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ABSTRACT

Lipoproteins and their subfraction profiles have been associated to diverse diseases including Cardio Vascular Disease (CVD). There is thus a great demand for measuring and quantifying the lipoprotein profile in an efficient and accurate manner.

Nuclear Magnetic Resonance (NMR) spectroscopy is uniquely able to measure the lipoprotein profile of a blood sample non-destructively due to its sensitivity to both lipid chemistry and lipid-micellar physics.

However, the NMR spectra must be scaled/regressed to a primary method of reference, such as ultracentrifugation, using multivariate regression methods.

This review provides an overview of the field and explains the methods at stake.

1. Introduction

Lipoproteins (LPs) are important constituents of the lipid fraction of the human body that function as carriers for water-insoluble lipids through the aqueous bloodstream. LP vehicles provide the active mobilization of endogenous and exogenous (dietary) lipids through the aqueous compartments within the cells as well as in the blood and body tissues where lipid molecules can be either stored (i.e. adipose tissue) or used as energy source.

Based on their buoyant densities, LPs can be classified in five major groups: ChyloMicrons (CM), Very Low Density Lipoproteins (VLDL), Intermediate Density Lipoproteins (IDL), Low Density Lipoproteins (LDL), and High Density Lipoprotein (HDL) with CM being the biggest and least dense LP particles [1] (see Table 1). Lipoproteins fractionation and quantification is a matter of primary interest in the field of clinical medicine since elevated concentrations of Cholesterol (Cho) and TriGlycerides (TG), in specific LPs, have been associated with significantly increased occurrence of Cardio Vascular Diseases (CVDs) [2]. In particular, studies on lipoprotein particle distributions have shown a highly consistent and direct correlation between plasma LDL and the development of atherosclerosis [2]. Even though such epidemiological investigations have shown a positive correlation between total cholesterol concentrations in LDL and Coronary Heart Disease (CHD) mortality, total LDL cholesterol does not accurately predict the risk of CHD in many patients [2]. The LDL/HDL cholesterol ratios are nowadays considered risk indicators with greater predictive value than single parameters, such as LDL [3]. Due to the so called “reverse cholesterol transport”, HDL prevents or reverses the formation of atherosclerotic plaques that may derive from LDL metabolism and thus may represent a non-casual integrative marker of CVDs [4]. Moreover, it has been proven that individuals

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Table 1
List of the source, function and main biochemical characteristics of the major LP particles classified according to their buoyant density. The density of LPs depends on the lipids to protein ratio: the greater the lipid to protein ration, the larger the size and the lower the density. Adapted from Crook (2012) [1].

Complex	Source	Function	Density (g/ml)	Apoprotein	% Pro[a]	% TG[b]	% PL[c]	% Cho[d]
ChyloMicrons (CM)	Intestine	Transport exogenous lipids from the intestine to all cells	<0.9500	A, B, C, E	1	90	5	4
Very Low Density Lipoproteins (VLDL)	Liver	Transport endogenous lipids from the liver to the cells	0.950–1.006	B, C, E	8	55	12	25
Low Density Lipoproteins (LDL)	VLDL via IDL	Transport of cholesterol to cells	1.019–1.063	B	20	5	20	55
High Density Lipoproteins (HDL)	Intestine, liver (chylomicrons and VLDLs)	Transport of cholesterol from cells back to the liver	1.063–1.210	A, C, E	50	5	25	20

[a] Protein.
[b] Triglycerides.
[c] Phospholipids.
[d] Free Cholesterol.

with predominantly small LDL particles experience greater CHD risk than those with large-size LDL [5], making an accurate quantification of the LP subfractions an essential screening tool for CVDs prevention and diagnosis.

Several analytical approaches can be used for accurately measuring blood LPs, such as gel electrophoresis and Gel-Permeation High Performance Liquid Chromatography (GP-HPLC), but density gradient Ultra-Centrifugation (UC) represents the "gold standard method" for lipoproteins isolation and quantification [6]. Nevertheless, LP analysis by UC is time consuming and labor intensive as it requires numerous sample handlings and specific enzymatic assays are needed to further estimate their composition (usually Cho or TGs content) [7]. High-field ^{1}H Nuclear Magnetic Resonance (^{1}H-NMR)-based lipoprotein profiling has proven to be a valuable alternative to the standard quantification methods of total lipoproteins. ^{1}H-NMR, which is normally used for structure elucidation and chemical mixture quantifications, has one more advantage, namely that it is sensitive to the size (translational and rotational diffusion) and density of macromolecules and supramolecular aggregates [8]. This makes NMR a unique platform for investigating Lipoprotein Particle Distributions (LPDs) primarily because different LP fractions and subfractions have different magnetic susceptibilities which will broadcast different signals whose amplitude reflects the particles concentration [9]. Moreover, the minimum sample pre-treatment and the possibility of gaining relevant biochemical information with a single rapid experiment make ^{1}H-NMR spectroscopy a preferable/valuable screening tool for diagnostics as well as for large scale epidemiological investigations [10]. When combined with multivariate regression, NMR spectroscopy can be used to efficiently and accurately determine LP concentrations as well as TG and Cho content in specific lipoprotein fractions. However, the NMR prediction methods still depend on calibration with reference methods such as UC, gel electrophoresis or GP-HPLC.

This review aims at providing an overview on the research conducted for developing NMR as an efficient tool for the quantification of lipoproteins and will have a special focus on studies with coherent NMR data and reference data form UC and HPLC.

2. Chemical and physical properties of lipoproteins

Lipoproteins are micelle-like particles made up of lipids and proteins whose main function is to render hydrophobic lipid molecules, such as Cho and TG, compatible with the aqueous environment of our blood. In order to facilitate their mobilization in the bloodstream, TG and Cholesteryl Esters (CE), which are the major constituents of the non-polar core of the LPs, are packed into a spherical structure with an outer shell of Free Cholesterol (FC), PhosphoLipids (PLs) (i.e. phosphatidylcholine and sphingomyelin) and polar apolipoproteins. Amongst the diverse chemical components of the LPs, apolipoproteins play an important role in regulating and controlling the metabolism of specific lipoprotein fractions [11]. Based on their size and distribution, apolipoproteins have been classified in five main groups: apoA, apoB, apoC, apoD and apoE. Several specific functions have been ascribed to these proteins. Besides mediating lipid transport and redistribution among various tissues, apolipoproteins act as cofactors for enzymes of lipid metabolism and cover an important role in the maintenance of the structure of the lipoproteins.

Plasma lipoproteins are usually classified according to their buoyant density, determined by the lipid composition itself (i.e. phospholipids have a higher density than the neutral lipids) and by the lipid to protein ratio. The classification is made into five main fractions: ChyloMicrons (CM), Very Low Density Lipoproteins (VLDL), Intermediate Density Lipoproteins (IDL), Low Density Lipoproteins (LDL) and High Density Lipoprotein (HDL). These fractions can be further refined by delicate separation procedures into subdivisions of the VLDL, LDL and HDL fractions. Each of these subfractions has distinctive apolipoprotein compositions and biological properties [1].

Even though the standard methods for lipoprotein classification are based on LPs density (i.e. UC), several methods based on lipid composition and size (diameter) have been devised [12]. This is for example the case for gel electrophoresis and GP-HPLC in which lipoproteins are classified based on size fractionation and charge. Fig. 1 shows the correlation between the relative size of plasma lipoproteins and their hydrated density. The insert in Fig. 1 shows the typical micellar structure of a lipoprotein.

3. Sample handling procedures

Lipoproteins are heterogeneous particles whose distribution in the blood depends on genotype-specific properties and reflects the dynamic response of the human body to changes in the external conditions (e.g. diet, lifestyle and environment) [13]. The multiple sample handling steps required before LPs quantification, such as sample collection, sample preparation, analysis and storage, can alter the LPs structure by destroying the natural equilibrium of the sample. For this reason, high-throughput protocols and Standard Operating Procedures (SOPs) and Quality Control (QC) criteria have been developed for minimizing as much as possible the inherent variability arising from the sample handling steps [14]. Fig. 2 shows a schematic diagram of the blood sample collection and handling for lipoprotein quantification.

Sample collection: fasting vs. non-fasting state. According to the standard protocols, fasting blood samples are required for

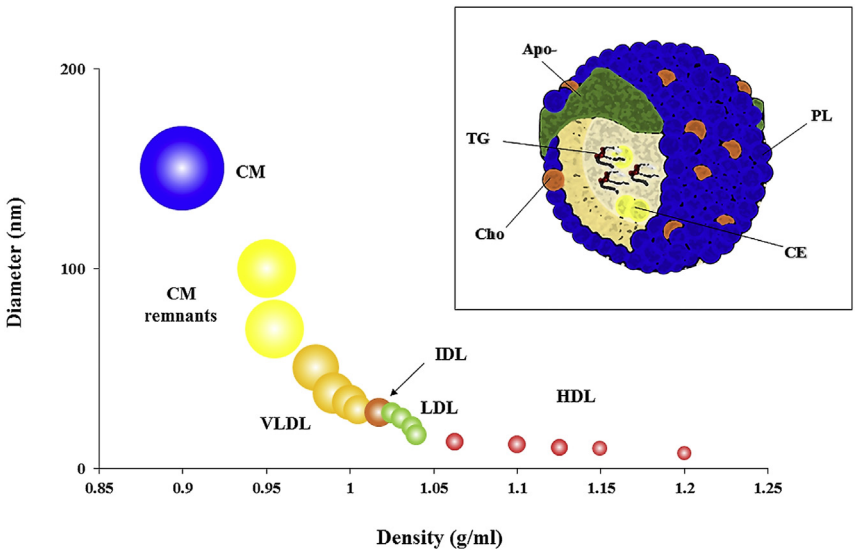

Fig. 1. The relationship between plasma lipoprotein size and hydrated density. The insert shows a schematic view of a typical lipoprotein micellar structure.

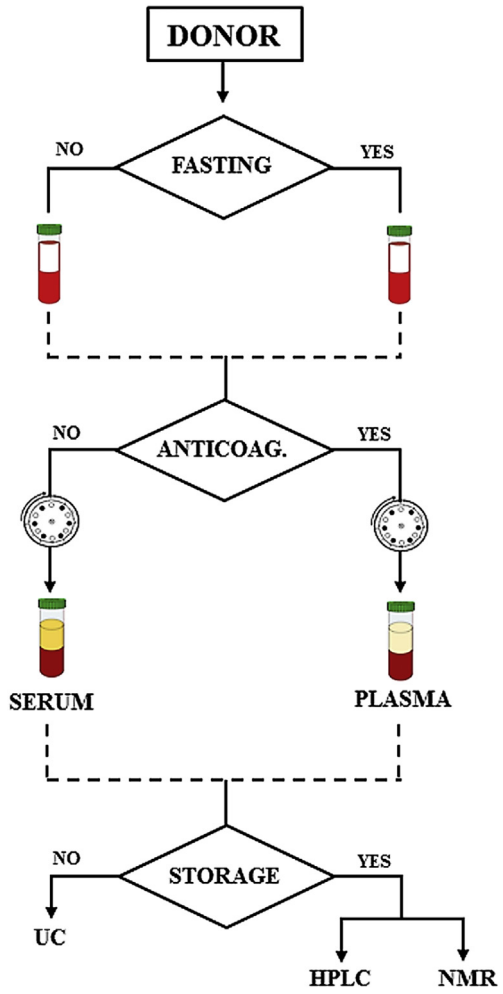

Fig. 2. The flow diagram shows the steps of the blood sample collection from the donor to storage for HPLC, NMR, and UC measurements. The different procedures for obtaining plasma and serum, from the addition of an anticoagulant agent (i.e. EDTA, heparin or sodium citrate) to the centrifugation step, are reported.

measuring lipoproteins since only the fasting state reflects the "homeostasis" of the human lipid profile in which the postprandial triglycerides in the form of CM are not dominating the measurements [15]. In fact, the presence of CM is a good measure of non-compliance to the fasting condition [16]. However, in subjects with metabolic syndrome CM remnants are also present in the fasting state [17]. Several studies have demonstrated that the TG concentrations of non-fasting healthy individuals may be stronger predictors of CVDs since the non-fasting state resembles better the day to day metabolism [18]. Indeed, chylomicrons adhesion on arterial walls may be as an important risk factor as LDL-Cho in causing strokes and heart attacks [19]. In order to determine the ability of an individual to clear postprandial lipids or the capacity of a food ingredient to reduce lipid uptake, TG-rich lipoproteins and, in particular, CM can be measured after an oral fat load [20]. It is thus dependent on the purpose of the investigation if fasting or non-fasting samples should be used. In fact, non-compliance to fasting is rapidly detected in the lipid profiles of the NMR spectra of blood such as have been shown in intervention studies with dietary fibers [16]. However, CM can also be present in the fasted state in subjects with metabolic syndrome.

Measurement matrix: serum vs plasma. Serum and plasma samples are routinely used for LP measurements. Both fractions derive from blood samples that have undergone different biochemical treatments after collection. In the serum case, coagulation factors (i.e. fibrinogen) along with blood cells are removed by centrifugation, while plasma is typically obtained from blood samples to which an anticoagulant agent (i.e. heparin or EDTA) is added before the removal of blood cells. The choice of the most appropriate anticoagulant agent is a crucial point in LP studies since, depending on the applied analytical technique, it may affect the measurement of the lipoproteins. Sample collection into an EDTA or heparin tubes will produce high intensity peaks (EDTA) or overlapping signals (heparin) in the NMR spectra [14,21].

Sample handling and storage. Standardized sample handling and storage conditions are highly important in order to obtain blood samples with stable physical lipoprotein characteristics (i.e. density and size). Due to the rapid increase in the number of studies aimed at biomarker discovery by profiling technologies (i.e. HPLC and NMR spectroscopy), detailed standardized procedures for sample handling and storage have been developed whose analytical and

experimental bias have been carefully assessed [22,23]. In particular, in the case of NMR spectroscopy, storage conditions have been shown to be critical and special attention has been paid to address the effect of common sources of analytical bias on serum and plasma profiles in terms of reproducibility and reliability of the obtained results [24]. According to the most recently developed SOPs, blood serum and plasma samples can be stored for 9 months at −80°C without leading to differences in the NMR profiles [23]. In contrast, fresh serum or plasma are recommended for UC measurements since fresh samples provide higher measurement accuracy and consistency for HDL-Cho, LDL-Cho, VLDL-Cho and TG concentrations thus suggesting that LP separation should be performed as soon as possible after sample collection [25]. In any case multiple freeze/thaw cycles must be avoided, as it has been shown that even a single freeze–thaw cycle can introduce 37% variability in HDL and LDL cholesterol determinations based on density fractionation [25]. It remains elusive why sample handling and storage have different effects on the results from different analytical platforms and obviously further research in the effects of sample handling and storage are required.

4. Analytical methods

The underlying complex multidimensional distribution of chemical and physical properties complicates lipoprotein isolation and measurement. Several innovative technologies, such as hyphenated size exclusion chromatography combined with mass spectrometry [26], have emerged over the last few years as useful tools for LP measurements. However, the four methods that are commonly used for lipoprotein profiling are density gradient Ultra Centrifugation (UC), Gel Electrophoresis (GE), Gel-Permeation High Performance Liquid Chromatography (GP-HPLC) and NMR spectroscopy [27,28]. Amongst these UC, gel electrophoresis and GP-HPLC require distinct separation and quantification steps, since quantification is based on determining the concentration of one or more of the chemical constituents of the particles. In contrast, NMR spectroscopy allows lipoprotein classification and quantification with a single measurement. Moreover, NMR is inherently quantitative and its sensitivity to LP chemical and physical properties, such as translational and rotational diffusion, chemical shielding and magnetic susceptibility leads to lipoprotein classification and subclass identification [9]. Moreover, the non-destructive nature of the NMR technique allows the natural chemical equilibria of the samples to be preserved. In the following sections, the principles and pros and cons of the main analytical methods for lipoprotein profiling will be briefly outlined with a more comprehensive treatment of the NMR method.

4.1. Proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy

By virtue of its numerous analytical advantages, ^{1}H-NMR spectroscopy has become an indispensable technique for the characterization of complex biological samples such as tissues and biofluids [29]. In particular, due to the possibility of conducting phase transitional studies, the use of ^{1}H-NMR spectroscopy has emerged as a valuable screening tool for measuring LPDs in plasma and serum samples [30–32]. The lipids inside the lipoproteins exist as liquid crystals of primarily triglycerides and cholesterol esters whose limited mobility gives rise to the broadening of the NMR signals of the methyl ($-CH_3$) and methylene ($-CH_2-$) groups centered around 0.8 and 1.2 ppm, respectively. Fig. 3 shows a representative NMR spectrum of a plasma sample. The region between 0.6 and 1.4 ppm is the spectral region used for determining lipoprotein profiles. The line shape distortion and off-set is due to the presence of broad non-lipoprotein signals arising from plasma proteins. In a 1D NOESY the lipoprotein signals between 0.6 and 1.4 ppm are overlaid with a broad signal from plasma proteins (i.e. albumin). This complicates lipoprotein profiling by NMR as the concentration of plasma proteins is individual as well as cohort dependent.

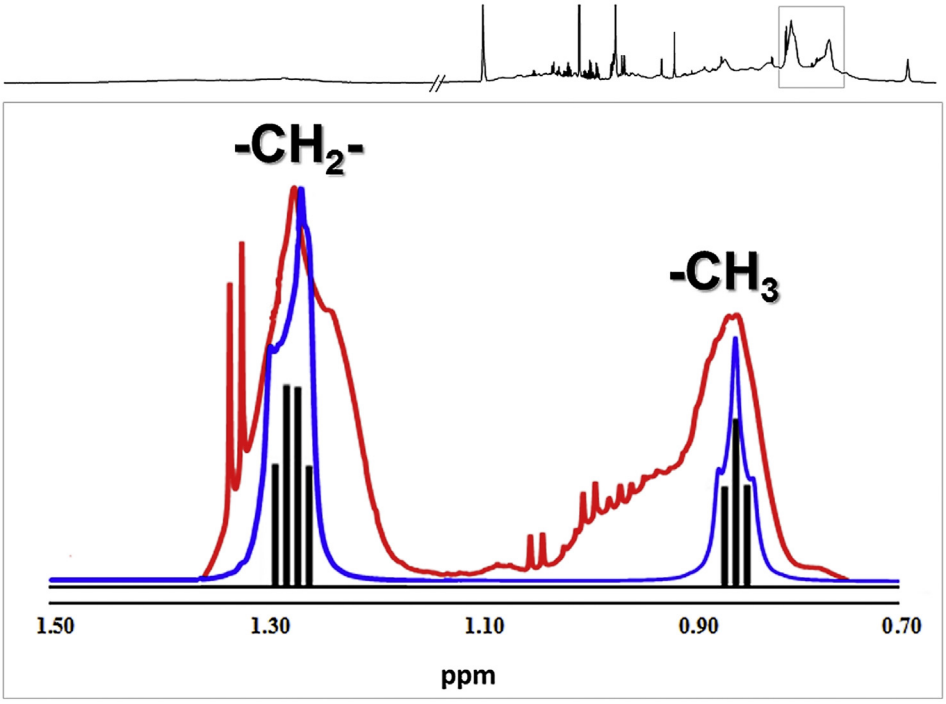

Fig. 3. A representative 600 MHz ^{1}H-NMR spectrum of human blood with an insert showing the ^{1}H NMR spectra of the methylene and methylgroups as theoretical lines of isolated $-CH_2-CH_3$ (black line), pure fat with conformational freedom (blue line) and from the interior of the lipoproteins in a blood sample (red line).

The main advantage of using NMR spectroscopy for the identification and quantification of LPs is due to the fact that different lipoprotein fractions and subfractions have different chemical compositions and sizes and therefore experience slightly different magnetic susceptibilities. This in turn gives rise to distinctive NMR signals whose chemical shift is mainly determined by the local electron density, and rotational diffusion of the lipoprotein vehicles. In particular, the methyl ($-CH_3$) signals arising from large and less dense LP particles (i.e. VLDL and LDL) are different in shape and resonate at lower field strength (higher frequency) than the lipid signals emitted by smaller LPs (i.e. HDL) [9]. Fig. 3 illustrates how the complexity of the lipid $-CH_3$ and $-CH_2-$ signals increases when increasing the complexity of the sample matrix from pure lipids to lipids within lipoproteins.

A typical NMR spectrum of plasma/serum is characterized by the presence of numerous metabolites (i.e. amino acids, organic acids), macromolecules (i.e. proteins) and lipoproteins whose signals are heavily overlapped. The entire up-field region from about 0.6 to 1.4 ppm can be used for lipoprotein quantifications, but in many cases only the methyl signal envelope at approximately 0.8 ppm is used [10,33]. The latter band contains the distinctive signals emitted by the terminal methyl group protons of phospholipids, cholesterol, cholesterol esters and triglycerides from the different lipoprotein main fractions and all the subfractions. The individual amplitudes of the NMR signals are directly proportional to the concentration of the LP particles giving NMR spectroscopy the unique capability of identifying and quantifying blood lipoproteins with one rapid experiment taking approximately 10 min in the case of 1D NOESY.

Several ^{1}H-NMR based protocols for lipoproteins fractions and subfractions identification and measurement have been developed. Amongst these, the commercial assays NMR LipoProfile® (LipoScience Inc.) [33], AXINON® lipoFIT® (Numares AG, Regensburg, Germany) [34], Brainshake Ltd. [35], Vantera® [36] and the Liposcale test (Biosfer Teslab SL) [37] are the pioneers for driving “the lipoprotein analysis by NMR” interest of the clinical and epidemiological community. Most recently, a new method, B.I.-LISA (Bruker IVDr LIpoprotein Subclass Analysis) (Bruker Biospin, Rheinstetten) (https://www.bruker.com) has been released by Bruker BioSpin, developed for Bruker's AVANCE IVDr (In-Vitro Diagnostics Research) system based on 600 MHz ^{1}H NMR which includes advanced hardware, software, automation, spectral libraries and Standard Operating Procedures (SOPs; Bruker IVDr Methods).

Lipoprotein profiling by NMR is different from most other quantitative and qualitative NMR applications as it is dependent on scrutinizing the shape of the signal envelopes of the methyl and methylene groups. Accordingly, the requirements for NMR reproducibility must be stressed to its limits. Careful SOPs have to be developed in order to increase the reproducibility and accuracy of the measurements and in order to make the measurements instrument lab independent. Full details on the methods for the study of LP profiles by NMR, as proposed by several authors, are given in the list of selected references in Table 2.

NMR sample preparation and data acquisition. Dona et al. devised a detailed set of updated protocols that carefully consider major experimental conditions such as sample preparation, spectrometer parameters and quality control at all stages is presented [14]. Amongst these, sample preparation is a crucial point for LP measurements since it might affect the reproducibility of the analytical results across different laboratories. Typically, sample preparation for lipoproteins analysis in plasma involves the following steps: a venous blood sample, previously collected into a tube containing an anticoagulant agent (i.e. EDTA or heparin), is spun down and the supernatant collected. Table 2 lists the diverse methods that have been proposed for sample preparation for NMR as well as the most commonly applied approaches for LP measurements by NMR spectroscopy.

Pre-processing of NMR data. While NMR is normally regarded as relatively low in sensitivity, it has a high sensitivity towards small changes in the physical properties of the solution, magnetic field strength and temperature whose alteration can dramatically affect the quality of the NMR spectra. In particular, signals misalignment is one of the major issues when measuring lipoproteins since it will compromise the subsequent identification. Although extremely detailed SOPs are followed when recording NMR spectra of blood, tiny spectral misalignments often persist and several signals have been proposed to be used as a reference for spectral alignment including the signal from the Ca^{2+} EDTA singlet at 2.52 ppm [38–40], the alanine doublet at 1.49 ppm [28,59] and the doublet from the glucose alpha-anomer at 5.24 ppm [20]. In the most recent protocols, the signal from the alanine doublet at 1.49 ppm is proposed as reference for alignment since it is independent of pH and close to the signals of interest, namely the methyl and methylene resonances. Several alignment methods have been compared and discussed in the review by Vu and Laukens [41] and, amongst these, *i*coshift [42] which can perform a simultaneous correlation optimized shifting of the alanine doublet (interval) for all the spectra in the cohort.

NMR quantification. NMR spectroscopy is inherently quantitative and can quantify baseline-resolved signals by simple integration. Even in complex chemical mixtures such as body fluids this principle often works. However, in the lipoprotein case this is not possible since it relies on deconvoluting the heavily overlapping signals of the lipid methylene and methyl envelopes. For this reason, the NMR quantification of lipoproteins requires a calibration to a reference method, for example the UC method (Table 2).

4.2. Reference methods

Ultracentrifugation. The UC technique is often described as the benchmark method for LPs measurement. Amongst UC techniques, the density gradient UC is referred to as the “gold standard” for lipoprotein measurement and has become a routine method for LPs separation [6]. Separation of CM, VLDL, LDL, and HDL is obtained by adjusting the density of the medium at each centrifugation step in order to allow sequential floatation of the individual lipoprotein fractions. A discontinuous gradient is then created and layers of solvents with different densities will cause the lipoproteins of different densities to be isolated in a cumulative fashion. The main disadvantage is that, due to the long spin times, the complete separation of all lipoprotein fractions may require from 2 to 5 days of centrifugation time which seriously limits the applicability of the method as a rapid screening tool for personalized medicine/nutrition and large epidemiological studies [7].

In order to reduce the experiment time, several improvements in the UC equipment have been introduced. Amongst these, the Vertical Auto Profiling (VAP) method allows lipoproteins to be separated in a single spin by using high centrifugal speeds (65,000 rpm, 1 h) [43]. This is achieved by using a vertical rotor in which the centrifuge tube remains perpendicular to the x-axis (ground) during centrifugation. This set-up will allow the lipoproteins separation across the shorter horizontal axis of the centrifuge. After centrifugation, all five lipoprotein fractions are analyzed for cholesterol content using the continuous flow VAP analyzer: every LP layer is mixed with a specific reagent for cholesterol and a red color will develop proportionally to the cholesterol concentration. A spectrophotometric detector (505 nm) will monitor the development of the enzymatic reaction. The recorded absorbance will be converted in cholesterol concentration.

Table 2
Human lipoprotein studies with coherent NMR spectra and Ultracentrifugation/HPLC measurements.

RM	NMR			Samples				Data analysis				Ref.
	Spectrometer	Probe	Experiment type and T	Cohort	Number	Storage	Sample type and preparation for NMR analysis	Data pre-pre processing	Fractions	Subfractions	Quantification method	
UC	400 MHz	10 mm	1D ^{1}H-NMR; 310 K	Diverse	58	Not given	Fasting; Plasma (2500 μl)[c]	Not given	VLDL IDL LDL HDL	– – – –	Line shape fitting on methyl region for TG, PL, Cho and CE quantification	[48]
UC and GGE	250 MHz	Not given	1D ^{1}H-NMR; 318 K	Healthy	30	UC: +4°C NMR: −70°C (storage time not given)	Fasting; not given	Not given	VLDL LDL HDL	– – –	Line shape fitting on the methyl signal for LP particles quantification	[9]
UC	600 MHz	5 mm	1D ^{1}H-NMR[a]; 316 K	Diverse Disease	44	+4°C UC: <2h NMR: <24h	Fasting; Plasma (500 μl)[c]	EDTA alignment; TSP scaling	VLDL IDL LDL HDL	– – – –	PLS on the aliphatic region from 0.4 to 1.4 ppm for Cho and TG quantification; NN analysis on the same region[b]	[36]
UC	500 MHz	5 mm	2D DOSY ^{1}H-NMR; 318 K	Diverse	17	−80°C (storage time not specified)	Fasting; Plasma (225 μl) + 225 μl of 0.9% NaCl in H_2O and 50 μl of D_2O	EDTA alignment	VLDL IDL LDL HDL	VLDL 1−2 – LDL 1−6 HDL 1−3	N-PLS and PARAFAC on methyl and methylene (only N-PLS) signals for LPs quantification	[38]
UC	600 MHz	120 μl flow	1D ^{1}H-NMR NOESY; 303 K	Diverse Obese− non-obese	103	−80°C (storage time not given)	Fasting; Plasma (100 μl) + 350 μl of 0.9% NaCl in H_2O and 50 μl of D_2O	EDTA alignment;	VLDL IDL LDL HDL	– – LDL 1-3 HDL 2a, 2b, 3	PLS on the spectral region from 0.2 to 5.7 ppm (EDTA excluded) for Cho and TG quantification	[37]
UC	500 MHz	5 mm	1D ^{1}H-NMR; 310 K	Diverse	75	Not given	Fasting; Serum (430 μl)[c]	TSP alignment; TSP scaling	VLDL IDL LDL HDL	– – – –	Bayesian model on the regions from 0.40 to 3.30 ppm for TG and Cho quantification	[51]
UC	500 MHz	120 μl flow	1D ^{1}H-NMR; 310 K	Males Healthy	153	+4°C UC: <24h NMR: <24h	Non-Fasting; Plasma (500 μl) + 60 μl D_2O	D-Glucose alignment;	CM	– – – –	iPLS for TG quantification	[19]
UC	600 MHz	5 mm	2D DOSY ^{1}H-NMR; 310 K	Healthy	4	Not given	Fasting Plasma (430 μl)[c]	TSP alignment;	VLDL IDL LDL HDL	– – LDL 1−2 HDL 2-3	Line shape fitting on methyl region for LPs distributions estimation	[18]
HPLC	600 MHz	3 mm	1D ^{1}H-NMR NOESY and DOSY; 310 K	Diverse Healthy	290	−80°C (storage time not given)	Fasting Serum (100 μl) + 100 μl phosphate buffer	Alanine alignment;	VLDL IDL LDL HDL	VLDL 3−7 – LDL 8−11 HDL 15−18	PLS on the methyl and methylene signals for Cho and TG quantification	[28]

[a] The spectra were run in the spinning mode (20 Hz).
[b] Signals from free amino acids and C-18 cholesterol where omitted from the neural network (NN) analysis.
[c] Sealed coaxial tubes used for external TSP referencing.

In spite of the recent advances, the UC technique compromises the natural chemical equilibria of the samples: the high salt concentrations and centrifugal forces can alter the chemical structure and destroy some of the most labile lipoproteins [44]. Moreover, UC methods are labor-intensive and technically demanding since they require a high level of technical pre-knowledge.

Gel-Permeation High Performance Liquid Chromatography. GP-HPLC is used to separate lipoproteins on the basis of the particle diameter, with small particles exhibiting a longer elution time than the big ones. The particle diameter of the lipoproteins is calibrated using stained lipoprotein standards with a known diameter whose size can be determined by gel gradient electrophoresis, dynamic light scattering or electron microscopy [45]. The elution position of the lipoproteins is derived from the relationship of calibration standards and its retention time. GP-HPLC can be coupled with enzymatic assays for lipid quantification [45].

Gel Electrophoresis. Gel electrophoresis is a method for macromolecules separation and analysis based on their size and charge. The main advantage in using GE for LP analysis is that, the migration regions of the serum proteins are the same regions as the lipoprotein main fractions. The migration regions of four serum proteins albumin, β-, pre-β- and α-globulins correspond to the regions of CM, VLDL, LDL and HDL, respectively [46]. The lipid concentrations per main lipoprotein class are calculated using peak integration. Gradient GE using nondenaturing conditions is commonly used to characterize the distribution of particles with very small differences in size [47].

5. Data analytical methods

Lipoprotein concentrations are normally given in equivalents of cholesterol or triglycerides. For the prediction of these lipoprotein concentrations from NMR spectra basically two methodologies have been used for the calibration to reference methods, namely Curve Fitting and Partial Least Squares (PLS) regression [48]. In curve fitting two different approaches have been applied: in the first one, the NMR spectra are fitted with individual Lorentzian line shapes while in the second one the NMR spectra are fitted with pure line profiles from predetermined LP subclass spectra. In the case of multivariate PLS regression, it has been applied either in the full spectral version or in the interval based version (*i*PLS) [49]. However, already in the full PLS version, the NMR spectral region is normally restricted to be from 0.6 to 1.4 ppm and 0.6–1.04 ppm which is to be regressed towards the response values of TG and Cho, respectively.

In the *i*PLS approach, the NMR spectra of the relevant region (typically 0.6–1.4 ppm) is divided into a number of small regions or intervals, for each of which a local PLS regression model is calculated. The abovementioned methods are described in the following sections and outlined in Fig. 4.

5.1. Curve fitting

Curve fitting using Lorentzians. The resonances of the NMR signals, including signals from lipoprotein lipids, have been shown to be well described by individual Lorentzian line shapes (see Fig. 4a). This method is based on mathematical line shape models for the lipid methyl resonances of the VLDL, LDL, and HDL fractions and, if successful, it will allow for accurate lipoproteins quantification. For the complex lipid signals of lipoproteins, several if not many Lorentzian are fitted simultaneously to the spectra. In the method proposed by Ala-Korpela et al. (1994) three individual Lorentzians were needed in the case of the VLDL and LDL fractions and one Lorentzian needed in the case of the HDL fractions in order to give an accurate description of the methyl resonances of the lipoprotein lipids [50]. The mathematical line shape models could be constructed for the methyl resonances of the abovementioned fractions by assuming the uniformity of the most relevant parameters of the individual Lorentzians: half line widths, relative resonance frequencies and intensity ratios of the individual components.

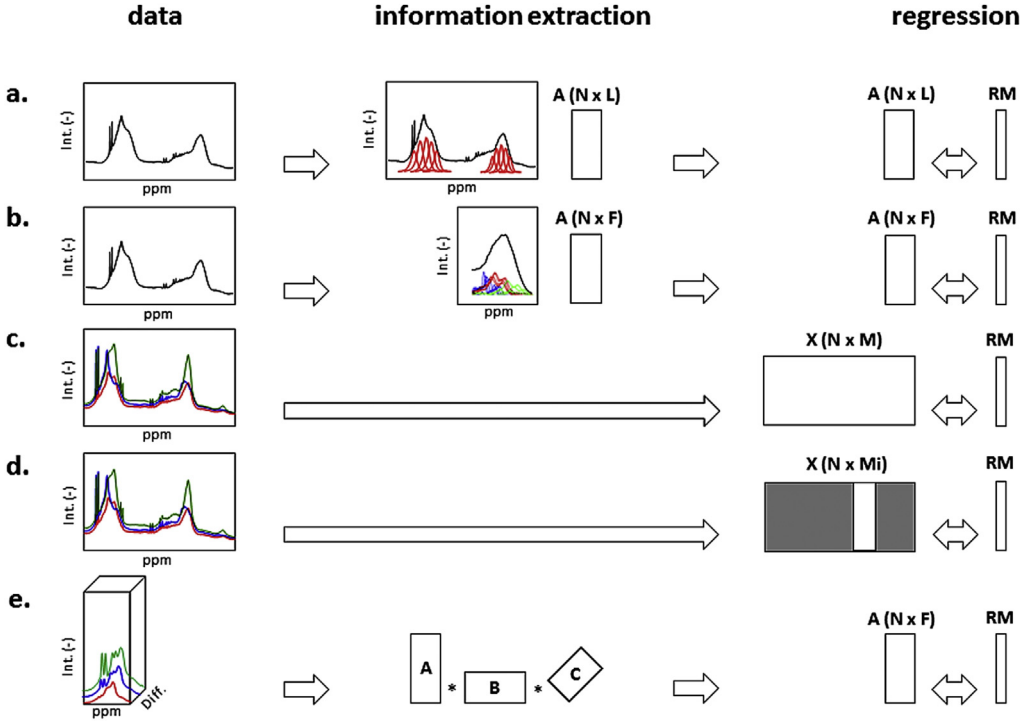

Fig. 4. Different approaches for LPs lipid quantification and LP particles determination. (a) Curve fitting using Gaussian-Lorentzian constraints; (b) Curve fitting using pure LP subfraction line shapes; (c) PLS regression, (d) PLS regression with variable selection exemplified with *i*PLS and (e) PARAFAC modeling of diffusion edited spectral dataset.

However, curve fitting with multiple Lorentzians is an ill-posed problem which is easily influenced by the shapes of the spectra, strongly overlapping peaks and the number of curves to be fitted. In recent applications, this issue has been attempted to be solved by applying a Bayesian approach for LPs quantification from ^{1}H NMR spectra [51].

Curve fitting with pure LP sub-fraction line shapes. A second curve fitting approach, based on the fitting of the methyl lipid resonance envelope, is commonly used for LPs quantification (see Fig. 4b). In this method, the complex line shapes of the overlapping NMR signals from different LPs are deconvoluted into multiple curves representing the NMR spectra of purified LP subfractions [52]. The hypothesis for this to work is that the line shapes of the sub-fractions do not differ amongst individuals or phenotypes. In this case the curve fitting algorithm uses a library of line shapes for each individual lipoprotein fraction and the simultaneous fit of all these curves can provide high-throughput quantification of lipoprotein fractions and subfractions.

5.2. Partial least squares regression (PLS)

PLS regression represents an effective alternative to curve fitting and was first applied to quantification of lipoproteins by Bathen et al. (2000) where PLS and Neuronal Network (NN) analysis were combined [38]. In this approach a PLS regression model is built to find the best linear association between the NMR spectra and the measured data from a reference method (i.e. UC) (see Fig. 4c). In contrast to the curve fitting approach, overlapping signals do not need to be deconvoluted directly since the PLS models focus on the relation between the NMR spectra and the reference UC data. PLS regression is an effective method for quantification, and most modern lipoprotein quantifications are based on this method. PLS calibrations need careful validation schemes since sample sets with coherent NMR spectra and UC determinations are rare and of relative small number of samples (see Table 2). Since PLS models are based on correlation (or covariance in the unscaled version), special care has to be taken to interpret the models in order to limit the effect of the so-called cage of covariance [53]. Such an interpretation may use selectivity ratios [54] since these ratios may give insight into which variables are important for the calibration [28].

Interval PLS. In order to develop a more parsimonious regression model, *i*PLS has been proposed for LPs quantification (see Fig. 4d). *i*PLS allows localization of relevant spectral regions that are correlated with the response variable, y, (i.e. Cho and/or TG concentrations as measured by a reference method) in the regression equation. This approach, which combines PLS with regional/interval variable selection, has proven to be successful for the prediction of postprandial chylomicrons [20] as well as for the determination of cholesterol in rodent plasma lipoprotein fractions [55]. However, in principle many other variable selection methods could be used for improving the parsimony, interpretability and performance of the prediction methods.

5.3. Augmenting the measurement dimensions

Several methods can be used for editing the NMR-detectable metabolite information in body fluids [32]. In particular, the accuracy of the line fitting method can be further enhanced by additionally taking into account the self-diffusion behavior of the lipoprotein particle sizes by performing diffusion edited NMR spectra [40,56]. In this approach, the information on the different lipoprotein particle sizes is encoded by both spectral position and diffusion weighting from the field gradients. Using this method, the interfering signals from small molecules and plasma proteins can be suppressed using gradients (see Fig. 4e). This is for example the case with the lactate doublet whose high sensitivity to pH adds random variation to the NMR spectra. Two major challenges are related to the diffusion encoding of metabolites. Firstly, and perhaps most importantly, it is nearly impossible to calibrate the gradients across different platforms and laboratories. Secondly, the long experiment time of 2D diffusion-edited measurements limits the applicability in large scale/epidemiological studies. However, the latter challenge can be partially circumvented by only recording one properly selected diffusion weighted spectrum together with the normal NOESY unweighted spectrum.

2D diffusion-edited NMR data becomes three dimensional (intensity × ppm × gradient) and can elegantly be modeled by multiway chemometrics methods such as PARAllel FACtor Analysis (PARAFAC) (see Fig. 4e). PARAFAC is the multiway analogue to Principal Component Analysis (PCA) without the constraint that the principal components are orthogonal. By using PARAFAC, the underlying pure lipoprotein profiles can be automatically recovered and the extracted concentrations represent real concentrations and thus no calibration is needed [40]. Unfortunately, this approach has so far only been able to recover the main fractions.

6. Concluding remarks

The lipoprotein content of a blood sample is difficult to characterize. The main reason for this is that the chemical composition, density and size of lipoproteins vary greatly, limiting the possibility of clearly establishing the relationships amongst these three fundamental properties. Several studies have been conducted with the aim of comparing different methods for lipoprotein subfractions determination. The systematic review by Chung et al. listed several limitations found in nine studies where different methods used for LDL subfraction determination were compared [57]. The wide variety of methodologies used, the non-uniform definitions or descriptions of LDL subfractions and the inappropriate statistical analyses limited the comparability amongst and within the analytical techniques. In addition, no comparison of diagnostic accuracy or clinical value was carried out.

Due to its inherent quantitative nature and sensitivity to size and density, NMR spectroscopy has increasingly gained attention as a valuable method for lipoproteins measurement, attracting several research groups to test and apply the methodology (Table 2). However, so far, the applications with coherent NMR and reference data (i.e. UC) have been limited to relatively small cohorts, and the lack of appropriate standards both for the UC and NMR measurements have made meta-studies extremely difficult if not impossible. Several commercial companies are now offering NMR-based lipoprotein analysis. For example, the commercial NMR-based lipoprotein quantification methodology of LipoScience, based on the method developed by Otvos et al. [9], has been widely applied in biomedical applications and has been approved by the US Food and Drug Administration (FDA) to directly quantify LDL particles.

Despite these advances, there is still some controversy about the introduction of NMR-based advanced lipoproteins technologies into clinical practice. In particular, the accuracy of the model prediction seems to be subfraction dependent limiting the applications in the diagnostic field [58].

Nevertheless, the current efforts towards extreme standardization of NMR measurements of blood samples [59] and similar efforts on standardization of the reference methods will become game changers that will revolutionize the field of lipoprotein profiling. In particular, the development of NMR- specific SOPs will allow the comparison of different cohorts and will generate new knowledge on the lipoprotein particle distribution with the possibility of turning it into a biomarker of lifestyle, diseases and healthiness.

The long-term application of NMR-based lipoprotein analysis in medical research is obviously an encouraging example for the epidemiological and clinical prospects of NMR-based technologies.

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