if a single size parameter rather than a distribution is to be used: it does however mean that comparisons between the two require care.

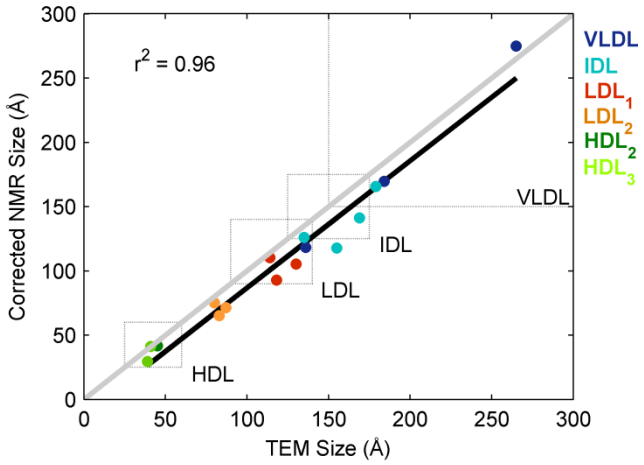

Figure 5. Regression between corrected NMR sizes and mean TEM sizes (*y=0.99x-12*). The gray line correspond to the identity line (*y=1.00x+0*).

3.1.5. Discussion

A previous study by O'Neal et al. used light scattering (LS) methods to determine the hydrodynamic radii of LDL lipoproteins **[37]**. LS methodology also determines the diffusion coefficients of lipoprotein fractions and uses the Stokes–Einstein equation to derive their hydrodynamic radii. However, in this study, a constant viscosity was considered. Sakurai et al. used the same technique to obtain the hydrodynamic radii of two LDL subclasses that were collected by ultracentrifugation **[38]**. Mean particle sizes of 108 and 102 Å for LDL_1 and LDL_2, respectively, were obtained. In our study, the mean particle sizes for the LDL_1 and LDL_2 subclasses were 114 and 87 Å, respectively. Thus, our approach seems to obtain relatively lower particle sizes for the LDL_2 fraction.

Although the experimental viscosity measured for this study, under stress conditions, does not necessarily correspond to the effective viscosity experienced by diffusing particle, the improvement in the correlation between NMR and TEM radii when the experimental viscosity is used in **Equation 3** suggests that this does provide an effective correction for the presence of solutes, including the lipoprotein. A more important reason

for systematic differences between radii estimated by TEM and by NMR, however, is the polydispersity evidenced in the TEM data (**see Figures S1-S7 in Section 3.1.8**). The methyl signal fitted to obtain the diffusion coefficient is a composite containing contributions from all the different lipoprotein sizes present in the sample. The diffusion coefficients obtained by NMR are thus the result of an averaging over all the lipoprotein present **[36]**. Here, we have demonstrated that the diffusion coefficients obtained by NMR accurately reflect the distribution of sizes seen in TEM data, the NMR results showing a greater contribution from slower (larger) particles. The method described here thus yields more reliable values than previous studies, in which a constant value for the viscosity was used and no corrections for polydispersity were attempted.

3.1.6. Conclusions

In this study, DOSY experiments were carried out on lipoprotein fractions to assess their average particle sizes. The diffusion coefficients thus obtained had low estimation errors, demonstrating the repeatability of this technique. The hydrodynamic radii found when using the experimental viscosity in the Stokes–Einstein equation were highly correlated with the mean TEM sizes, although there was a systematic difference between the TEM and NMR-derived sizes. This systematic difference was shown to be explained by the polydisperse distributions found by TEM; once this was taken into account, a high degree of agreement was obtained between the two techniques. We propose that NMR is a potentially useful alternative to other available approaches for measuring lipoprotein fraction particle sizes, due to its inherent robustness and minimal sample manipulation.

3.1.7. Acknowledgments

We acknowledge CIBER de Diabetes y Enfermedades Metabólicas Asociadas (ISCIII, Ministerio de Ciencia e Innovación), for partially funding this work, as well as the FIS (project PI 081409). This work was partly supported by the Engineering and Physical Sciences Research Council (Grant Numbers EP/H024336/1 and EP/I007989/1). We also acknowledge Dr. M. Moncusí and Dr. R. Marimon for their assistance with the TEM analysis of the lipoprotein fractions as well as Dr. S. Pujol for her assistance with viscosity measurements.

3.1.8. Supplemental material

The content of this section is divided into three parts. First, a table listing the biochemical data of the ultracentrifuged fractions of the four patients is presented. This biochemical data correspond to the cholesterol, triglycerides, Apo-A and Apo-B values for each lipoprotein fraction. The concentration factor used to perform such analysis is reported as well. Second, TEM images corresponding to different fractions and patients are shown. Finally, the last section includes an appendix with a detailed description of the correction applied to the NMR-derived diffusion coefficients.

Table S1. Biochemical results from ultracentrifugation.

Fraction	Patient	Conc. Factor	Chol. (mmol/L)	Trig. (mmol/L)	ApoA-I (mg/dl)	ApoB-100 (mg/dl)
VLDL	1	1	1,31	3,66	-	-
	2	1	2,27	2,86	-	14
	3	2	3,03	5,40	-	38
	4	2	0,35	0,72	-	3
IDL	1	1	0,21	0,25	-	-
	2	1	0,61	0,18	-	3
	3	2	0,55	0,30	-	2
	4	2	0,37	0,18	-	3
LDL1	1	2	0,26	0,15	-	3
	2	2	1,08	0,18	-	16
	3	2	0,79	0,2	-	19
	4	2	2,95	0,29	-	72
LDL2	1	2	0,22	0,11	-	4
	2	1	0,15	0,05	-	1
	3	2	0,98	0,14	-	21
	4	2	0,5	0,08	-	7
HDL2	1	2	0,23	0,17	26	-
	2	2	0,36	0,10	32	-
	3	2	0,30	0,08	19	-
	4	2	0,56	0,08	31	-
HDL3	1	2	0,16	0,09	28	-
	2	2	0,72	0,17	109	-
	3	2	0,47	0,14	84	-
	4	2	0,75	0,09	97	-

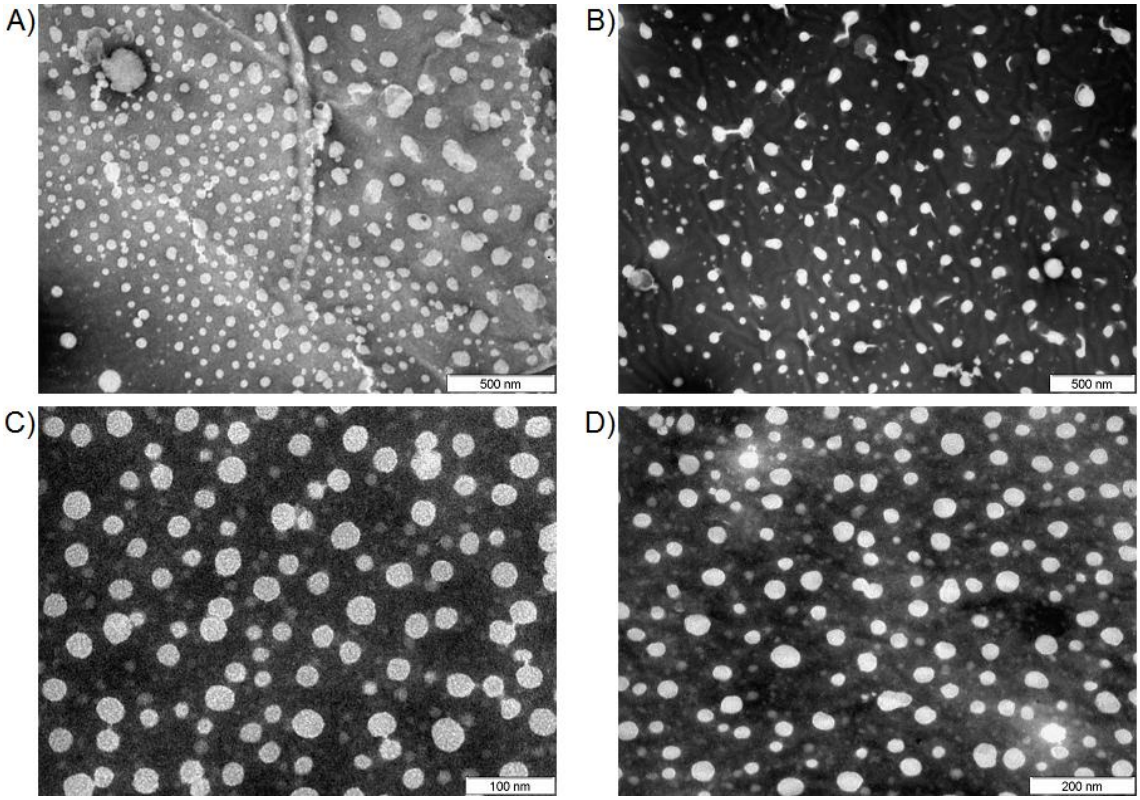

Figure S1. TEM images of VLDL particles for patients A) 1 (magnification of 50k), B) 2 (magnification of 40k), C) 3 (magnification of 200k), and D) 4 (magnification of 120k).

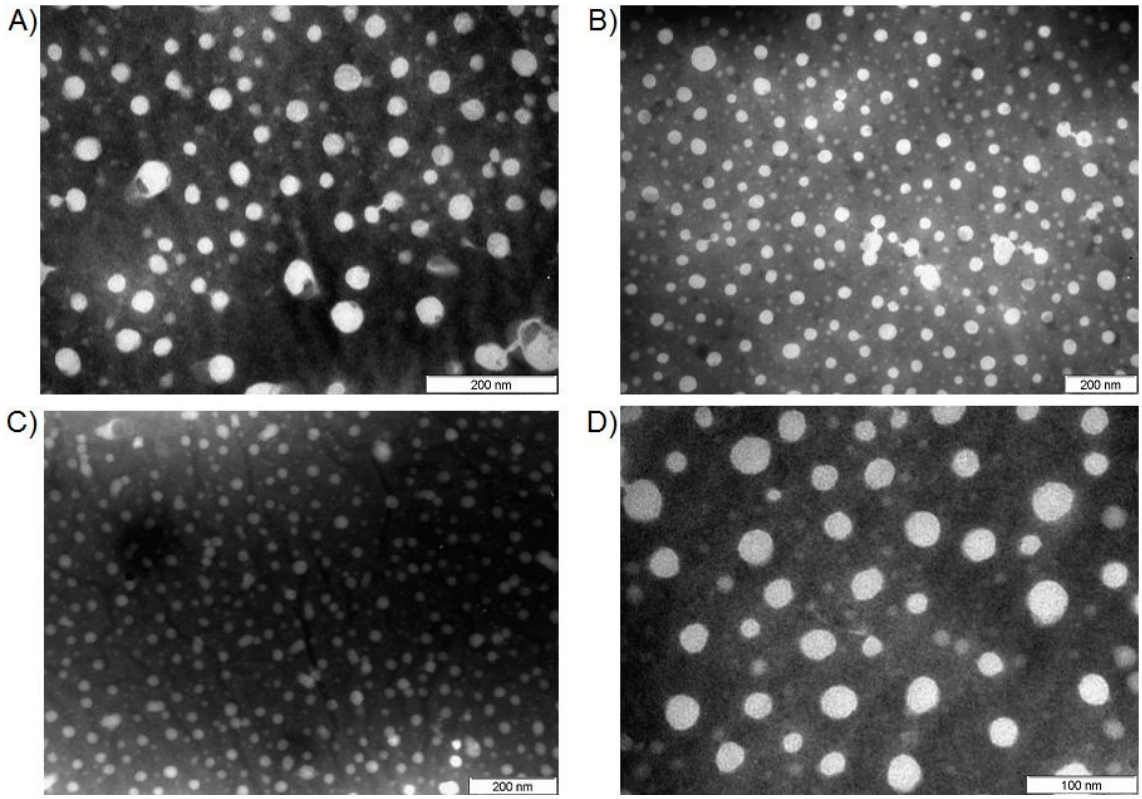

Figure S2. TEM images of IDL particles for patients A) 1 (magnification of 150k), B) 2 (magnification of 80k), C) 3 (magnification of 100k), and D) 4 (magnification of 250k).

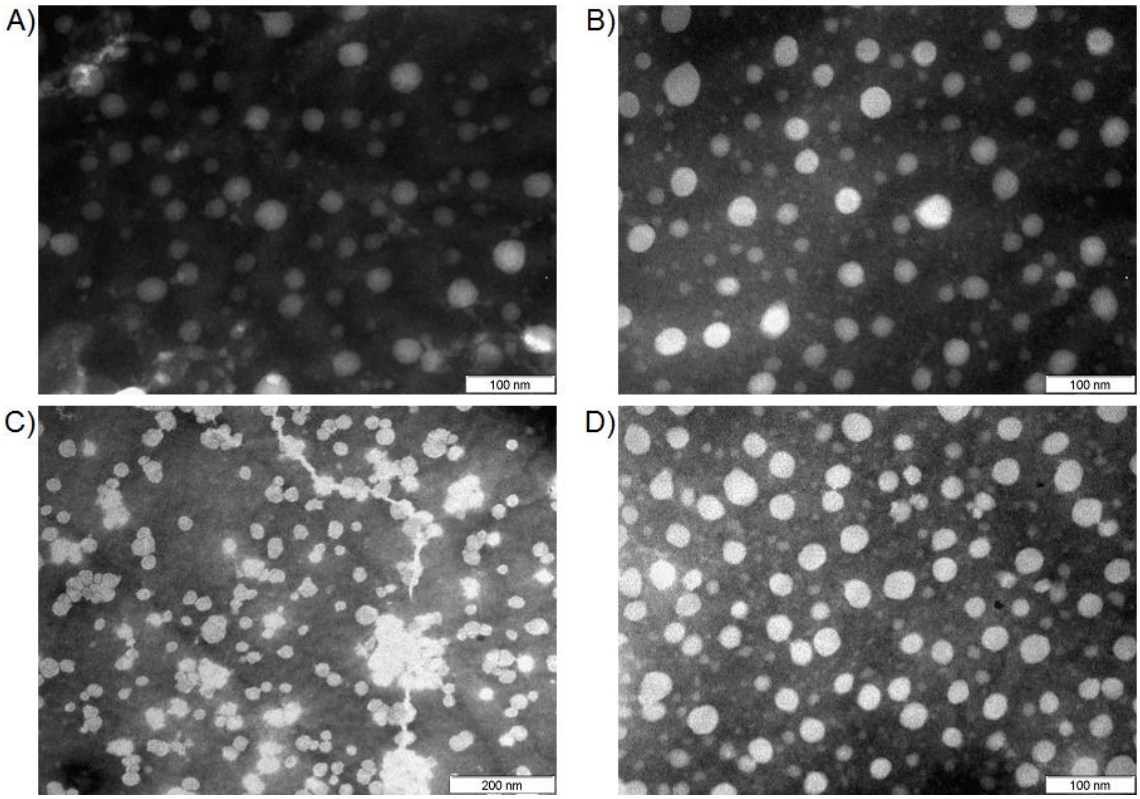

Figure S3. TEM images of LDL_1 particles for patients A) 1 (magnification of 200k), B) 2 (magnification of 200k), C) 3 (magnification of 120k), and D) 4 (magnification of 200k).

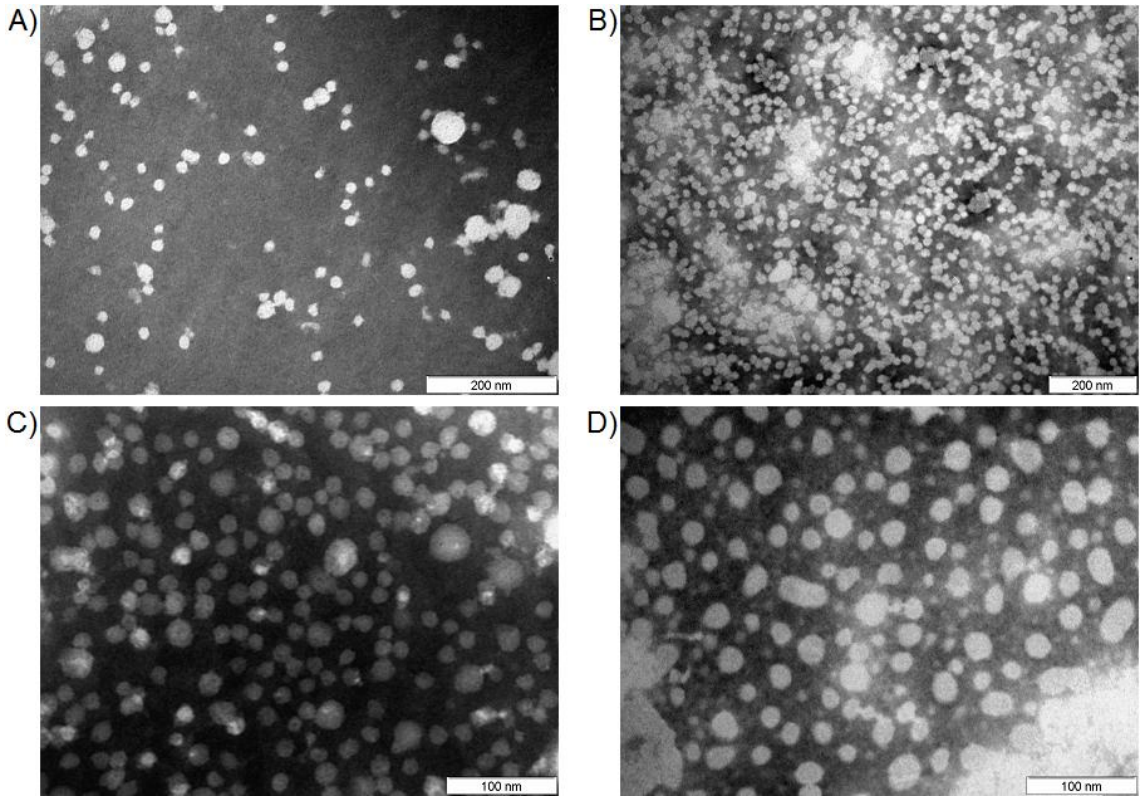

Figure S4. TEM images of LDL_2 particles for patients A) 1 (magnification of 150k), B) 2 (magnification of 100k), C) 3 (magnification of 250k), and D) 4 (magnification of 250k).

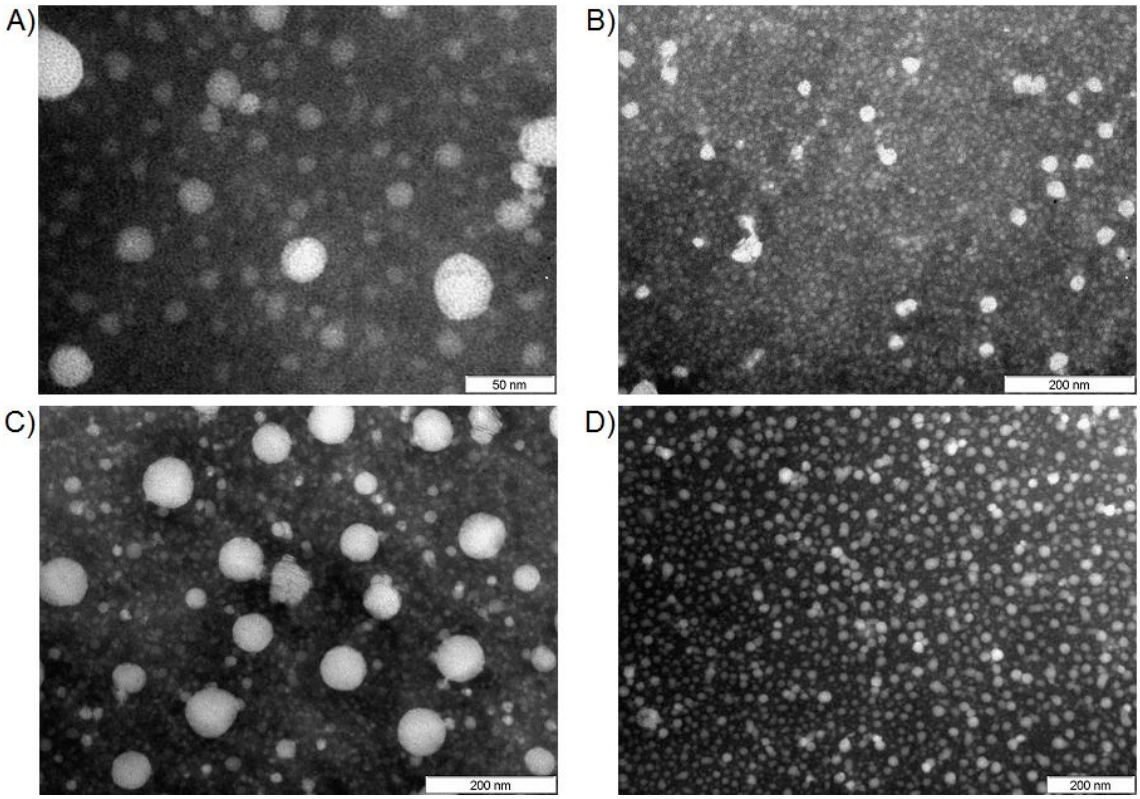

Figure S5. TEM images of HDL_2 particles for patients A) 1 (magnification of 400k), B) 2 (magnification of 150k), C) 3 (magnification of 150k), and D) 4 (magnification of 100k).

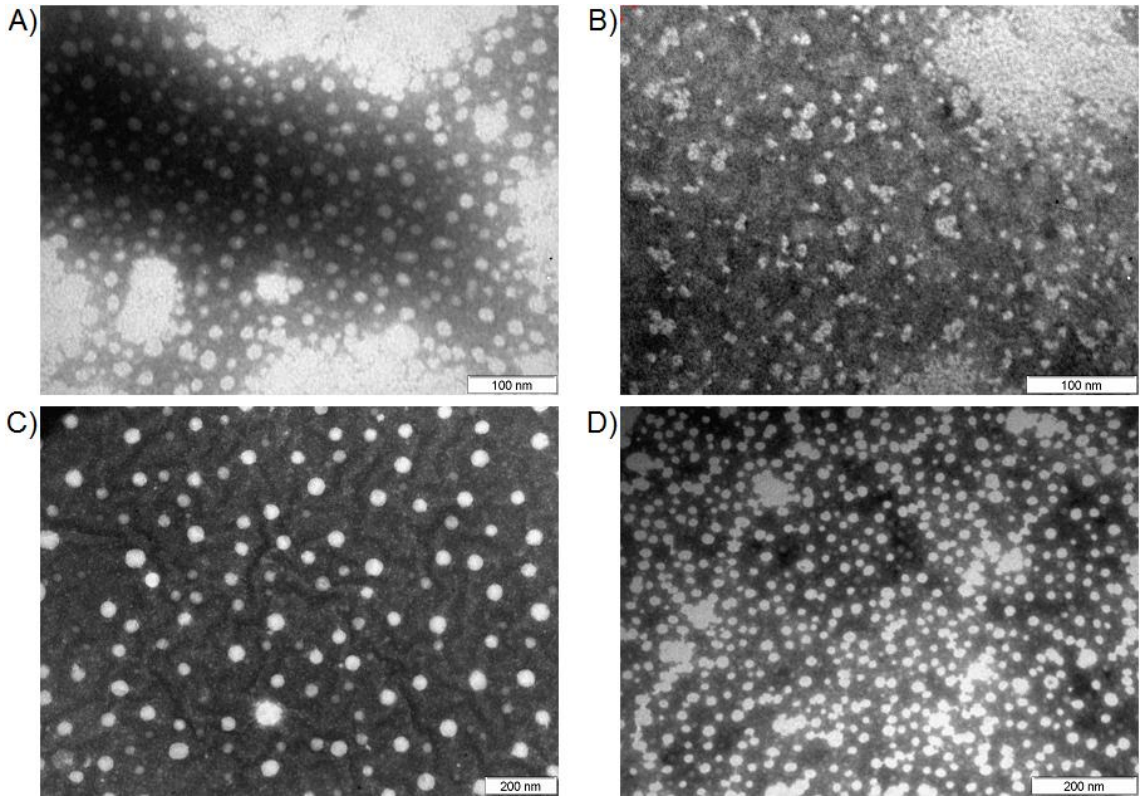

Figure S6. TEM images of HDL_3 particles for patients A) 1 (magnification of 200k), B) 2 (magnification of 250k), C) 3 (magnification of 80k), and D) 4 (magnification of 120k).

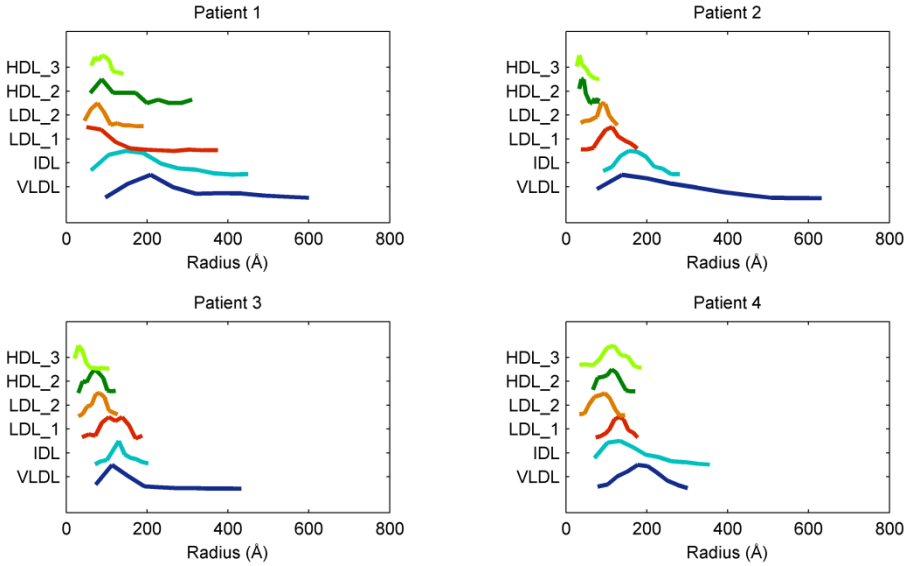

Figure S7. Lipoprotein particle size distributions.

Appendix

For each lipoprotein fraction, we obtained a weighed distribution of lipoprotein particles sizes using the experimental distributions $N(R_{TEM})$ found by TEM:

$$F(R_{TEM}) = \frac{N(R_{TEM})dR_{TEM}}{\int_0^\infty N(R_{TEM})dR_{TEM}} \quad \textbf{(1)}$$

where R_{TEM} are the lipoprotein sizes. Moreover, we obtained a diffusion coefficient for each lipoprotein particle:

$$D(R_{TEM}) = \frac{kT}{6\pi\eta R_{TEM}} \quad \textbf{(2)}$$

Then, we simulated the signal attenuation for each lipoprotein particle assuming that the NMR signal is proportional to the volume of a given lipoprotein particle and using the diffusion coefficient obtained previously:

$$I(G, R_{TEM}) = \alpha {R_{TEM}}^3 e^{-\gamma^2 G^2 \delta^2 \Delta' D(R_{TEM})} \quad \textbf{(3)}$$

where α is a proportionallity factor. Finally, integrating the signal attenuation of all lipoprotein particles within a given fraction taking into account its weighed size distribution we can obtain a simulated average signal attenuation:

$$I_{av}(G, R_{TEM}) = \int_0^\infty F(R_{TEM}) \alpha {R_{TEM}}^3 e^{-\gamma^2 G^2 \delta^2 \Delta' D(R_{TEM})} dR_{TEM} \quad \textbf{(4)}$$

Fitting this simulated average attenuation to Eq. 5 we obtained a corrected diffusion coefficient for each lipoprotein fraction.

3.1.9. References

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3.2. "Surface fitting of 2D diffusion-edited ^{1}H NMR spectroscopy data for the characterization of human plasma lipoproteins"

3.2.1. Abstract

Determining the concentration and size of lipoprotein complexes is very important due to their role in cardiovascular diseases and metabolic disorders. However, standard methods for lipoprotein fractionation are manual and time consuming and cannot be used as standard diagnostic tools. Because different subclasses of lipoproteins have different radii and, hence, different diffusion velocities, we propose a fast and reliable method that uses 2D diffusion-edited ^{1}H NMR spectroscopy to acquire a set of 2D spectra of plasma samples, followed by a surface fitting algorithm based on Lorentzian functions to estimate the sizes and the relative proportions of different lipoprotein subclasses. We were able to demonstrate that the derived sizes and positions related to the Lorentzian functions follow an exponential relationship for normolipidaemic and dislipaemic samples with coefficients of determination (r^2) of 0.85 and 0.81, respectively. Moreover, we found a linear relationship between the width and size of the Lorentzian functions for normolipidaemic samples (r^2=0.88) while for dislipaemic samples this relation was nonlinear (r^2=0.62). Dividing our samples set into four different lipoprotein profiles (normal lipid values, low HDL/LDL ratio, high triglycerides values and both risk factors) and using principal component analysis (PCA) followed by multivariate analysis of variance (MANOVA), our method was able to statistically discriminate between those groups, with *p*-values of 0.0016, 0.0006, <1e-4 and 0.0035, respectively. These parameters are characteristic and indicative of different lipoprotein profiles and can be used to distinguish between normolipidaemic, hypercholesterolaemic, hypertriglyceridaemic and chylomicronaemic profiles.

3.2.2. Introduction

Cholesterol esters and triglycerides are packed within the liver into round macromolecules surrounded by a monolayer of phospholipids, free cholesterol and proteins that give rise to lipoprotein complexes. Lipoproteins are divided into five main classes depending on their size and density: chylomicrons (Q; radii 400–2500 Å), very low density lipoproteins (VLDL; radii 150–400 Å), intermediate density lipoproteins (IDL; radii 125–175 Å), low density lipoproteins (LDL; radii 90–140 Å) and high density lipoproteins (HDL; radii 25–60 Å) **[1]**. Furthermore, these main classes can be divided into different subclasses to obtain a more detailed lipoprotein profile **[2]**.

The concentration of blood plasma lipids transported by lipoproteins is a widely used metric for cardiovascular disease (CVD) risk assessment **[3-5]**. In addition, recent studies have shown that CVD risk is also related to the size distribution of lipoprotein molecules **[6, 7]**. For instance, LDL particles are known as bad cholesterol, but small and dense LDL particles are considered more atherogenic than larger ones. Among HDL particles, the most buoyant are more protective than the smallest and most dense HDL particles. Chylomicrons are not usually taken into account in medical screening because they are not found in fasting samples and because the manipulation of chylomicron-rich samples is more difficult and their biochemical analysis is less reliable. However, it has been suggested that postprandial triglyceride concentrations are better at predicting the risk of CVD **[8]**. Thus, it is important to develop a fast and reliable method for measuring a complete lipoprotein profile, including lipid and lipoprotein concentrations and the size distributions of different lipoprotein subclasses, from both fasting and non-fasting subjects.

The main classes and subclasses of lipoproteins are usually characterised by density gradient ultracentrifugation **[9]**, gel electrophoresis **[10, 11]** or precipitation **[10]**. These techniques are manual and time consuming and cannot be used as standard analyses for

clinical purposes. Their use has been summarised by the NIH in the USA **[12-14]**. In routine clinical analysis, where ultracentrifugation is impractical, the level of LDL cholesterol is estimated from HDL cholesterol measurements (measured after a precipitation process). The total cholesterol and triglycerides are estimated with the Friedewald formula **[15]**. This method may only be used when the total triglyceride concentration is less than 4.5 mmol/l. Recently, new techniques have been developed to allow the direct measurement of LDL cholesterol from plasma; however, these procedures are neither widely used nor clinically validated **[16, 17]**.

Alternatively, plasma lipoproteins can be measured by ^{1}H NMR spectroscopy **[18]**. This methodology has been used for disease risk assessment and diagnostics **[19]**. The NMR spectra contain many peaks originating from both medium and low molecular weight metabolites, as well as broad signals coming mainly from cholesterol and triglycerides associated with lipoproteins **[20]**. The intense signals arising at 0.8 and 1.3 ppm correspond to the terminal methyl and methylene groups, respectively. These signals are frequently used for lipoprotein characterisation. Jeyarajah et al. used the methyl lipid signal for lipoprotein subclass quantification **[21]**. Currently, they can obtain the concentration and size of up to nine lipoprotein subclasses **[22]**. Their method involves the spectral deconvolution of the methyl signal, where a leastsquares curve-fitting algorithm is used to extract the individual lipoprotein signal amplitudes. This test has been commercialised by Liposcience Inc. (North Carolina, NC, USA) and distributed since 1997 under the commercial name of NMR Lipoprofile. To date, more than two million analyses have been performed using Liposcience technology.

Other studies have used statistical correlations between ^{1}H NMR spectra and biochemical data by means of PLS regression models to quantify blood plasma lipids **[23, 24]**. Suna et al. quantified eleven lipoprotein subclasses and used self-organising maps

(SOM) to classify lipoprotein profiles according to different clinical situations **[25]**. In addition, researchers have also used Bayesian methods to build robust models that correlate NMR spectra with cholesterol and triglycerides of the main lipoprotein classes **[26]**.

However, none of the aforementioned methods is capable of directly estimating lipoprotein size distributions. For this purpose, there has been increasing interest in 2D diffusion-edited ^{1}H NMR spectroscopy for the analysis of plasma samples **[27, 28]**. Briefly, by applying a gradient strength iteratively, different NMR spectra are acquired while their intensities are attenuated. Changes in the NMR intensity of a given peak depend not only on the strength of the gradient applied, but also on the diffusion coefficient of the metabolite from which such resonance originates. Thus, using diffusion NMR experiments it is possible to obtain estimated diffusion coefficients of the lipoproteins within a plasma sample. Once the diffusion coefficients have been derived, they can be entered into the Stokes–Einstein equation to assess the hydrodynamic radius.

Several prior works have used diffusion experiments. Liu et al. measured one serum sample at 298 K to obtain six Lorentzian functions from the deconvolution of the methyl and methylene peaks **[29]**. Each function was associated with an average diffusion coefficient (or a mean lipoprotein size). The same group has shown that the use of diffusion-edited experiments improved their assessments of hepatotoxicity in rat blood serum compared to assessments derived from traditional ^{1}H NMR spectra **[30]**. Dyrby et al. performed a diffusion-edited experiment on plasma samples at 318 K and applied three-way chemometrics algorithms such as N-PLS and PARAFAC models to quantify lipoprotein subclasses in human plasma samples **[31]**. Additionally, Numares (Regensburg, Germany) described a method to quantify lipoprotein subclasses based on the line-shape fitting of the methyl and methylene peaks of ^{1}H NMR spectra measured at different gradient strengths

and temperatures **[32]**.

In this study, a method for the deconvolution of the methyl peaks of plasma samples with different dislipaemias is presented. The areas and averaged diffusion coefficients from the deconvoluted functions obtained from our method are then used as input parameters for a multivariate comparison of the different lipoprotein profiles under study. Compared with ^{1}H-NMR line-shape fitting methods **[23-26]**, this approach is much more robust because the function parameters are adjusted using the spectra of n gradients **[29, 31, 32]**. Although Liu et al. proposed a similar approach to the one applied in this work, only one serum sample was used, and the potential benefits of the method were neither demonstrated nor exploited **[29]**.

3.2.3. Materials and methods

3.2.3.1. Plasma sample handling and analytical methods

In our study, we used two sample sets.

3.2.3.1.1. Sample set No. 1

Blood samples (n=8) were obtained from patients attending the Lipid Unit of HUSJ (Hospital Universitari Sant Joan de Reus, Spain) because of lipid metabolism disturbances: normolipidaemia (n=3), hypercholesterolaemia (n=1), hypertriglyceridaemia (n=1) and hyperchylomicronaemia (n=3). Written informed consent was obtained from the volunteers. **Table S1** in **Section 3.2.7** details the analytical lipoprotein profile values obtained from patients in sample set No. 1. Samples were obtained after a 12-h overnight fasting period. Venous blood (20 ml) was withdrawn into EDTA tubes and centrifuged immediately for 15 min at 4 ºC and 1500xg to obtain plasma. From the plasma, total

cholesterol, triglycerides, direct LDL and HDL cholesterol, Apo A1 and Apo B100 were determined by standard enzymatic and immunoturbidimetric assays adapted for the Cobas-Mira-Plus autoanalyzer (SPINREACT S.A.U, Spain).

Seven lipoprotein fractions were isolated from the plasma by sequential preparative ultracentrifugation using density ranges of <0.95 g/ml for Q, 0.95–1.006 g/ml for VLDL, 1.006–1.019 g/ml for IDL, 1.019–1.044 g/ml for LDL1, 1.044–1.063 g/ml for LDL2, 1.063–1.0125 g/ml for HDL2 and 1.125–1.210 g/ml for HDL3. The samples were ultracentrifuged in a Kontron ultracentrifuge T-1075 rotor TFT 45.6 at 4 ºC for 30 min at 17,000 rpm to obtain Q; for 20 h at 40,000 rpm to obtain VLDL, IDL, LDL1 and LDL2 sequentially; and 40 h at 40,000 rpm to isolate HDL2 and HDL3 **[33]**. The fractions were retrieved by aspiration and dialysed against PBS for 24 h to remove NaCl and NaBr excess.

The lipids and apolipoprotein contents in the lipoprotein fractions were also measured using enzymatic and immunoturbidimetric assays adapted for the Cobas-Mira-Plus autoanalyzer (SPINREACT S.A.U, Spain).

3.2.3.1.2. Sample set No. 2

Human blood (n=18) was obtained from healthy and mild-to-moderate hypercholesterolaemic volunteers, and the plasma was prepared as described above. Samples were kept at -80 ºC until NMR spectroscopic analysis was performed.

Total cholesterol (TC), triglycerides (TG), HDL cholesterol (HDL-C), ApoA1, and ApoB100 were quantified using standard methods on an autoanalyzer (Beckman Coulter-Synchron, Galway, Ireland). LDL cholesterol (LDL-C) was calculated using the Friedewald formula.

3.2.3.2. 2D diffusion-edited ^{1}H NMR measurements

For the NMR measurement, 430 µl of serum or buffered isolated subclass was transferred to a 5-mm NMR tube. A double tube system was used. The external reference tube (O.D. 2 mm, supported by a Teflon adapter) containing the reference substance (9.9 mmol/l sodium 3-trimethylsilyl[2,2,3,3-d_4]propionate (TSP) and 0.47 mmol/l $MnSO_4$ in 99.9% D_2O) was placed coaxially into the NMR sample tube (O.D. 5 mm). This double tube system was kept at 4 ºC in the sample changer until the moment of analysis.

^{1}H NMR spectra were recorded at 310 K on a Bruker Avance III 600 spectrometer operating at a proton frequency of 600.20 MHz (14.1 T) equipped with a 10 A GREAT 1/10 gradient amplifier unit (Bruker®) using a 5-mm CPTCI triple resonance (^{1}H, ^{13}C, ^{31}P) pulse field gradient cryoprobe. Diffusion measurements were performed at 310 K to obtain a full signal from the melted lipids in the lipoproteins and to avoid serum degradation during the measurement.

One-dimensional reference ^{1}H pulse experiments were carried out using the nuclear Overhauser effect spectroscopy (NOESY)-presaturation sequence (RD-90º-τ_1-90º-τ_m-90º ACQ) to suppress the residual water peak. The time τ_1 was set to 4 µs, and the time τ_m (mixing time) was 100 ms. The 90º pulse length was calibrated for each sample and varied from 10.5 to 13.1 µs **[34]**. The result of the 90º pulse calibration of each sample was then used for the diffusion experiment. The spectral width was 30 ppm (18,000 Hz), and a total of 64 transients were collected into 64,000 data points for each spectrum.

The double stimulated echo (DSTE) pulse program (**Figure 1**), including bipolar gradient pulses and a longitudinal eddy current delay (LED), was used **[35]**. This pulse program is composed of a stimulated echo sequence with z-storage of the magnetisation, allowing for longer diffusion delays limited only by the longitudinal relaxation of the molecules. This requirement is compulsory if the pulse sequence is to be applied to

lipoproteins, where $T_1 >> T_2$. A double stimulated echo sequence efficiently compensates for flow convection currents that typically develop at elevated temperatures. Additionally, three orthogonal homospoil gradients are employed during the z-storage periods and during the repetition delay to shorten the phase cycle and eliminate accidental refocusing or unwanted magnetisation.

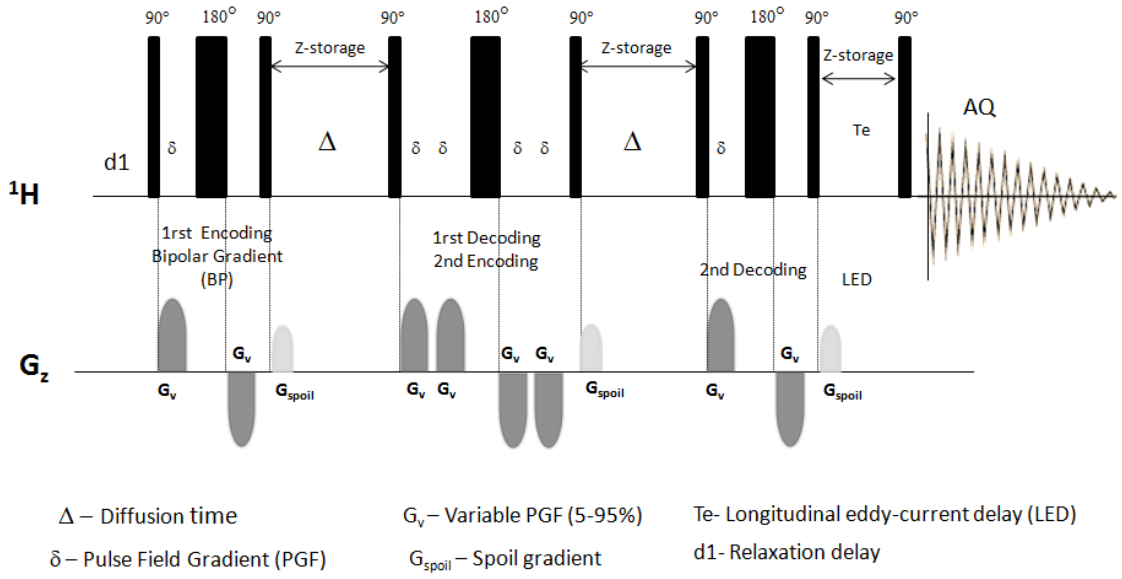

Figure 1. 2D double stimulated echo experiment with bipolar gradients and longitudinal eddy-current delay.

To further minimise the occurrence of convection currents, the heating air flow rate was set to 670 l/h. A spectral window of 18,000 Hz was accumulated in an acquisition time of 1.82 s to compare the attenuation with the one-dimensional NOESY.

The relaxation delay was 2 s, the FIDs were collected into 64,000 complex data points, and 64 scans were acquired on each sample. The gradient pulse strength was increased from 5 to 95% of the maximum strength of 53.5 G cm^{-1} (0.535 T m^{-1}) in 50 steps, where the squared gradient pulse strength was exponentially distributed. This distribution yields a concentration of experimental points around low-intensity gradients, where all

subclasses contribute to the spectra, and scattered measures in high intensity gradients, where the signals of small subclasses are attenuated **[27]**. A diffusion time of 104 ms and bipolar sine shaped gradient pulses of length 12 ms were applied to obtain a reasonable amount of diffusion of the lipoprotein signals in the raw serum. The total experiment time was 1 h 45 min per sample.

3.2.3.3. Surface fitting

When a DSTE pulse sequence is applied, the NMR signal obtained can be described using the following equation according to the applied gradient G **[27]**:

$$\boldsymbol{I(G) = I_0 e^{-\left[2\frac{2}{\pi}\gamma\delta G\right]^2 D\left(\Delta+\frac{4\delta}{3}+\frac{3\tau}{2}\right)}} \quad \textbf{(1)}$$

where γ is the ^{1}H magnetogyric ratio, δ (6 ms) is the length of the gradient pulse, G is the gradient strength (Gauss cm^{-1}), D is the diffusion coefficient (cm^2 s^{-1}), Δ (120 ms) is the effective diffusion time, τ (100 μs) is a short delay, 2/π is a gradient shape factor for the sine-bell-shaped gradient, and I and I_0 are the NMR signal intensities in the presence and absence of the gradient pulses, respectively.

For fully resolved resonances, the calculated diffusion coefficient in a given chemical shift is directly related to a single molecule. However, the methyl envelope is a broad region in which the responses of the different lipoprotein subclasses overlap. In our model, we decomposed the methyl envelope peak by a sum of a series of Lorentzian functions:

$$\boldsymbol{S_0(G,s) = \sum_{i=1}^{n} \frac{h_i}{1+\left(\frac{s-s_i}{\sigma_i}\right)^2} e^{-kD_iG^2}} \quad \textbf{(2)}$$

where S_0 is the methyl envelope intensity, $\boldsymbol{k = \left[2\frac{2}{\pi}\gamma\delta\right]^2\left(\Delta+\frac{4\delta}{3}+\frac{3\tau}{2}\right)}$, n is the number of

Lorentzian functions used to fit the envelope, h_i, s_i and σ_i are the height, shift and width of a Lorentzian function, respectively, and D_i is the diffusion coefficient related to the *i*th Lorentzian function. Once the diffusion coefficients have been estimated, the Stokes–Einstein equation can be applied to derive the radius associated with each function according to the following relationship:

$$\boldsymbol{D = \frac{kT}{6\pi\eta R_H}} \quad \textbf{(3)}$$

where k (J K^{-1}) is the Boltzmann constant, T (K) is the temperature, η (Pa s) is the plasma viscosity and R_H (Å) is the hydrodynamic radius. We have used a constant value of 1.1 mPa s as it has been reported that normal viscosity values in control individuals range from 1.10 to 1.30 mPa s at 37 ºC **[36]**.

Eight Lorentzian functions (F1–F8) were chosen to fit the NMR spectra of blood plasma. Function F1 was used to fit the background within the region 0.7–1 ppm. Functions F2–F8 were used to fit the methyl region within the range 0.8–0.9 ppm. We chose to run the algorithm using a constrained approach in order to avoid obtaining multiple solutions across different runs. Thus, the lower and upper bounds for the function heights were set to 0 and to the maximum of the methyl signal of the corresponding sample, respectively. The lower and upper bounds for the widths were set between 25 and 50 Hz for function F1 and between 3 and 10 Hz for functions F2–F8. The lower and upper bounds of the positions were set between 0.9 and 1 ppm for function F1. The region between 0.8 and 0.9 ppm was divided into seven intervals of equal width, and the limits of each interval were used as constraints to estimate the positions of functions F2–F8, placing each function in a different interval. Finally, the lower and upper bounds for the diffusion coefficients were set to 0 and to 15 cm^2 s^{-1}, respectively. The initial parameters taken for each surface fitting were randomly chosen between the constraints considered above, and spectra acquired at a gradient strength less than 13% of the maximum strength were discarded to avoid

interferences from low molecular weight metabolites.

To measure the goodness of the fit, we used the normalized root mean squared error (NRMSE) expressed as a percentage:

$$\boldsymbol{NRMSE}(\%) = \frac{\sqrt{\frac{\sum_i^n \sum_j^m (S_{exp} - S_{est})^2}{n \times m}}}{\boldsymbol{max}(S_{exp}) - \boldsymbol{min}(S_{exp})} \times \mathbf{100} \quad \textbf{(4)}$$

where S_{exp} and S_{est} are the experimental and estimated surfaces, respectively, n is the number of points considered in the interval length (0.7–1 ppm), and m is the number of gradients used. In our experiment, n and m have the same values for each sample and those are 300 data points and 34 gradients, respectively.

3.2.3.4. Statistical analysis

Principal component analysis (PCA) was carried out to determine the relationships between the NMR-derived areas. In addition, we performed MANOVA between pairs to test the discriminatory power of the resulting PCA model, obtaining the statistical significance (p-value) of the clustering. All statistical analyses were computed in MATLAB, Ver. 7.9.0.529 (The MathWorks) using the PLS-Toolbox, Ver. 5.2.2 (Eigenvector Research).

3.2.4. Results and discussion

The methyl region of a 2D diffusion-edited ^{1}H NMR spectrum from Sample 7 is shown in **Figure 2a**. The optimal Lorentzian functions for the same sample are shown in **Figure 2b**, and the logarithm of the attenuation of the intensity of each function is plotted against the squared gradient strength in **Figure 2c**. From **Equation 1** it can be derived that the slope of the logarithm of attenuation is related to the diffusion coefficient for a given

chemical shift. **Table 1** lists the means and standard deviations of the intensities, widths, chemical shifts, diffusion coefficients and sizes of the eight plasma samples of sample set No. 1. The estimated sizes clearly fall within the lipoprotein size ranges found in the literature **[18, 37]**. The NRMSEs for each sample were: 0.71, 1.04, 0.74, 1.19, 1.08, 1.22, 0.59 and 0.80%, respectively. Higher errors correspond to samples containing higher concentrations of either cholesterol or triglycerides. Function F1, which is associated with background lipoprotein responses, was excluded from further analysis.

An important issue was the number of functions used for the surface fitting. We have found that the minimum number that allows a good fitting for all the samples is eight, where the first one (F1) is used to fit the broad contributions from proteins (mainly albumin and albuminrelated compounds). If the number of functions is increased, the convergence time also increases, and more than one good solution appears. However, most of the additional solutions have no biophysical meaning (*i.e.*, the sizes for components F2–F8 are not sorted).

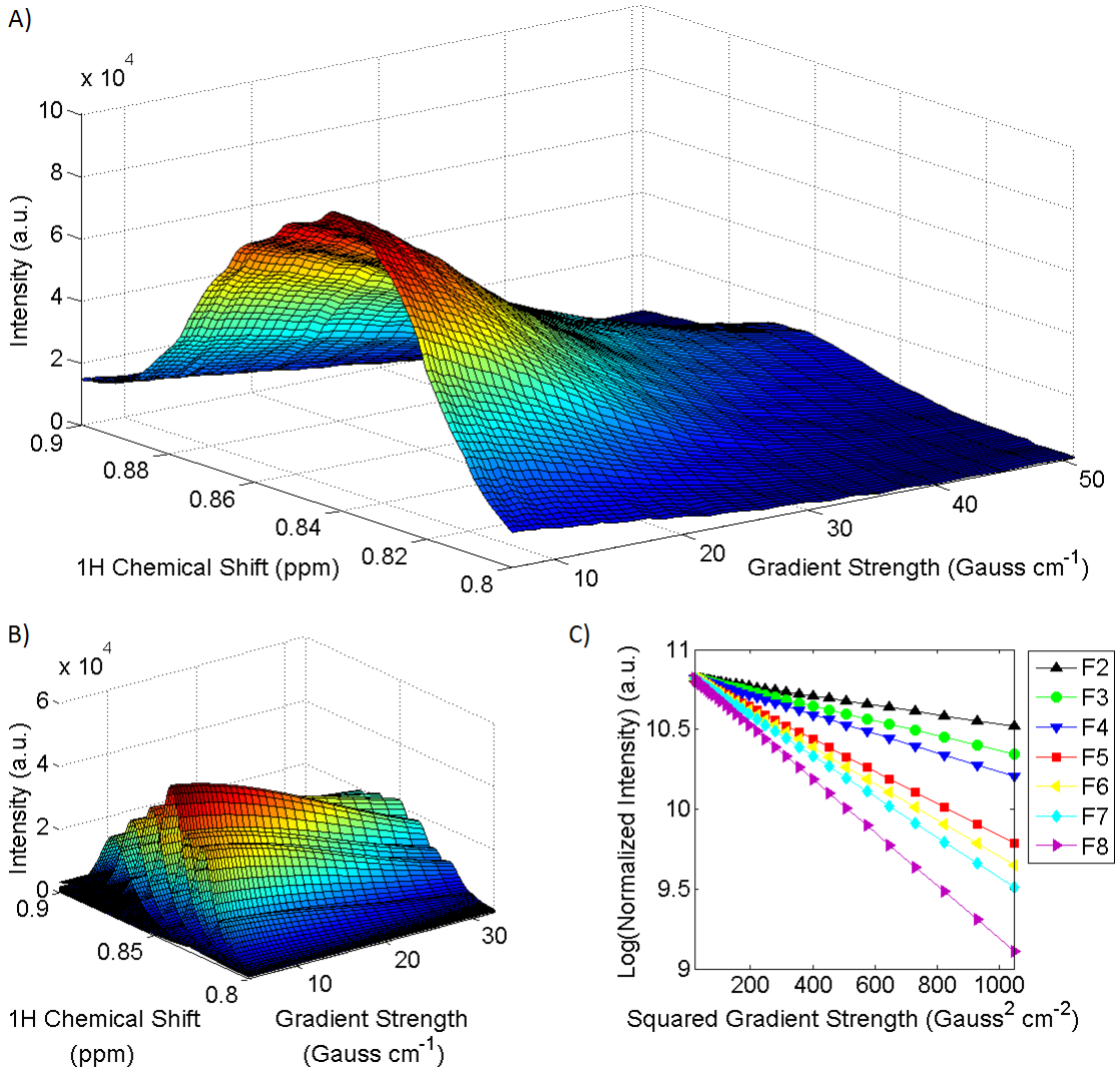

Figure 2. a) Example of 2D diffusion-edited ^{1}H NMR spectra (sample 7); b) optimal functions used to fit the surface; and c) representation of the logarithm of the intensity of each function against the square gradient strength (Gauss^{2} cm^{-2}).

Table 1. Average and standard deviation values of position (ppm), width (Hz), intensity (x10^5 a.u.), diff. coeff. (cm^2 s^{-1}), radius (Å) and rel. area (%) for functions F2 to F8.

Function	Position (ppm)	Width (Hz)	Intensity (a.u.)	Diff. Coeff. (10^{-7} cm^2 s^{-1})	Radius[a] (Å)	Rel. Area (a.u.)
F2	0.875 ± 0.002	12.5 ± 0.2	1.64 ± 1.59	0.8 ± 0.2	271 ± 64	43.7 ± 25.9
F3	0.862 ± 0.001	8.6 ± 2.0	0.61 ± 0.29	1.2 ± 0.3	176 ± 44	14.6 ± 4.5
F4	0.853 ± 0.002	8.1 ± 1.8	0.39 ± 0.13	1.9 ± 0.5	118 ± 32	10.5 ± 6.5
F5	0.841 ± 0.002	9.3 ± 0.4	0.39 ± 0.14	3.7 ± 1.1	60 ± 18	11.7 ± 7.0
F6	0.832 ± 0.001	8.3 ± 1.0	0.38 ± 0.12	4.4 ± 0.6	48 ± 6	9.6 ± 4.3
F7	0.823 ± 0.002	7.2 ± 1.0	0.31 ± 0.07	5.1 ± 0.5	41 ± 4	7.3 ± 4.0
F8	0.814 ± 0.002	5.8 ± 1.2	0.14 ± 0.03	6.8 ± 0.8	31 ± 4	2.7 ± 1.6

[a]A viscosity value of η=1.10 mPa s was used to derive the estimated sizes related to each Lorentzian function according to the Einstein-Stokes equation.

Another important concern is related to the application of the Stokes–Einstein equation to plasma samples, which is used to derive the radius of the lipoproteins. The viscosity of the sample is needed to determine the radius value. Though normal values in control individuals range from 1.10 to 1.30 mPa s at 37 ºC, patients with chylomicrons, hypertriglyceridaemia or mixed hyperlipidaemia have been found to have mean plasma viscosities significantly higher than those of controls **[38, 39]**; thus, the actual radius depends on the specific sample and should be measured for every sample. On the other hand, the Stokes–Einstein equation is strictly valid for a spherical particle with a radius, R_H, in a continuum solvent. It is often used to estimate the sizes of molecules in solution under dilute conditions **[28]**. These conditions are not satisfied in the case of plasma samples, and therefore, the calculated radius could require a further correction taking into account all these factors. Indeed, the viscosity parameter does not directly determine the diffusion coefficient in concentrated systems, where obstruction effects are important. All these

aspects have not been discussed in previous works related to diffusion NMR experiments and plasma **[29, 31, 32]**. For all the above reasons, the calculated radius values of lipoprotein subfractions should be taken as estimates.

In **Figures 3A-B**, we plot the dependence between the chemical shifts of functions F2–F8 and their derived sizes for normolipidaemic and dislipaemic samples, respectively. Our model shows that there is a decrease in particle size along the chemical shift axis for all the samples. This tendency can be modelled by the following equation:

$$\boldsymbol{r}(\text{Å}) = \boldsymbol{a}\boldsymbol{e}^{\boldsymbol{bs}} \quad \textbf{(5)}$$

where a=1.2 x 10^{-16} Å and *b*=-48.5 seg for normolipidaemic samples, *a*=6.3 x 10^{-17} Å and b = -49.4 seg for dislipaemic samples, and s represents a given chemical shift. The coefficient of determination for normolipidaemic and dislipidaemic samples is r^2=0.85 and r^2=0.81, respectively. In addition, there is evidence that functions with maxima at the same chemical shift can have different sizes, depending on the sample, suggesting that the size of an individual subclass depends not only on the position of the maximum but also on its width. Indeed, when analysing the relationship between the chemical shift and the width of a given function (**Figures 3C-D**), there is an almost linear dependence for normolipidaemic samples (r^2=0.88), whereas for pathological samples, this relationship is non-linear (r^2=0.62).

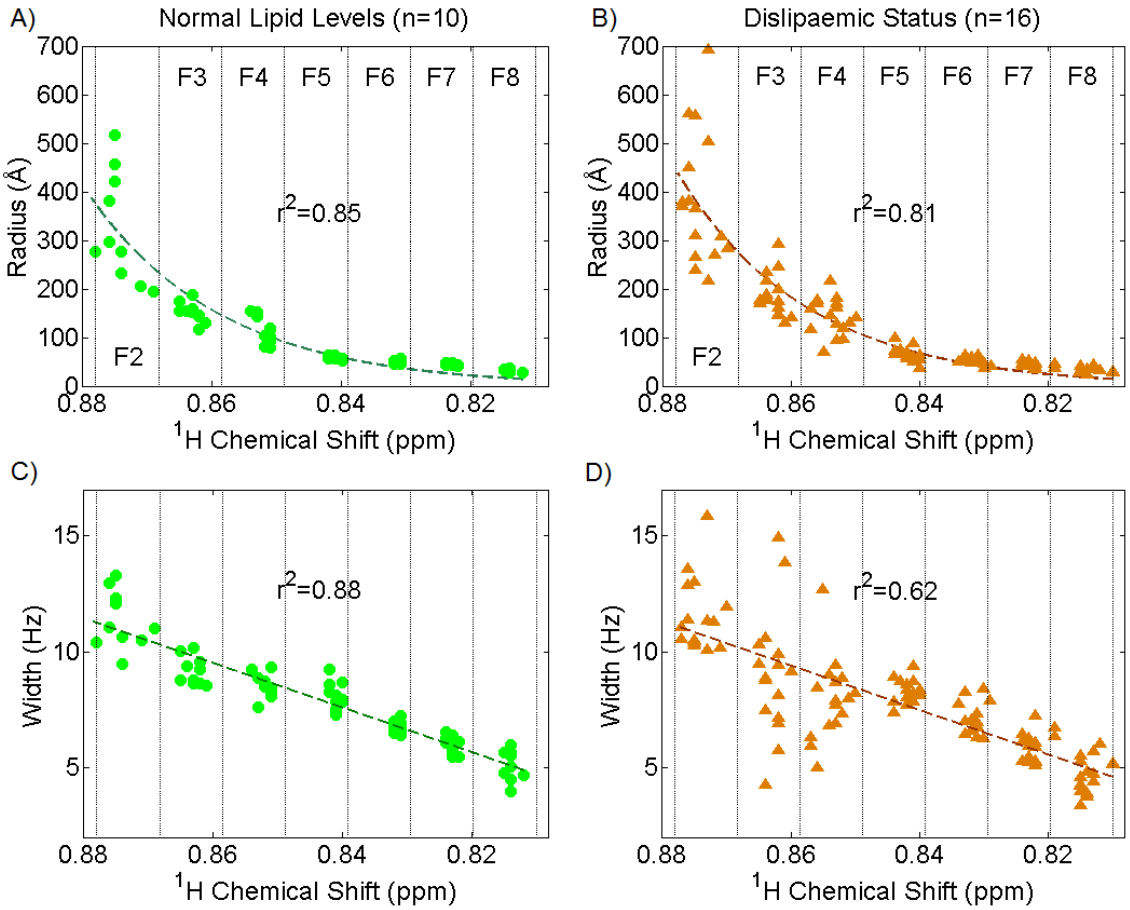

Figure 3. Radius (Å) and width (Hz) plotted against the chemical shift (ppm) related to the fitting functions (F2-F8) for sample set No. 1: a-c samples with normal lipid values and b-d samples with a dislipaemic status.

From our experimental data, we have shown that the radius associated with the Lorentzian functions decreases exponentially along the chemical shift axis. It is worth noting that the diffusion coefficients and the positions of the functions are not fixed before the fitting. This means that our method is able to find this relationship in an unsupervised way. These results are in good agreement with those of **[21, 40, 41]**. However, we have observed that functions related to dislipidaemic samples presented associated radii that were significantly different from the theoretical values. This is especially evident in the chylomicron-rich samples, where some functions have higher associated radii, which makes sense from a biological point of view. This finding reveals that the slower diffusion

coefficients associated with functions F2–F4 are indicative of the presence of chylomicrons in the sample.

On the other hand, we have observed that there is an almost linear increase in the widths of functions coming from normolipidaemic samples, whereas for dislipaemic samples this linear relationship no longer holds. This has been already investigated by other authors and they found that the averaged methyl lineshape is significantly narrower in cancer patients than in control patients **[42, 43]**. However, our method reveals that this behaviour is mostly due to the functions arising at higher chemical shifts (i.e. functions with larger associated radii). For chylomicron-rich samples, the widths of functions F2 and F3 are much lower than those of normolipidaemic samples. Thus, we have demonstrated that the chemical shifts of the functions are related not only to the average size of lipoproteins but also their widths.

In **Figure 4**, we present the relative contributions (%) of the areas associated with the functions for each sample of Sample set No. 1. Note the high differences between the different lipoprotein profiles (chylomicron-rich, hypertriglyceridaemic, hypercholesterolaemic and normolipidaemic samples). For instance, the radii associated with function F2 in samples 5 and 6 are indicative of the presence of chylomicrons, because of their very high values (>350 Å), which fit perfectly with large lipoproteins.

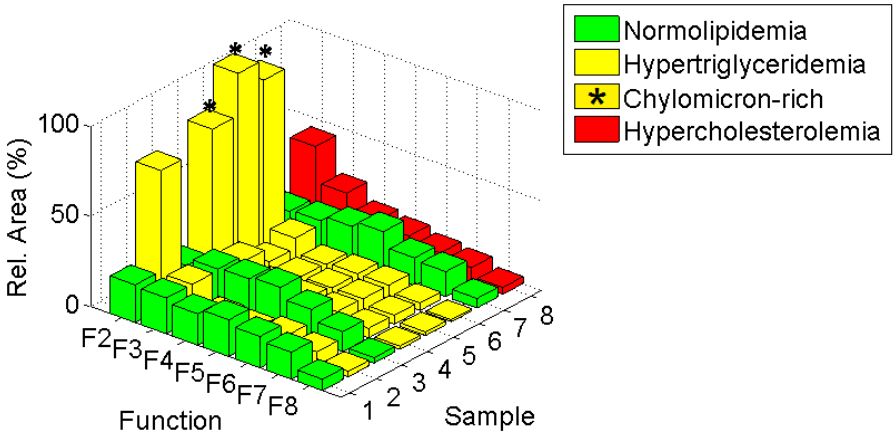

Figure 4. Relative areas (%) of functions F2-F8 for sample set No. 1. Samples are coloured according to their lipidaemic status.

Finally, we performed the same surface fitting procedure on sample set No. 2, and PCA of all 26 samples (sample sets Nos. 1 and 2), was carried out using the relative areas of functions F2–F8. **Figure 5A** shows the scores of PC1 versus PC2, representing 88% of the total variance. The samples were colour-coded according to the NCEP guidelines for CVD risk assessment for biochemistry values for every sample (**Table 1**), resulting in four different groups: normolipidaemia, low HDL/LDL ratio, high triglycerides and samples displaying both risk factors. Along PC1, which represents 71% of the total variation, samples tend to cluster according their triglyceride content. Thus, all triglyceride-rich samples present negative values for PC1, whereas normolipidaemia samples hold positive PC1 values. PC2, explaining 17% of the variance, explains differences in the HDL/LDL ratios among the groups considered. The statistical reliability of the lipoprotein combination subset determined was established by MANOVA testing in a pair-wise comparison. The results are shown in **Figure 5A** as a network connecting the centroids of each cluster and the statistical significance of the discriminatory power of each pair of groups. **Figures 5B-C** show the loading plots for PC1 and PC2, respectively. From this figure it is clear that the separation along PC1 is mainly due to the relative area of function F2 (mean radii of 362 Å)

and functions F4–F8 (mean radius from 133 to 32 Å, respectively), which seem to be inversely correlated. On the other hand, the variable that contributes most to the separation along PC2 is the relative area of F3 (mean radius of 166 Å). Furthermore, functions F7 and F8 also contribute to that separation and are inversely correlated to F3. This result would suggest that the ratio HDL/LDL is mainly represented by large LDL particles and small HDL particles. Moreover, function F4 (mean radius of 133 Å) does not contribute to the discrimination of samples in terms of their HDL/LDL ratio.

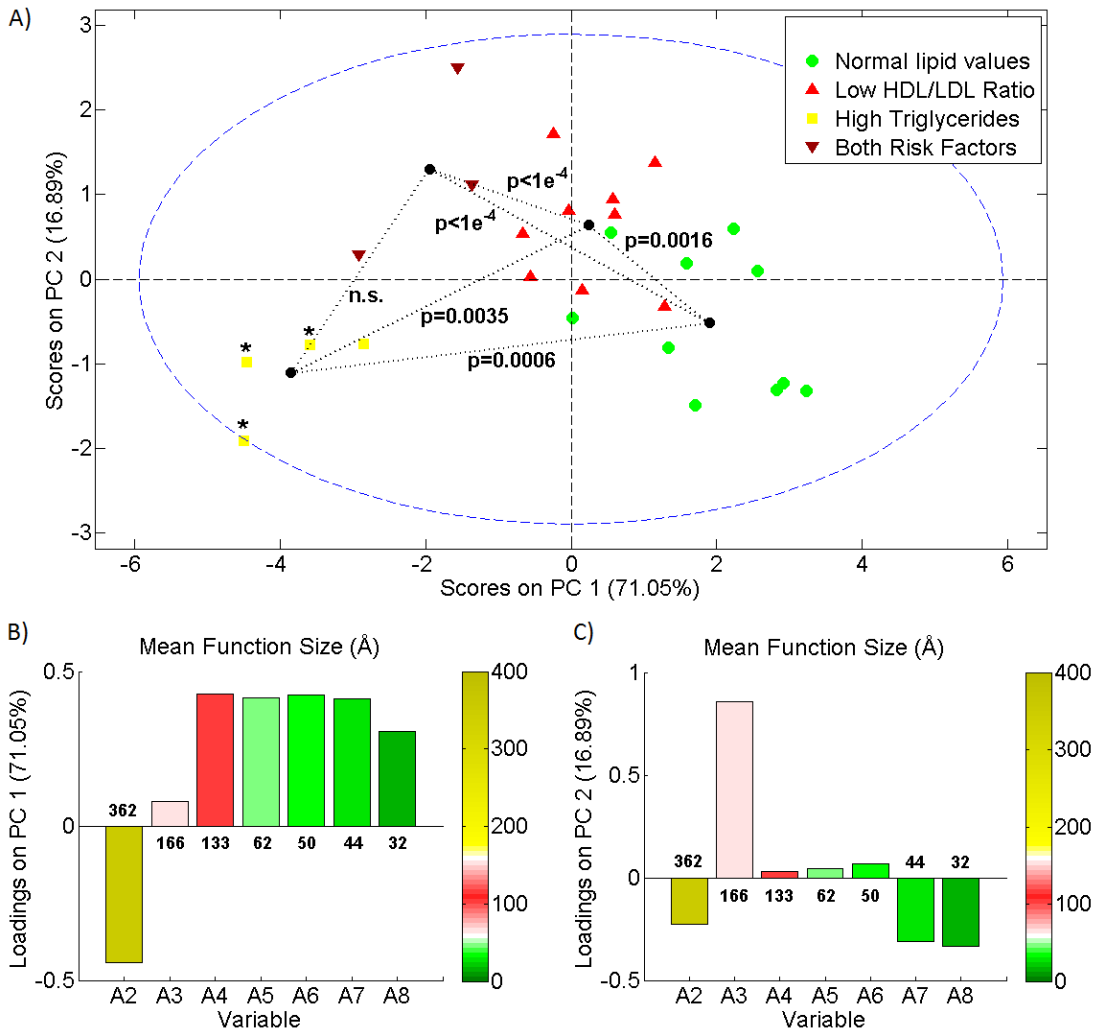

Figure 5. PCA of samples from sample sets No. 1 and No. 2 (*n*=26) plotted against with the areas of functions F2-F8. a) Score diagram showing the distribution of lipoprotein profiles; and b) loading diagram of PC1 and PC2, showing the most relevant variables involved in the above distribution.

Using an unsupervised algorithm such as PCA fitted on the area under the curve of each of the seven functions considered, we observed that samples belonging to different classes of dislipaemias, and thus presenting different lipoprotein profiles, tend to cluster according to their dislipaemia group. For instance, we confirm the results of Kumpula et al. who used SOM analysis with complete lipid profiles to show that large LDL particles are associated with relatively high LDL cholesterol and that small dense LDL particles are related to low plasma LDL cholesterol concentrations **[41, 44]**. In addition, the fact that function F2 (mean radius of 362 Å) and functions F4–F8 (mean radius from 133 to 32 Å, respectively) are inversely correlated agree with what we know of the metabolic pathways of lipoprotein particles, as it is known that higher triglyceride concentrations are inversely correlated with HDL cholesterol **[40]**. In order to test the need for a feature extraction method based on the surface fitting of the methyl signal with a collection of Lorentzian functions, we performed a PCA using only one gradient increment where attenuation of low molecular weight metabolites has been achieved (**Figure S1**). In this case, the clustering is not as strong as before. From these results, it can be determined that the area of each of the functions fitted with the methodology presented might be further used in fingerprinting strategies as a complementary tool to diagnose dislipaemias.

One important issue addressed by our method was fixing the measurement temperature. Some authors reported a temperature of 298 K, where the core cholesterol ester of LDL particles is not completely fluidised, but the temperature convection effects produced by the gradients are minimised **[29]**. Nevertheless, we have chosen to measure the plasma at 310 K for two main reasons. First, this is the physiological temperature, and all the lipids in the lipoproteins are melted, resulting in a stronger signal **[18, 19]**. A second consideration was that there is some consensus using this measuring temperature as the standard for serum **[19]**. Jeyarajah et al. and Dyrby et al. used 318 K, well above the phase-transition temperature, but we were concerned that some protein degradation processes

in the serum samples may be initiated at this temperature **[21, 45]**. We used a DSTE experiment to avoid convection currents that could arise at 310 K **[31]**.

In our approach, we fitted the surface of the methyl peaks of plasma samples of 2D diffusion-edited ^{1}H NMR spectra using eight Lorentzian functions; each was characterised by an amplitude, width, chemical shift and diffusion coefficient (*i.e.*, size). NMR-derived areas are associated with a lipoprotein fraction, with a concentration value given by the area of the function (related to the width and amplitude) and an average lipoprotein size, which is inversely related to the extracted diffusion coefficient through the Stokes–Einstein equation (**Equation 3**).

3.2.5. Conclusions

In this study, we used 2D diffusion-edited ^{1}H NMR experiments on plasma samples to characterise plasma profiles. A total of eight Lorentzian functions were used to fit the methyl peak at different gradient strengths. We demonstrate that the derived areas are useful in differentiating lipoprotein profiles. In agreement with previous studies **[36]**, we have experimentally demonstrated that when the lipoprotein plasma profiles are normolipidaemic, there is a perfect exponential relationship between the radii of the lipoproteins derived from the diffusion coefficient of the function and the shift positions of the maxima of the Lorentzian functions used to fit the surface. For non-normolipidaemic plasma profiles, this relationship deviates from the ideal exponential behaviour. In contrast with the previous line-fitting approaches, our diffusion-based method is able to overcome the non-ideal behaviour of non-normolipidaemic samples and to give results in accordance with classical biochemical analysis.

Thus, the surface fitting of 2D diffusion-edited ^{1}H NMR experiments provides a

robust way in which to characterise lipoproteins in plasma samples, yielding discrete size distributions for an arbitrary number of NMR-derived lipoprotein subclasses.

3.2.6. Acknowledgments

We acknowledge CIBER de Diabetes y Enfermedades Metabólicas, an initiative of ISCIII (Ministerio de Ciencia e Innovación) for partially funding this work, as well as FIS (project PI 081409). We acknowledge Dr. Gareth Morris for fruitful discussions about the diffusion experiments.

3.2.7. Supplemental material

Table S1. Analytical values of patients from sample set No. 1.

		Sample							
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Cholesterol (mmol/l)	**Total**	4.52	5.17	5.80	5.14	4.79	8.95	4.85	6.79
	VLDL	0.31	0.95	0.15	2.14	1.48	2.86	0.15	0.39
	IDL	0.19	0.19	0.13	0.60	0.21	0.65	0.16	0.21
	LDL1	1.10	0.72	2.30	0.50	0.49	0.84	1.93	3.40
	LDL2	0.51	1.77	0.49	0.14	0.89	1.54	0.36	1.17
	HDL2	0.72	0.20	0.97	0.12	0.10	0.41	0.64	0.12
	HDL3	0.76	0.58	0.45	0.99	0.44	0.57	0.64	0.67
Triglycerides (mmol/l)	**Total**	1.24	2.66	0.82	3.64	6.3	8.37	0.8	1.45
	VLDL	0.60	1.94	0.31	2.30	3.46	4.76	0.30	0.81
	IDL	0.06	0.14	0.08	0.15	0.18	0.26	0.06	0.08
	LDL1	0.09	0.06	0.22	0.06	0.09	0.18	0.19	0.32
	LDL2	0.01	0.06	0.02	0.00	0.05	0.15	0.01	0.04
	HDL2	0.05	0.00	0.06	0.02	0.01	0.06	0.03	0.03
	HDL3	0.08	0.07	0.13	0.07	0.12	0.16	0.06	0.07
Apo B (mg/dl)	**LDL1**	81	54	146	32	39	73	124	192
	LDL2	58	167	50	8	106	186	36	118
Apo A (mg/dl)	**HDL2**	147	7	152	40	18	30	127	18
	HDL3	186	176	163	194	162	182	170	180

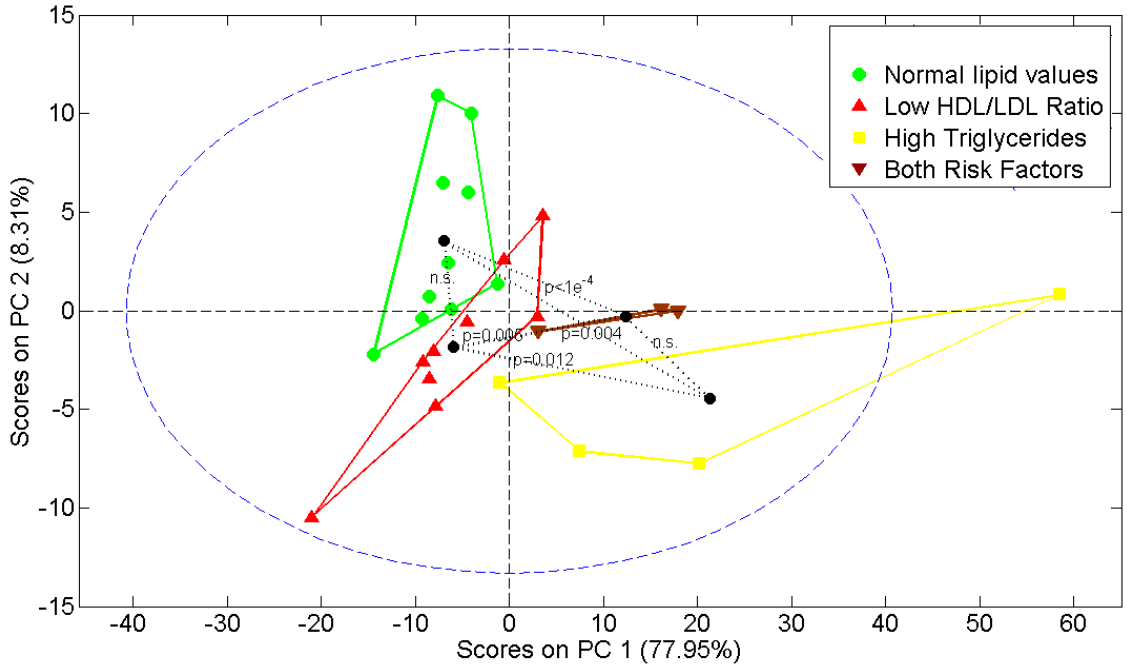

Figure S1. PCA of the methyl signal using only a gradient strength where low molecular metabolites have been completely attenuated.

3.2.8. References

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3.3. "Liposcale: a novel advanced lipoprotein test based on 2D diffusion-ordered ^{1}H NMR spectroscopy"

3.3.1. Abstract

Determination of lipoprotein particle size and particle number using advanced lipoprotein analyses is of particular interest since the LDL particle number has been shown to improve cardiovascular disease risk prediction. Advanced lipoprotein tests (ALT), however, are not yet routinely introduced in clinical practice partly due to the lack of standardization. Here we present the Liposcale test, a novel ALT based on 2D diffusion-ordered ^{1}H NMR spectroscopy. Our method uses diffusion coefficients to provide a direct measure of the mean particle sizes and particle numbers of the main lipoprotein classes and nine subclasses, namely large, medium and small VLDL, LDL and HDL. Using 177 plasma samples from healthy individuals and the concentration of apolipoprotein B and A from isolated lipoprotein fractions (as surrogates of particle numbers), we compared the accuracy of our NMR test to the Lipoprofile® test commercialized by Liposcience. Our test showed a stronger correlation between the NMR-derived lipoprotein particle numbers and VLDL-ApoB, LDL-ApoB, and HDL-ApoA concentrations (r=0.87, r=0.91, and r=0.68, respectively) than the Lipoprofile® test (r=0.78, r=0.75, and r=0.60, respectively). We also converted LDL particle numbers to ApoB equivalents (mg/dL) to allow a direct comparison with an established biochemical parameter. Our test yielded more accurate values of LDL-ApoB than the Lipoprofile® test (absolute mean bias of 1.7 and 7.4 mg/dL, respectively). Finally, a second cohort of 322 subjects, including type 2 diabetic patients with and without atherogenic dyslipidemia (AD) was characterized using the Liposcale test. Principal component analysis distinguished between both phenotypes (AUC=0.87) and showed concordant relationships between variables explaining AD. Altogether, our method provides reproducible and reliable characterization of lipoprotein classes and subclasses and it is

applicable to pathological states such as AD.

3.3.2. Introduction

Abnormal levels of blood lipids such as high concentrations of LDL cholesterol (LDL-C) and low concentrations of HDL cholesterol (HDL-C) increase the risk of cardiovascular disease (CVD), the first cause of death in developed countries **[1]**. Accordingly, the European Atherosclerosis and Cardiology Societies, and The National Cholesterol Education Program (NCEP) through their third report of the Adult Treatment Panel (ATP), established LDL-C and non-HDL-C as the primary and secondary target, respectively, of cholesterol-lowering therapy to reduce cardiovascular risk **[2]**. LDL-C lowering therapy has been shown to reduce the rate of cardiovascular events in patients with or without cardiometabolic risk (CMR) **[3, 4]**. However, a large proportion of patients under treatment, as well as undiagnosed individuals, may suffer cardiovascular events despite showing normal LDL-C levels. Cardiovascular events are more likely to occur in patients with diabetes and metabolic syndrome. These pathologies share a common phenotype characterized by high content of triglycerides, a preponderance of small, -dense LDL particles, and low HDL levels. In individuals with this particular phenotype, LDL-C has been shown to be a poor predictor of cardiovascular risk, so standard lipid panels that measure the cholesterol or triglyceride content of lipoproteins seem to be insufficient to predict risk of CVD. To fill this gap, advanced lipoprotein tests (ALT) **[5]** that allow for an extensive characterization of lipoprotein particles through a range of additional parameters such as size and particle number have been proposed for improving assessment of risk of CVD and for guiding lipid-lowering therapies **[6]**.

NMR spectroscopy is a technique that enables analysis of lipoprotein particles **[5]**. Briefly, depending on the size of the particle, the methyl moieties of the lipids in lipoprotein

particles resonate at slightly different frequencies, the smaller particles resonating at lower frequencies. Therefore lipoproteins can be quantified either by decomposing the methyl signal of the core lipids into individual signals **[7, 8]** or using statistical methods on the entire methyl envelope to estimate lipid concentrations **[9]**. Currently three methodologies use NMR to characterize lipoprotein particles. The method described by Jeyarajah et al. provides the size and particle number of the main lipoprotein classes (i.e., VLDL, LDL, and HDL) and the particle number of nine lipoprotein subclasses **[7]**. This method is built on a library of 1D NMR spectra from previously isolated lipoprotein fractions and on an algorithm that fits their NMR methyl signal with those of lipoproteins from serum or plasma samples. The particle sizes of the isolated lipoprotein fractions were determined by transmission electron microscopy and gradient gel electrophoresis. A second method described by Kaess et al. characterizes fifteen lipoprotein subclasses measuring the samples by magnetic field gradient intensities and temperatures **[8]**. The latter NMR methodology was reported by Ala-Korpela and colleagues **[9]**, and it estimates lipid content, size and particle numbers of the main lipoprotein classes, as well as the particle number of fourteen lipoprotein subclasses based on regression models calibrated using the lipid content and size obtained by high performance liquid chromatography.

NMR-based ALTs have demonstrated that LDL and HDL particle numbers (LDL-P and HDL-P) are more powerful than classical cholesterol markers as indices of cardiovascular risk **[10]**. For instance, LDL-P better indicated atherosclerotic risk than LDL-C in individuals with discordant LDL-P and LDL-C levels **[11]**. This discordance is usually explained based on the large variability in the amount of cholesterol per LDL particle and to differences in LDL particle size. In another study, HDL-P, but not HDL-C, was inversely associated with carotid intima-media thickness after adjusting for covariates **[12]**. Moreover, the use of NMR-derived lipoprotein subclasses improved risk stratification for subclinical atherosclerosis in comparison to conventional lipids **[13]**. Altogether, these and other evidences have

precipitated two significant events: 1) the 510(k) clearance from the U.S. Food and Drug Administration to market the Vantera(R) Clinical Analyzer commercialized by Liposcience, the first NMR-based diagnostic platform that determines LDL-P, and 2) the recommendation from the AACC Lipoprotein and Vascular Diseases Division Working Group on Best Practices to include LDL-P in the guidelines used to manage CMR based on the evidences collected from twenty-five clinical studies **[14]**.

Despite these advances, there is still some controversy about the introduction of NMR-based ALTs into clinical practice partly due to the fact that current methods do not provide a direct measure of lipoprotein sizes. As an alternative to current NMR methods, here we present the Liposcale test, a novel method for characterizing lipoprotein particles based on 2D diffusion-ordered ^{1}H NMR spectroscopy (DOSY). DOSY allows measuring the diffusion coefficients and directly calculating lipoprotein sizes through the Stokes-Einstein equation **[15]**. Noteworthy, a direct measure of lipoprotein sizes is of particular importance since they are used to compute lipoprotein particle numbers by dividing the spatial volume of the total lipid molecules by the mean volume (*i.e.* size) of the lipoprotein particles. Our rationale is that using DOSY to directly calculate lipoprotein sizes should yield more accurate measurements of lipoprotein particle numbers than current NMR-based ALT methods. To develop our new DOSY-based ALT we used a cohort of 177 healthy individuals and then we compared the lipoprotein particle numbers obtained using the Liposcale test with those obtained using the FDA-cleared ALT commercialized by Liposcience. Finally, we applied the Liposcale test to characterize a second cohort of 322 type 2 diabetic patients with and without atherogenic dyslipidemia. Our results demonstrate that our methodology can be applied to study samples with aberrant lipid and lipoprotein concentrations and can add insight into the understanding of metabolic diseases.

3.3.3. Materials and methods

3.3.3.1. Study subjects

We used samples from the VITAGE project to develop the Liposcale test **[16]**. Briefly, 177 healthy non-smoking men (0 cigarettes/day for >6 months) were enrolled in Clermont-Ferrand (France) and Reus (Spain). Exclusion criteria were familial hypercholesterolemia, chronic diseases (including diabetes, cancer, cardiac insufficiency, inflammatory diseases, and unstable hypertension), or alcohol abuse. Mean age was 45.8 ± 15.5 years, including a minimum age of 19 years and a maximum age of 75 years. Fasting venous blood samples were collected in EDTA tubes and centrifuged immediately for 15 min at 4ºC at 1500 x g. Plasma samples were then kept at -80ºC until further analysis. The ethics committee of the two recruiting centers approved the study protocol, and written informed consent was obtained from all volunteers.

We used a second cohort to validate the results provided by the Liposcale test. Eligible participants were 322 type 2 diabetic men and women, ranging 30-80 years of age, with (n=100) or without (n=222) atherogenic dyslipidemia (AD). AD was defined as having triglyceride levels over 150 mg/dL and HDL-C levels under 40 mg/dL (men) or 50 mg/dL (women). Exclusion criteria were the presence of cardiovascular disease, chronic hepatic or renal alterations. Patients under lipid-lowering medication entered a wash-out period of 4 weeks. Samples were kept at -80ºC until NMR analysis. The study protocol was also approved by the ethics committee of the participating institution.

3.3.3.2. Lipid and lipoprotein measurements

Lipoproteins were separated by sequential preparative ultracentrifugation, using a Kontron 45.6 fixed-angle rotor in a Centrikon 75 (Kontron Instruments, Italy). The lipoprotein fractions isolated were VLDL (d<1.006 g/ml), IDL (d=1.006-1.019 g/ml), LDL

(d=1.019-1.063 g/ml), and HDL (d=1.063-1.21 g/ml). Their cholesterol and triglyceride content was quantified using standard enzymatic assays adapted to the Cobas-Mira-Plus autoanalyzer (SPINREACT S.A.U., Spain) **[17]**. The concentration of ApoB100 and ApoA1 in the ultra-centrifuged fractions were also quantified using an immunoturbidimetric assay adapted to the Cobas-Mira-Plus auto-analyzer (SPINREACT S.A.U., Spain). The assay and the value of the calibrator concentration were standardized against the Certified Reference Material ApoA1 WHO/IFCC SP1-01 and ApoB100 WHO/IFCC SP3-07. Plasma samples were also analyzed by Liposcience (Raleigh, USA) to obtain reference values of VLDL, LDL, and HDL sizes and particle numbers **[7]**.

3.3.3.3. Advanced lipoprotein testing using DOSY

2D diffusion-ordered ^{1}H NMR spectroscopy (DOSY): Plasma samples were analyzed by NMR spectroscopy using a modified existing protocol **[18]**. Briefly, ^{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 310 K. We used the double stimulated echo (DSTE) pulse program with bipolar gradient pulses and a longitudinal eddy current delay (LED). The relaxation delay was 2 seconds, the finite impulse decays (FIDs) were collected into 64K complex data points and 32 scans were acquired on each sample. The gradient pulse strength was increased from 5 to 95% of the maximum strength of 53.5 Gauss cm^{-1} in 32 steps. The squared gradient pulse strength was linearly distributed.

Surface fitting: The methyl signal was surface fitted using a previously reported procedure **[18]**. The number of functions was increased to account for the nine lipoprotein subclasses. The uniqueness of the solutions was studied by fitting each sample ten times with randomly chosen initial values of the signal intensities. As a result, we obtained unique solutions for all samples after 10 runs. Normalized root mean squared errors (NRMSE) of the fittings were calculated as previously described **[18]**. The initial values of the signal

intensities were taken to be the mean solution values of all samples.

Prediction of lipid concentration: PLS regression models were calibrated to predict the cholesterol and triglyceride concentration of the main lipoprotein fractions (VLDL, LDL, and HDL). For the VLDL fraction, only the concentration of triglycerides was determined because cholesterol concentrations of VLDL particles were very low compared with the concentrations of triglycerides. 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. The coefficients of determination of the validation step ranged from 0.81 to 0.98 and are in the range of other reported studies **[19]**.

Lipoprotein sizing and particle number determination: The NMR functions were associated with a given lipoprotein class (VLDL, LDL or HDL) according to their associated NMR size. The main lipoprotein fractions were defined as VLDL (38.6-81.9 nm), LDL (14.7-26.6 nm) and HDL (6.0-10.9 nm). The mean particle size of every main fraction (VLDL, LDL, and HDL) was derived by averaging the NMR area of each fraction by its associated size. To obtain particle-weighted lipoprotein sizes, each NMR area was divided by their associated volume. Then a mean particle size was obtained for each lipoprotein class by multiplying the NMR lipoprotein particle sizes by their fractional particle concentration relative to the total particle concentration of a given class:

$$\boldsymbol{VLDL\ Size}\ (\text{Å}) = \frac{\sum_{i=1}^{n} \boldsymbol{R_i} \cdot \frac{\boldsymbol{A_i}}{\boldsymbol{R_i^3}}}{\sum_{i=1}^{n} \frac{\boldsymbol{A_i}}{\boldsymbol{R_i^3}}}, \boldsymbol{i = 1, \dots, 3}$$

$$\boldsymbol{LDL\ Size}\ (\text{Å}) = \frac{\sum_{i=1}^{n} \boldsymbol{R_i} \cdot \frac{\boldsymbol{A_i}}{\boldsymbol{R_i^3}}}{\sum_{i=1}^{n} \frac{\boldsymbol{A_i}}{\boldsymbol{R_i^3}}}, \boldsymbol{i = 4, \dots, 6}$$

$$\boldsymbol{HDL\ Size}\ (\text{Å}) = \frac{\sum_{i=1}^{n} \boldsymbol{R_i} \cdot \frac{\boldsymbol{A_i}}{\boldsymbol{R_i^3}}}{\sum_{i=1}^{n} \frac{\boldsymbol{A_i}}{\boldsymbol{R_i^3}}}, \boldsymbol{i = 7, \dots, 9} \quad \textbf{(1)}$$

where A_i and R_i are the area (au) and radius (Å) of a given lipoprotein particle *i*. The particle numbers of each lipoprotein main fraction were calculated by dividing the lipid volume by the particle volume of a given class. The lipid volumes were determined by using common conversion factors to convert concentration units obtained from the PLS models into volume units **[7]**. The relative areas of the lipoprotein components used to decompose the 2D spectra were used to derive the particle numbers of the nine lipoprotein subclasses.

3.3.3.4. Spiking experiments

We performed spiking experiments to validate the grouping of the NMR functions. Serum samples were obtained from three volunteers. Lipoprotein fractions (VLDL, LDL and HDL) were obtained by sequential ultra-centrifugation as described above. Then, serum samples were spiked with the lipoprotein fractions from the same subjects one by one. A serum sample made up with buffer to equal volumes as the spiked samples was also analyzed.

3.3.3.5. Analytical performance

The analytical performance of the Liposcale test involved blood samples from two volunteers. Briefly, fasting venous blood samples were collected in EDTA tubes and centrifuged immediately for 15 min at 4ºC at 1500 x g. Then, 5 aliquots were obtained for each subject and kept at -80ºC until the NMR analysis. The repeatability of the method was studied based on the analyses of the 5 aliquots of the two subjects in the same day, while the reproducibility was based on the analyses of the 5 aliquots of the two subjects through 3 consecutive days.

3.3.3.6. Statistical analyses

The Bland-Altman plot was used to measure the agreement between both NMR techniques in assessing particle sizes and numbers **[20]**. This plot uses the differences between observations made by the two methods on the same subjects and it analyses the agreement between two methods in terms of bias and precision. When there is a relationship between difference and magnitude, the standard Bland-Altman analysis can be extended either with a logarithmic transformation approach or a more general regression approach. As a measure of agreement we calculated the percentage error (PE) between both techniques, as described previously **[21]**:

$$\boldsymbol{PE} = \sqrt{\boldsymbol{CV}_{\boldsymbol{new}}^{\boldsymbol{2}} + \boldsymbol{CV}_{\boldsymbol{old}}^{\boldsymbol{2}}}\ (\%) \qquad \textbf{(2)}$$

We also defined a maximum PE by using the CV of the old method twice in **Equation 2**.

Spearman analysis was used to assess the relationship between continuous variables. All the analyses were performed with MATLAB version 7.10.0.499 R2010a (MathWorks). PLS and PCA models were built using PLS Toolbox (Eigenvector Research).

3.3.4. Results

3.3.4.1. Assignment of DOSY NMR functions to the main lipoprotein classes

The Liposcale test provides lipid concentrations (i.e., triglycerides and cholesterol), sizes and particle numbers for VLDL, LDL and HDL classes, as well as the particle numbers of nine subclasses, namely large, medium and small VLDL, LDL and HDL, respectively. **Figure 1** provides an overview of the Liposcale test showing the most important processes on which the characterization of lipoprotein classes is based. The use of the diffusion dimension represents the main difference between our approach and the NMR-based platform commercialized by Liposcience. The Liposcale test uses 2D spectra from DOSY experiments (**Figure 1A**) to decompose the lipoprotein peak from (CH_3) proton resonances into nine

Lorentzian functions (i.e., F1 to F9) (**Figure 1B**). This approach largely prevents multiple solutions and, consequently, enhances robustness of the measurements. Indeed, we obtained an average NRMSE for the surface fittings of less than 1.5% (see Materials and Methods section for details), which indicates that our deconvolution is highly reproducible. According to the Einstein-Stokes equation, the larger the diffusion coefficient the smaller the size of a given lipoprotein particle it will be. Thus, we associated the nine NMR functions with the three main lipoprotein classes based on their described size ranges (**Figure 1C**) **[22]**: F1 to F3 (38.6-81.8 nm) were associated with the VLDL particles, functions F4 to F6 (15.2-27.6 nm) with the LDL particles, and functions F7 to F9 (5.6-11.6 nm) with the HDL particles. These associations were validated using spike-in experiments. We isolated VLDL, LDL and HDL particles from serum samples of three volunteers by ultracentrifugation (see Materials and Methods section for details), and each isolated lipoprotein fraction was mixed one by one with the serum sample of the same individual. **Figure 2** shows the average relative change of every lipoprotein class in serum, represented as the total NMR area corresponding to VLDL (i.e., F1 to F3), LDL (F4 to F6) and HDL (F7 to F9) particles by applying our surface-fitting algorithm. Our results indicate that only the area of the lipoprotein fraction that has been spiked-in shows a substantial increment, demonstrating that our NMR functions are correctly assigned to VLDL, LDL and HDL particles.

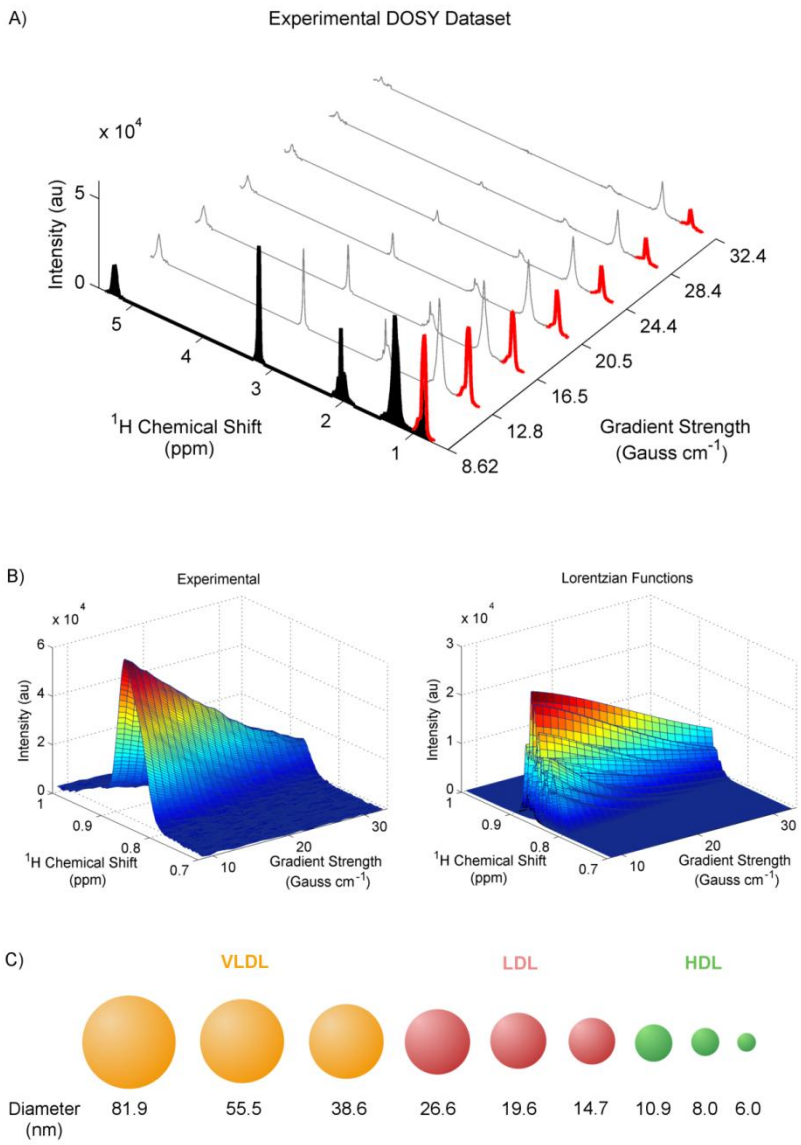

Figure 1. Overview of the Liposcale test. A) Experimental DOSY spectra. Seven gradients out of thirty-two total are shown for visualization purposes. The first gradient has been filled in black to show the NMR regions of the spectra that are used to calibrate and predict cholesterol and triglyceride concentrations using PLS regression. In red we show the attenuation of the methyl NMR signal along the gradient axis to show the region used to characterize the different lipoprotein subclasses. B) Surface fitting process for a given sample. The experimental attenuation of the methyl NMR signal (left) and the nine NMR functions used to fit the experimental surface (right). C) Using this methodology we can elucidate nine lipoprotein subclasses, namely large, medium, and small VLDL, LDL and HDL.

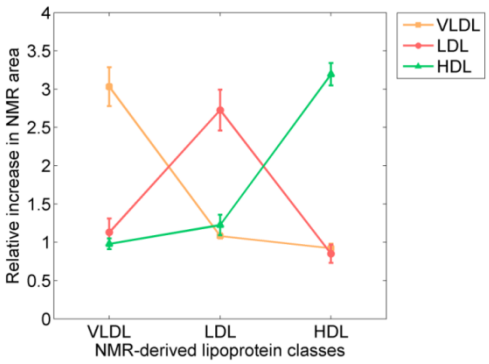

Figure 2. Spike-in experiments with isolated VLDL, LDL and HDL particles in serum samples. Mean relative changes in the total area under the NMR functions for each lipoprotein class (VLDL=F1+F2+F3, LDL=F4+F5+F6, and HDL=F7+F8+F9). Each color represents spiked serum samples either with isoalted VLDL, LDL, or HDL fractions.

3.3.4.2. Correlation between lipoprotein particle numbers and apolipoprotein concentrations

Next, we explored the degree of correlation between the VLDL, LDL and HDL particle numbers calculated using the Liposcale and the LipoProfile® tests, and the apoliproprotein content of each lipoprotein class, since the apolipoprotein concentrations in each class can serve as a surrogate of the number of particles. **Figure 3A** shows a strong positive linear relationship between the concentration of VLDL-ApoB determined using biochemical methods and the VLDL-P values determined using the Liposcale test (r=0.91) and the LipoProfile® test (r=0.75). Similarly, the correlation coefficients between LDL-P determined via the Liposcale and LipoProfile® tests and its respective apolipoprotein concentration were 0.86 and 0.78, respectively (**Figure 3B**). Finally, the correlation coefficients between HDL-P and HDL-ApoA were 0.68 for the Liposcale test and 0.60 for the LipoProfile® test (**Figure 3C**). Thus, the Liposcale test showed a stronger correlation between lipoprotein particle number and apolipoprotein concentration than the FDA-cleared NMR method that we took as the benchmark for the measurement of the number of particles in each lipoprotein class.

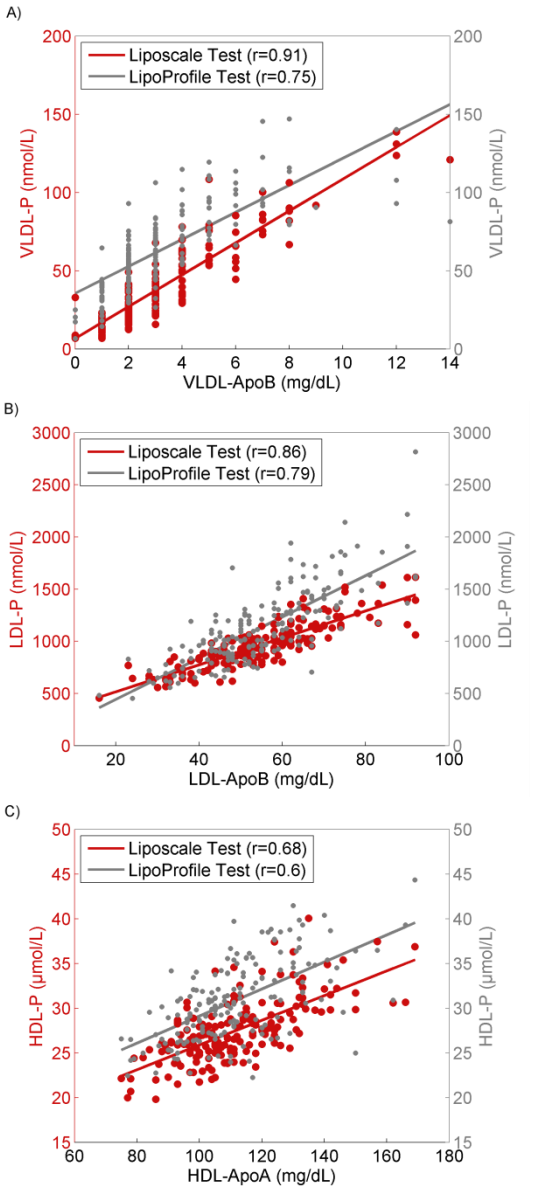

Figure 3. Regression analyses that examine the relationship among lipoprotein particle numbers using the Liposcale and LipoProfile® tests, and the apolipoprotein concentrations of A) VLDL, B) LDL, and C) HDL particles.

3.3.4.3. LDL particle size and number agreement between the Liposcale and the *LipoProfile*® NMR tests

LDL-P is the most validated and clinically useful parameter that advanced lipoprotein tests can determine. In this regard, three consensus reports **[3, 23, 24]** have all recommended that apoB and/or measurements of LDL-P shall be incorporated into existing consensus guidelines for advanced cardiometabolic risk management. Thus, here we

measured the agreement between our 2D-NMR test (i.e., Liposcale) and the 1D-NMR test (i.e., LipoProfile®) (see **Figures 4A-B**). From the VITAGE cohort, the mean LDL-size and LDL-P were 17.5 nm and 967.1 nmol/L using Liposcale, and 21.3 nm and 1133.1 nmol/L using LipoProfile®, respectively. This results in an average LDL-size and LDL-P difference (i.e., bias) between both methods of 3.7 nm and 166 nmol/L, respectively. We further analyzed the agreement between both methods using the Bland-Altman plot. The plot is characterized by a proportional bias in the form of a negative linear relationship between the two methods resulting from fewer number of small LDL particles using the Liposcale test in comparison with the LipoProfile® test. The logarithmic transformation of data did not correct this proportional error. Thus, to narrow down the limits of agreement the difference between the methods was regressed on the average of the two methods (**Figure 4B**).

The coefficient of variation (CV) of LDL size and LDL-P was 3.6% and 22.5% using the Liposcale test, and 3.4% and 32.3%, respectively, using the LipoProfile® test. The percentage error (PE) obtained between both methods was 4.8% for LDL size, and 39.4% for LDL-P. In short, we consider that the agreement between both NMR tests is acceptable based on a PE below 5% for LDL-Size and 50% for LDL-P.

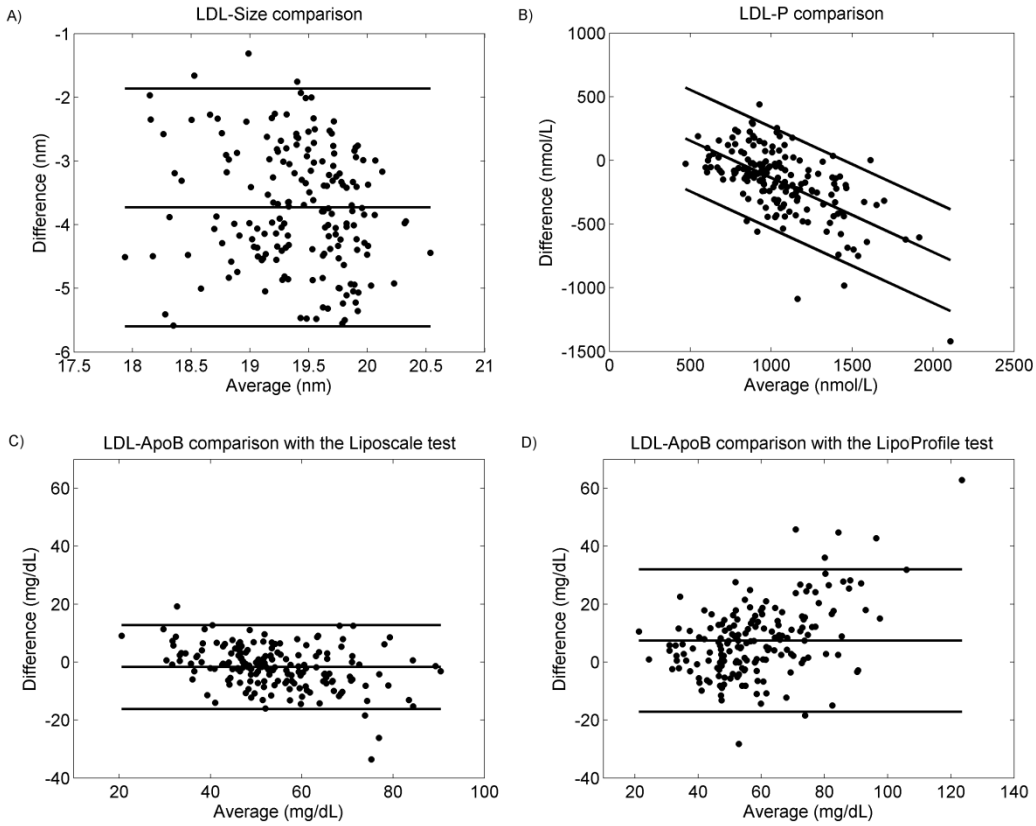

Figure 4. Bland-Altman analyses to measure the agreement between both NMR tests in assessing particle sizes and numbers. Comparison of A) LDL-size and B) LDL-P as assessed by the Liposcale and the LipoProfile® tests. Comparison of LDL-ApoB concentrations measured from isolated LDL fractions using a classical biochemical method and the same concentration estimated using C) the Liposcale test and D) the LipoProfile® test.

The concentration of ApoB-100 serves as a reference value of LDL particle number due to the fact that each LDL particle contains only one molecule of ApoB-100. In this regard, some authors have suggested that LDL particle numbers (nmol/L) should be converted to apolipoprotein B (ApoB) equivalents (mg/dL) to allow a direct comparison with an established biochemical parameter **[14]**. Thus, to investigate which NMR test yielded more accurate LDL-P values, we converted LDL particle concentration (nmol/L) into ApoB concentration (mg/dL) on the basis of a molecular weight for ApoB-100 of 550 kDa **[14]**. Once again, we assessed the agreement between the LDL-ApoB concentrations estimated by the Liposcale and LipoProfile® tests, and the same concentration assayed biochemically from LDL fractions isolated by ultracentrifugation (see Materials and Methods for details)

using the Bland-Altman plot. The Bland-Altman analysis revealed a mean difference between the LDL-ApoB concentration estimated using the LipoProfile® test and biochemical methods of 7.4 mg/dL, while for Liposcale this mean difference was -1.7 mg/dL (**Figures 4C-D**). The CV of LDL-ApoB concentration biochemically assayed was 26.5%, yielding a maximum PE of 40%. LDL-ApoB concentration obtained using the Liposcale and the LipoProfile® tests yielded a CV of 22.5% and 32.3%, respectively. Thus, the PE between the biochemical assay and Liposcale was 34.8%, and 41.8% when comparing with LipoProfile®.

3.3.4.4. Analytical Performance of the Liposcale test

Next we assessed the precision of the Liposcale test understood as the ability of the assay to consistently reproduce the same result when different sample aliquots are taken from the same specimen. The repeatability of the method, calculated from the analysis of five different aliquots from two different subjects within the same day (i.e., n=10), on the determination of cholesterol and triglyceride concentrations, and particle numbers for the LDL class and its small, medium and large subclasses, was ≤ 7%. The reproducibility on the same parameters, calculated from the same five aliquots ran on three consecutive days, was ≤ 10% (see **Tables S1-S2** in **Section 3.3.7**). Similarly, the repeatability and reproducibility for cholesterol and triglycerides concentration, and VLDL and HDL particle number, were ≤ 6%. Finally, both repeatability and reproducibility for the mean particle size for every lipoprotein class were ≤ 1%. Overall, the precision values of our 2D-NMR method are within the range of those reported by Liposcience Inc. for the LipoProfile® test **[7]**.

3.3.4.5. Characterization of atherogenic dyslipidemia in an independent cohort of diabetic patients

To demonstrate that the Liposcale test can be used to characterize and discriminate individuals with aberrant lipid and lipoprotein values, we implemented our 2D-NMR

method on a cohort study of 322 subjects that includes type 2 diabetic patients (T2DM) with (n=100) and without (n=222) atherogenic dyslipidemia (AD). A principal component analysis (PCA) using the input variables cholesterol and triglycerides concentrations, mean sizes and mean particle numbers of VLDL, LDL and HDL classes, and particle numbers of nine subclasses of lipoproteins, shows two separate clusters along PC1 (41.42% of the variance) corresponding to the two patient groups, that is, T2DM and T2DM with AD (**Figure 5A**). In order to evaluate which are the main variables responsible for the separation of the T2DM patients with and without AD, the resulting loadings were visualized in a variables-loading plot (**Figure 5B**). The plot reveals that the triad of AD, namely increased blood concentrations of small, dense LDL particles, decreased HDL particles, and increased triglycerides concentration, contributes to the separation of the two patient groups. Importantly, the loadings plot also shows that T2DM patients with AD have a smaller number of large and medium LDL and HDL particles relative to T2DM patients without AD. Moreover, patients with T2DM and AD have a greater number of total LDL and, in particular, of small LDL particles. Patients with AD also have greater number of small HDL particles but, on the contrary, it does not lead to an increase in total HDL particle numbers.

Considering the first principal component as the output of a putative classifier, we calculated the receiver-operating curve (ROC) in order to evaluate the classificatory power of our PCA model. As can be observed in the ROC curve depicted in **Figure 6**, our approach showed an area under the curve (AUC) of 0.87, showing an excellent classification performance to discriminate between T2DM patients with and without AD. All together, these results demonstrate that the Liposcale test is clinically useful to classify individuals showing an abnormal lipid and lipoprotein pattern that is typical of AD, which has emerged as an important risk factor for myocardial infarction and cardiovascular disease.

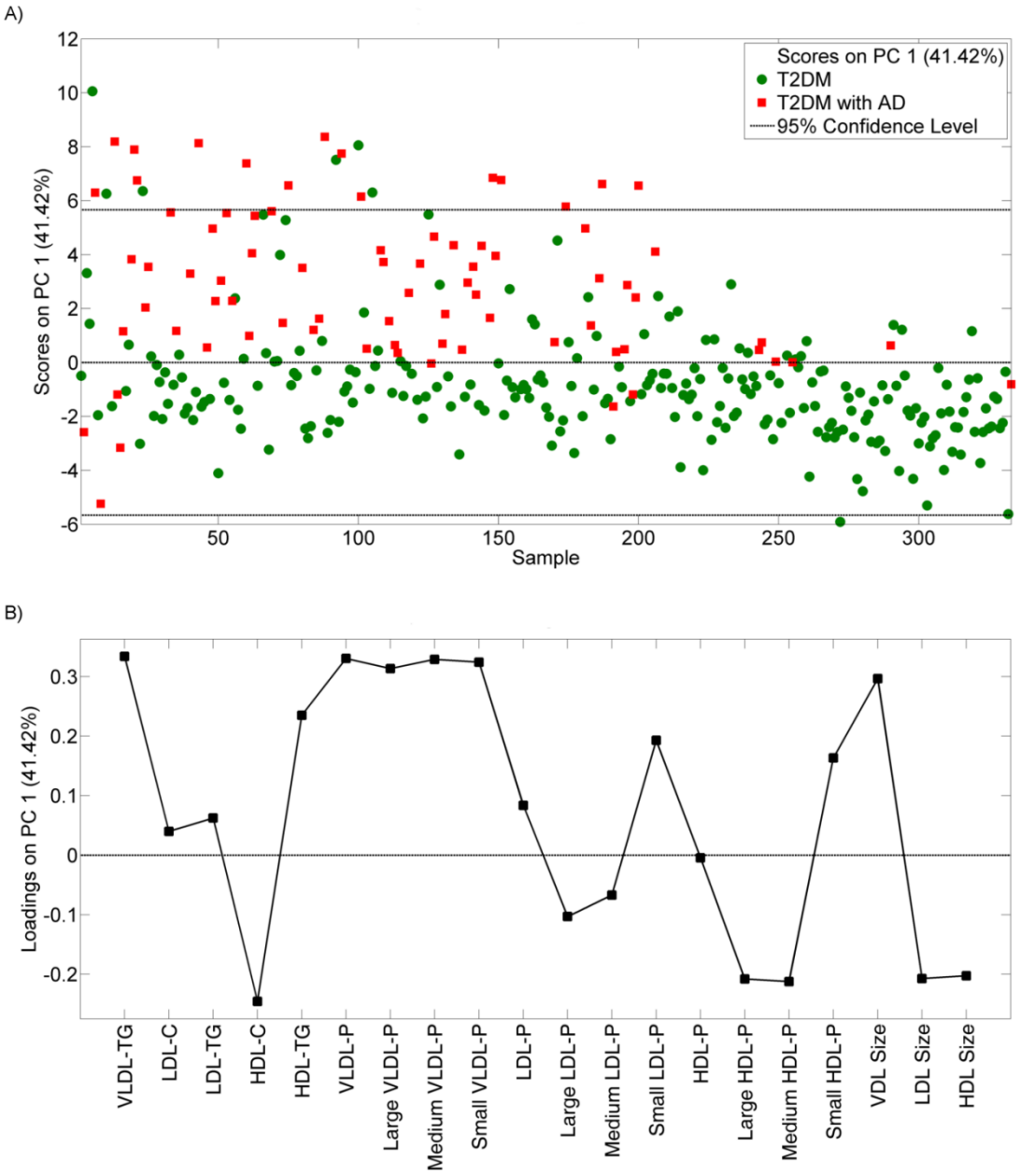

Figure 5. A) Scores plot and B) loadings plot for a PCA model built to discriminate between T2DM with and without AD.

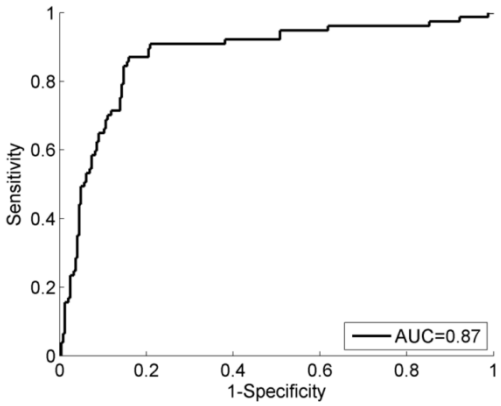

Figure 6. ROC curve evaluating the discriminatory power of the PCA model.

3.3.5. Discussion

The methyl moieties of the lipids in lipoprotein particles resonate at slightly different frequencies depending on the size of the particle, the smaller particles resonating at lower frequencies **[25]**. Though different lipoprotein subclasses may in theory be deconvoluted within the 1D-NMR spectrum according to their chemical shift positions, it has proven to be complicated in practice due to significant spectral overlap as different lipoprotein subclasses contribute to NMR resonance at the same frequency **[18]**. This is of great importance for LDL subclasses, which are particularly difficult to characterize due to the effect of neighboring lipoprotein classes (i.e., VLDL and HDL) **[26, 27]**. Provided that LDL-P has been validated as a better CVD predictor than LDL-C when these measures are discordant, the addition of a second dimension in the NMR spectrum by means of a diffusion experiment (i.e., DOSY) helps to better characterize this lipoprotein class. DOSY allows the separation of the lipoprotein subclasses according to their diffusion coefficient, and with the use of the Stokes-Einstein equation, DOSY NMR yields an objective separation of lipoprotein subclasses based on their size and favors the uniqueness of mathematical solutions compared to 1D-NMR.

In this study we present a novel advanced lipoprotein test based on DOSY NMR spectroscopy called Liposcale, which has been compared to the established NMR method

developed and commercialized by Otvos et al. and Liposcience (i.e., LipoProfile®) **[7]**, respectively, which has become the only *FDA*-cleared blood test that directly quantifies LDL particles. Our study constitutes the first attempt to compare two ^{1}H NMR methodologies, which is particularly important within the framework of standardized advanced lipoprotein tests **[14, 28]** if LDL particle number is intended to be used in cardiovascular risk management.

The agreement between the 2D-NMR (Liposcale) and 1D-NMR (LipoProfile®) methods for measuring LDL-P revealed a linear dependency between the difference and the magnitude of the measurements. However, the LDL-ApoB values obtained using our 2D-NMR method were more in conformity with biochemical values than those obtained using the *FDA*-cleared test. This disagreement between the two NMR methodologies may come partly from the small bias between the lipoprotein sizes determined by the two methods, through which our 2D-NMR method estimates smaller LDL particle sizes in comparison with the 1D-NMR method. This might be a consequence of the method used to derive the particle concentrations. While the LipoProfile® test computes mass-weighted particle numbers, our method computes particle-weighted particle numbers. From our point of view, the particle-weighted approach is more realistic since the concentration of small particles exceed the concentration of larger ones. A previous study also reported a linear dependence between the difference and magnitude of LDL-size measured by Liposcience and gradient gel electrophoresis (GGE) **[29]**. On the other hand, no method-comparison study has evaluated different LDL-P assays. As well as the Liposcience test, ion mobility (IM) analysis has been shown to be an alternative method for directly measuring LDL-P **[30]**. However, concerns have been raised about its lack of accuracy **[31]**.

Concerning HDL particles, our 2D-NMR method showed a better correlation between HDL-P and HDL-ApoAI concentration relative to the LipoProfile® test. Even so, regardless of which NMR method is used, the correlation between HDL-P and HDL-ApoAI is

not as good as when correlating LDL-P and the ApoB content. This could be partially explained by the lack of a direct relationship between HDL particles and ApoAI molecules. Unlike in LDL where each particle contains one molecule of ApoB, HDL particles contain between 2 and 4 molecules of ApoAI. A recent study has shown that the values of HDL-P estimated using an updated version of the Shen model, that relates the HDL-size and the ratio of HDL-C to ApoA-I concentration, are approximately 50%-60% lower than the HDL-P measurements obtained using the established NMR technique commercialized by Liposcience **[32]**. As can be seen in **Figure 3C**, the Liposcale test estimated HDL-P values that are lower than the ones estimated by the LipoProfile® test and consequently are more concordant with the number of HDL particles estimated by the modified Shen model **[32]**.

3.3.6. Conclusions

We evaluated a new methodology for quantifying lipoprotein subclasses based on 2D diffusion-ordered ^{1}H NMR spectroscopy (DOSY), which directly measures the sizes of lipoprotein particles. This methodology can provide the lipid concentration, lipoprotein size and lipoprotein particle numbers of the main fractions and subclasses. We found very similar correlations between our test and a reference NMR technique, although our derived particle numbers measured yielded higher correlations with external validations such as the concentration of VLDL-ApoB, LDL-ApoB, and HDL-ApoA. Moreover, the characterization of AD on type 2 diabetic patients further demonstrated the applicability of our methodology in a population with pathological states.

3.3.7. Supplemental material

Table S1. Intra-assay precision.

		Subject 1			Subject 2		
		Mean	**SD**	**CV (%)**	**Mean**	**SD**	**CV (%)**
Lipids (mg/dL)	**VLDL-TG**	23,7	0,7	3,0	18,1	0,5	3,0
	LDL-C	52,1	3,4	6,5	72,5	5,2	7,1
	LDL-TG	15,0	0,5	3,1	11,4	0,1	0,9
	HDL-C	67,8	1,2	1,8	49,3	0,8	1,7
	HDL-TG	10,8	0,2	1,6	6,3	0,1	1,1
Particle Number*	**VLDL-P**	17,6	0,6	3,4	13,3	0,5	3,4
	Large VLDL-P	0,3	0,0	10,0	0,2	0,0	10,6
	Medium VLDL-P	1,5	0,1	4,3	1,4	0,1	6,0
	Small VLDL-P	15,8	0,6	3,9	11,7	0,5	3,9
	LDL-P	539,6	29,3	5,4	669,4	41,9	6,3
	Large LDL-P	40,6	2,4	5,9	59,5	4,4	7,3
	Medium LDL-P	147,5	7,6	5,2	179,6	8,9	4,9
	Small LDL-P	351,4	22,2	6,3	430,4	29,8	6,9
	HDL-P	32,6	0,7	2,3	27,9	0,7	2,5
	Large HDL-P	2,6	0,1	2,9	1,1	0,1	9,1
	Medium HDL-P	12,1	0,3	2,6	6,2	0,5	8,0
	Small HDL-P	18,0	0,9	4,8	20,6	1,2	5,9
Size (nm)	**VLDL**	39,0	0,1	0,3	39,1	0,1	0,3
	LDL	19,8	0,0	0,1	19,8	0,0	0,1
	HDL	8,2	0,0	0,3	8,0	0,0	0,4

*VLDL/LDL: nmol/L; HDL: µmol/L

Table S2. Day-to-day precision based on the analyses of 5 aliquots of two different subjects for 3 days.

		Subject 1			Subject 2		
		Mean	**SD**	**CV (%)**	**Mean**	**SD**	**CV (%)**
Lipids (mg/dL)	**VLDL-TG**	24,2	1,3	5,5	18,2	0,7	3,7
	LDL-C	53,8	4,2	7,7	70,3	8,2	11,6
	LDL-TG	15,0	0,5	3,6	11,4	0,5	4,3
	HDL-C	67,8	1,1	1,7	49,5	0,9	1,9
	HDL-TG	10,8	0,2	1,5	6,4	0,1	1,5
Particle Number*	**VLDL-P**	17,9	0,7	4,2	13,4	0,5	3,6
	Large VLDL-P	0,3	0,0	8,9	0,2	0,0	23,4
	Medium VLDL-P	1,5	0,3	16,9	1,4	0,2	11,7
	Small VLDL-P	16,0	0,5	3,4	11,9	0,5	4,4
	LDL-P	552,3	35,0	6,3	654,0	67,2	10,3
	Large LDL-P	42,2	4,6	11,0	57,3	7,7	13,4
	Medium LDL-P	152,0	11,4	7,5	169,7	20,2	11,9
	Small LDL-P	358,2	26,0	7,3	426,9	43,1	10,1
	HDL-P	31,8	1,7	5,3	28,2	1,3	4,7
	Large HDL-P	2,7	0,2	7,3	1,1	0,1	10,8
	Medium HDL-P	12,4	0,8	6,4	6,1	0,7	11,1
	Small HDL-P	16,8	2,5	15,1	20,9	2,1	9,8
Size (nm)	**VLDL**	39,0	0,2	0,5	39,0	0,3	0,7
	LDL	19,8	0,0	0,2	19,8	0,0	0,2
	HDL	8,3	0,1	0,9	8,0	0,1	0,7

*VLDL/LDL: nmol/L; HDL: µmol/L

3.3.8. References

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4. DISCUSSION

4.1. NMR measurements of serum/plasma samples to characterize lipoprotein particles

Nuclear magnetic resonance (NMR) spectroscopy enables us to obtain a comprehensive analytical profile including the most abundant chemical components such as lipoproteins, aminoacids, organic acids, carbohydrates and albumin found in biological samples **[1, 2]**. In this thesis, we focused on the analysis of lipoprotein particles either in serum or plasma since detailed characterization of these macromolecular complexes can be obtained quickly without the need to apply complex sample preparation procedures. In fact, the most important reason for the use of NMR in the analysis of lipoproteins is to avoid the tedious physical isolation of the different lipoprotein fractions and subfractions from serum or plasma. In addition, another important advantage of using NMR spectroscopy to study plasma and serum is that measurements can often be made with minimal sample preparation, usually with only the addition of 5–10% D_2O for locking and a suitable standard in the sample or in an inner capillary for referencing or quantification purposes. Finally, measurements between different labs are comparable. Indeed, the coefficient of variation associated with inter- and intra-laboratory NMR experiments is typically very low and biological differences between individuals are far greater than the variations induced by experimental factors **[3]**.

Nevertheless, the results obtained when analyzing lipoprotein particles by NMR will be rendered worthless if samples are not collected, stored, and prepared correctly. For this reason, protocols for handling biofluids, such as plasma and serum, have recently been proposed **[4]**. In this regard, **Section 2.3.2** offers an overview of the different handling and conservation aspects that must be taken into account when dealing with serum or plasma samples for the analysis of lipoprotein particles by NMR. Whenever possible, the analyst

should be involved throughout the design and decision-taking-process of all protocols, from the experimental design and sample collection to final data results. This involvement is becoming more and more important in serum and plasma analysis, since it can lead to good inter-laboratory reproducibility.

Once serum or plasma samples are ready to be analyzed, the choice of the NMR pulse sequence to be used in order to obtain the desired information is decisive. Thus, Sections **2.3.3** and **2.3.4** describe a series of NMR pulses that can be applied to serum and plasma samples depending on the sort of molecules under investigation. Because our interest is the study of lipoprotein particles, this thesis is focused on the use of pulsed field gradient experiments. In fact, the single pulse spectrum of human serum or plasma samples is very complex. The highly overlapped resonances from metabolites, proteins, lipids and lipoproteins in the narrow chemical shift range of 1–13 ppm make it very difficult to successfully identify components even at a high magnetic fields **[1]**. Briefly, the resonance frequency of NMR signals of lipoprotein particles depends on their size and composition, but the neighbouring NMR signals of each class differ only slightly in chemical shift, thus limiting the reproducibility and accuracy of lipoprotein quantification in blood plasma by NMR line-shape fitting approaches. Therefore, to overcome the problem of extensive lipoprotein signal overlap in the analysis of NMR spectra of serum or plasma, another physical and independent measurement, such as diffusivity, could be desirable in addition to the chemical shift information. In this regard, different pulse sequences can be used so that results can be more accurate and reliable. A wide variety of useful spectroscopic techniques can be used to edit the NMR-detectable metabolite information in body fluids. In the case of lipoprotein particles, a diffusion-edited NMR spectrum enhances the NMR signal of lipoproteins and removes the signal of small metabolites.

Once the proper NMR pulse sequence has been selected, one have to configure a

set of acquisition parameters to obtain high quality raw data. The most crucial parameters to bear in when using diffusion experiments are the number of gradients used, their spatial distribution, the number of scans, and the temperature acquisition. In order to let the low molecular weight metabolites diffuse so only the signals from lipoprotein particles remain, one have to select a minimum gradient strength at which all the signals from low molecular weight metabolites are suppressed. Liu et al. used gradient values of 30% to 95% of the maximum gradient strength **[5]**. In the case of Dyrby et al., they used gradient strength values of 5% to 95% of the maximum strength, although they discarded the first gradients in further analyses since signals from low molecular weight metabolites are still visible at percentage of gradient strength below 25% **[6]**. Regarding the number of gradients and scans used, in the first case they used 64 gradients and 64 scans, while in the second case they used 24 gradients an 256 scans.

Another important issue addressed by our method was fixing the measurement temperature. Some authors reported NMR measurements below physiological temperature (298 K **[5]** or 303 K **[7]**), where the core cholesterol ester of LDL particles is not completely fluidised, minimizing its NMR visibility **[8]** but the temperature convection effects produced by the gradients are minimised. On the other hand, Jeyarajah et al. **[9]** and Dyrby et al. **[6]** used 318 K, well above the phase-transition temperature. In the case of the NMR LipoProfile® test, plasma samples are heated to 318 K to facilitate the deconvolution of highly overlapped lipoprotein subclass signals **[9, 10]**. However, we were concerned that some protein degradation processes in the serum samples may be initiated at this temperature. The method described by Kaess et al. characterizes fifteen lipoprotein subclasses **[11]**. The serum/plasma samples are measured at different temperatures (typically 278 K, 298 K, 308 K and 318 K). As the temperature rises, the relaxation times T1 and T2 increase and the lineshape narrows. These measuring conditions, combined with

three diffusion weighted NMR measurements carried out with a modified STE–LED (stimulated echo and longitudinal eddy-current delay) pulse sequence, produce a range of different spectra, which depend on the attenuation of the lipoprotein fractions under each of the measuring conditions. In our case, we have chosen to measure the plasma at 310 K for two main reasons. First, this is the physiological temperature, and all the lipids in the lipoproteins are melted, resulting in a stronger signal **[12, 13]**. A second consideration was that there is some consensus using this measuring temperature as the standard for serum **[13]**. The use of the DSTE experiment avoids convection currents that could arise at 310 K **[6]**.

4.2. Data processing and analysis of raw NMR spectra

After the acquisition of the NMR spectra, they have to be processed in a manner that important biological and clinical information is obtained. For this purpose, we have developed the Liposcale test composed by a set of mathematical and computational steps that yield a comprehensive profile of lipoprotein particles. These computational steps involve using the NMR spectra in different ways so different characteristics of the lipoprotein particles are obtained in one shot. **Section 2.4** describes the most important methods to process NMR data to characterize lipoprotein particles. In general, NMR-derived lipoprotein information can be obtained either by decomposing the methyl signal of the core lipids into individual signals **[5, 9, 11, 14]** or using statistical methods on the entire methyl envelope to estimate lipid concentrations **[2, 7, 15]**. Moreover, the mathematical and computational treatment of the raw NMR data will depend on its dimensionality nature (1D or 2D) **[5, 6, 9, 11, 16]**. Finally, the resulting NMR-derived information will vary depending on the calibration data used and the degree of data processing that is

performed. Briefly, the different NMR methodologies yield very different profiles, which can be composed by lipid parameters, particle size, particle numbers and/or raw NMR areas. Overall, NMR has proven to be a straightforward and useful technique for the qualitative and quantitative measurement of lipoproteins, giving the cholesterol and triglyceride concentration **[7]**, as well as the particle number of different subclasses and their size **[9]**. This is a great achievement because even though the behaviour of the different lipoprotein subclasses is modulated by their size, the overlap between them in the spectra is severe, and sophisticated deconvolution algorithms are required for data analysis **[9]**.

Section 3.2 describes the first attempt to characterize lipoprotein particles directly from plasma using DOSY. Briefly, we applied a mathematical deconvolution algorithm to decompose the DOSY spectra of 26 plasma samples into eight Lorentzian functions. We further used the obtained NMR areas to demonstrate that the extracted areas could separate the different lipoprotein profiles (normal, high triglycerides, low HDL/LDL ratio, and both risk factors) in a principal component analysis (PCA) score plot much better than the original spectra **[17]**. The results of this study also showed that the relationship between the shift position of the Lorentzian functions and the lipoprotein radii calculated from the experimental diffusion coefficient agrees with results found by other authors **[18]**, particularly in normolipidemic samples. An important issue in this first attempt was fixing the number of functions used for the surface fitting. We have found that the minimum number that allows a good fitting for all the samples is eight, where the first one (F1) is used to fit the broad contributions from proteins (mainly albumin and albumin related compounds). If the number of functions is increased, the convergence time also increases, and more than one good solution appears. However, most of the additional solutions have no biophysical meaning (e.g., the sizes for components F2–F8 are not sorted).

Then, **Section 3.3** describes the Liposcale test, the second version of our algorithm

to characterize lipoprotein particles directly from either serum or plasma samples. The Liposcale test provides lipid concentrations (i.e., triglycerides and cholesterol), sizes and particle numbers for VLDL, LDL and HDL classes, as well as the particle numbers of nine subclasses, namely large, medium and small VLDL, LDL and HDL, respectively. The number of particles in each lipoprotein class (VLDL, LDL, and HDL) were calculated by dividing the volume occupied by cholesterol and triglycerides (after a conversion of concentration units into volume units), by the volume of the mean particle size obtained by NMR for every class (see Materials and Methods for details). The concentration of cholesterol and triglycerides in VLDL, LDL, and HDL classes were obtained by calibrating a partial least squares (PLS) regression model. The rationale for calibrating the PLS models was to use them to further predict the cholesterol and triglycerides concentrations on the second cohort. The mean particle sizes were derived by averaging the NMR area of each fraction by its associated size. In this version, we used nine Lorentzian functions to decompose the lipoprotein peak from (CH_3) proton resonances of the DOSY experiments (i.e., F1 to F9). We assigned a fixed diffusion coefficient value, and a fixed amplitude and chemical shift to each function on the basis of their mean values obtained from a previous deconvolution step with all the parameters free, avoiding multiple solutions and, consequently, enhancing robustness of the measurements.

We then compared the Liposcale test with the LipoProfile®, the established NMR method developed and commercialized by Otvos et al. and Liposcience (i.e., LipoProfile®) **[9]**, respectively. The LipoPorfile test has become the only *FDA*-cleared blood test that directly quantifies LDL particles. Our study constitutes the first attempt to compare two NMR methodologies, which is particularly important within the framework of standardized advanced lipoprotein tests **[19, 20]** if LDL particle number is intended to be used in cardiovascular risk management. In fact, the reliable application of risk cutpoints and

treatment goals, which are based on data derived from epidemiologic trials, requires precise and accurate analytical measurements to be medically useful. The accuracy and precision of a method is established by standardization, a process involving a reference system of primary and secondary measurement procedures and reference materials, which assures that reported results for an analyte will agree with other laboratories over time, independent of methodology. At this time, no performance goals have been established by the NCEP or any other expert panel for LDL-P. However, standardization is not a prerequisite for the establishment of an association of a biomarker with a clinical outcome within any single laboratory and study, but it is critical for comparison between laboratories and studies or if data from several laboratories will be pooled for the establishment of disease risk assessments or treatment cutpoints. In this regard, LDL-P could conceivably conformed to the apo B Lipid Standardization Program (LSP) by adding the VLDL particle number to the LDL-P and converting nanomoles per liter (nmol/L) to mass units (mg/dL) based on the molecular weight of apo B (550.000 Da).

The agreement between the 2D-NMR (Liposcale) and 1D-NMR (LipoProfile®) methods for measuring the two LDL-P revealed a linear dependency between the difference and the magnitude of the measurements. However, the LDL-ApoB values obtained using our 2D-NMR method were more in conformity with biochemical values than those obtained using the LipoProfile test. This disagreement between the two NMR methodologies may come partly from the small bias between the lipoprotein sizes determined by the two methods, through which our 2D-NMR method estimates larger and smaller LDL particle sizes in comparison with the 1D-NMR method. This is a consequence of the wider dynamic range of lipoprotein sizes that allows DOSY spectra and the surface-fitting algorithm of the Liposcale test. Similarly, our 2D-NMR method showed a better correlation between HDL-P and HDL-ApoAI concentration relative to the LipoProfile test. Even so, regardless of which

NMR method is used, the correlation between HDL-P and HDL-ApoAI is not as good as when correlating other lipoprotein classes and the ApoB content. This could be largely due to the lack of a direct relationship between HDL particles and ApoAI molecules, unlike LDL particles and ApoB. In this case, each HDL particle contains between 2 and 4 molecules of ApoAI. Finally, the precision values of our 2D-NMR method are within the range of those reported by Liposcience Inc. for the LipoProfile test.

An important concern is related to the application of the Stokes–Einstein equation to serum or plasma samples, which is used to derive the radius of the lipoproteins. The viscosity of the sample is needed to determine the radii of the lipoprotein particles. Though normal values in control individuals range from 1.10 to 1.30 mPa s at 37ºC, patients with chylomicrons, hypertriglyceridaemia or mixed hyperlipidaemia have been found to have mean plasma viscosities significantly higher than those of controls **[21, 22]**. Thus, the radius depends on the specific sample and should be measured for every sample. On the other hand, the Stokes–Einstein equation is strictly valid for a spherical particle with a radius, R_H, in a continuum solvent (infinite dilution conditions). It is often used to estimate the sizes of molecules in solution under dilute conditions **[23]**. These conditions are not satisfied in the case of serum or plasma samples, and therefore, the calculated radius could require a further correction taking into account all these factors. Indeed, the viscosity parameter does not directly determine the diffusion coefficient in concentrated systems, where obstruction effects are important. **Section 3.1** describes a method for measuring lipoprotein sizes from isolated lipoprotein fractions. In this case, the viscosity values for each fraction were measured experimentally. Although the experimental viscosity measured for this study, under stress conditions, did not necessarily correspond to the effective viscosity experienced by diffusing particle, the improvement in the correlation between NMR and TEM radii when the experimental viscosity was used suggested that this

does provide an effective correction for the presence of solutes, including the lipoproteins. Another correction would imply the dilution of the samples being analyzed. However, because the inherent limitation of NMR is its low sensitivity, the dilution of the samples to avoid the obstruction effects commented above would lead to a decrease in signal to noise ratio and thus to noisier and poorer spectra. To compensate for this effect, one could increase the number of scans in each acquisition, but the acquisition time would become compromised.

4.3. Clinical utility of NMR-based advanced lipoprotein tests

Finally, the main application of NMR spectroscopy in the study of the metabolism of diabetes is the quantification of lipoprotein subclasses. This analytical technique has made it possible to relate different lipoprotein parameters and diabetes, and also other risk factors, in a univariate fashion (see **Section 2.5**). In case-control studies, the common conclusion was that VLDL size increased and LDL size decreased in diabetic patients. Most of the studies agree that HDL size appears to decrease in diabetic patients. On the other hand, there is general agreement that there is an increase in the values of total, large and small VLDLs, and total, medium and small LDLs. There is also general agreement that the values of large LDL and total HDL are lower in diabetic patients. A more recent application has focused on the classification and characterization of diabetic states in order to highlight patho-physiological mechanisms and come to a holistic understanding of the disease.

In this thesis, we have combined both approaches by developing a novel NMR-based advanced lipoprotein test to characterize lipoprotein subclasses and then we have applied clustering methods to distinguish between different pathological states. **Section 3.3.4.5** demonstrates that the Liposcale test can be used to characterize and discriminate

individuals with aberrant lipid and lipoprotein values. For this purpose, we implemented our 2D-NMR method on a cohort study of 322 subjects that includes type 2 diabetic patients (T2DM) with and without atherogenic dyslipidemia (AD). A PCA using the input variables cholesterol and triglycerides concentrations, mean sizes and mean particle numbers of VLDL, LDL and HDL classes, and particle numbers of nine subclasses of lipoproteins, showed two separate clusters corresponding to the two patient groups, that is, T2DM and T2DM with AD. In order to evaluate which were the main variables responsible for the separation of the T2DM patients with and without AD, the resulting loadings were visualized in a variables-loading plot. The plot revealed that the triad of AD, namely increased blood concentrations of small, dense LDL particles, decreased HDL particles, and increased triglycerides concentration, contributed to the separation of the two patient groups. Importantly, the loadings plot also showed that T2DM patients with AD had a smaller number of large and medium LDL and HDL particles relative to T2DM patients without AD. Moreover, patients with T2DM and AD had a greater number of total LDL and, in particular, of small LDL particles. Patients with AD also had greater number of small HDL particle but, on the contrary, it did not lead to an increase in total HDL particle numbers. Considering the first principal component as the output of a putative classifier, we calculated the receiver-operating curve (ROC) in order to evaluate the classificatory power of our PCA model. Our approach showed an excellent classification performance to discriminate between T2DM patients with and without AD. All together, these results demonstrate that the Liposcale test is clinically useful to classify individuals showing an abnormal lipid and lipoprotein pattern that is typical of AD, which has emerged as an important risk factor for myocardial infarction and cardiovascular disease.

NMR-based ALTs have demonstrated that LDL and HDL particle numbers are more powerful than classical cholesterol markers as indices of cardiovascular risk **[16, 24-26]**.

These and other evidences have stressed the importance of adding other parameters when analyzing lipoproteins such as the Apo B concentration or the number of lipoprotein particles and their size. In this regard, the National Lipid Association Expert Panel has recently advised testing of LDL-P to assess and manage cardiovascular risk **[27]** and the AACC Lipoprotein and Vascular Diseases Division Working Group on Best Practices recommended to include LDL-P in the guidelines used to manage CMR based on the evidences collected from twenty-five clinical studies **[20]**.

At the same time, there is some controversy about whether the LDL and HDL subclass parameters improve cardiovascular risk prediction in comparison with the standard lipid panel guidelines. It has been reported that the relationship between LDL particle size and cardiovascular risk is not inherent to this parameter but related to the fact that smaller particles lead to an increase in the particle number of this type of LDL **[27]**. Moreover, many of these tests are of limited accessibility and because of their high cost they cannot be applied to the general population. In addition, it may be difficult to identify the groups that might benefit from them. But in the context of personalized medicine, they could be used in selected groups of patients who have been identified as having some risk factors. As has been mentioned above, diabetic individuals characterized by a preponderance of small-dense LDL particles, high triglyceride levels and low HDL-C levels are a target group that is well-suited for an LDL-P measurement.

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5. CONCLUSIONS

In this thesis it has been demonstrated that 2D diffusion-ordered ^{1}H NMR spectroscopy (DOSY) is a suitable technique for the characterization of lipoprotein particles. We have tested this hypothesis within different contexts and approximations, from the analysis of lipoprotein fractions previously isolated using ultracentrifugation to the analysis of serum/plasma samples without the need for separation protocols and with minimal sample manipulation. Regarding the data processing approaches after acquisition of the NMR spectra, we used a surface fitting algorithm to characterize the 2D signal attenuation from DOSY experiments to obtain the average particle sizes in the case of the lipoprotein fractions and a discrete number of NMR areas in the case of the plasma samples.

The diffusion coefficients obtained in the first study presented in **Section 3.1** had low estimation errors, demonstrating the repeatability of this technique. The hydrodynamic radii found when using the experimental viscosity in the Stokes–Einstein equation were highly correlated with the mean TEM sizes, although there was a systematic difference between the TEM and NMR-derived sizes. This systematic difference was shown to be explained by the polydisperse distributions found by TEM; once this was taken into account, a high degree of agreement was obtained between the two techniques.

In the study presented in **Section 3.2** we demonstrated that the derived areas are useful in differentiating lipoprotein profiles. In fact, we demonstrated experimentally that when the lipoprotein plasma profiles are normolipidaemic, there is a perfect exponential relationship between the radii of the lipoproteins derived from the diffusion coefficient of the function and the shift positions of the maxima of the Lorentzian functions used to fit the surface. For non-normolipidaemic plasma profiles, this relationship deviates from the ideal exponential behaviour. In contrast with the previous line-fitting approaches, our diffusion-based method is able to overcome the non-ideal behaviour of non-normolipidaemic samples and to give results in accordance with classical biochemical analysis.

Finally, in **Section 3.3** we presented the final version of the Liposcale test, the NMR-based advanced lipoprotein test developed within the context of this thesis. The Liposcale test also uses a surface fitting algorithm to characterize the 2D signal attenuation of serum/plasma samples. In this case, the algorithm was extended to provide average particle sizes of the main lipoprotein classes (VLDL, LDL and HDL) directly from serum/plasma samples and the particle numbers of nine subclasses (namely large, medium and small VLDL, LDL and HDL). We found very similar correlations between our test and a reference NMR technique, although our derived particle numbers measured yielded higher correlations with external validations such as the concentration of VLDL-ApoB, LDL-ApoB, and HDL-ApoA. Moreover, the characterization of AD on type 2 diabetic patients further demonstrated the applicability of our methodology in a population with pathological states.

Importantly, the main difference between our 2D NMR approach and current 1D NMR methods is that the addition of a second dimension in the NMR spectrum by means of a diffusion experiment allows the separation of the lipoprotein subclasses according to their diffusion coefficient, and with the use of the Stokes-Einstein equation, DOSY NMR yields an objective separation of lipoprotein subclasses based on their size and favors the uniqueness of mathematical solutions compared to 1D NMR.

Altogether, 2D diffusion-ordered ^{1}H NMR spectroscopy provides a robust way in which to characterise lipoproteins either in lipoprotein fractions or in serum/plasma samples. Indeed, we propose that DOSY is a potentially useful alternative to other available approaches for measuring lipoprotein fraction particle sizes or discrete size distributions for an arbitrary number of NMR-derived lipoprotein subclasses due to its inherent robustness and minimal sample manipulation.

In the clinical setting, although advanced lipoprotein testing is not yet ready for routine clinical use, there is a need to account for a complete lipoprotein subclass

composition, size and functionality analysis to determine whether (i) progressive changes in lipoproteins are useful as therapeutic targets and (ii) certain subgroups of individuals can benefit from these analyses. These methods may also be important for advancing research and understanding the pathophysiology of CVD.